### Detailed program for the 18th Symposium on Purine and Pyrimidine Metabolism in Man

*The program can be modified*

<table>
<thead>
<tr>
<th><strong>Tuesday June 11th 2019</strong></th>
<th></th>
</tr>
</thead>
</table>
| 18.00-22.00 | Welcome cocktail at Le Sherrington  
204B avenue des Frères Lumière, 69008 Lyon |

<table>
<thead>
<tr>
<th><strong>Wednesday June 12th 2019</strong></th>
<th><strong>The Auditorium, IARC</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>8.30-8.40</td>
<td>Opening</td>
</tr>
</tbody>
</table>

| **8.40-10.40** | **Session 1**  
**Enzymes in purine and pyrimidine metabolism 1**  
**Chairs:** Santiago Ramón-Maiques & Wolfgang Knecht |
|---------------|----------------|
| **S1.1** 8.40-9.05 | Mark Rider  
Targeting purine nucleotide metabolizing enzymes to achieve AMP-activated protein kinase activation for the treatment of type 2 diabetes |
| **S1.2** 9.05-9.20 | Jim Dompierre  
The purine intermediate AICAR impairs the mitotic spindle assembly checkpoint by affecting the *de novo* pyrimidine biosynthesis pathway |
| **S1.3** 9.20-9.40 | Doreen Dobritzch-Lohkamp  
Crystal structure and pH-dependent allosteric regulation of human β-ureidopropionase |
| **S1.4** 9.40-9.55 | Marta Tomczyk  
An unexpected link between ecto-5′-nucleotidase deficiency and impaired cardiac vitamin B12 metabolism |
| **S1.5** 9.55-10.10 | Juan Manuel Orozco Rodriguez  
Reconstitution of human dihydroorotate dehydrogenase for neutron scattering studies |
| **S1.6** 10.10-10.25 | Blanca Martinez-Arribas  
DCTPP1 preserves genomic integrity through the modulation of dCTP and dTTP homeostasis |
| **S1.7** 10.25-20.40 | Patrycja Jablonska  
Extracellular metabolism of NAD+ and NMN on the surface of murine lung endothelial cells |
| 10.40-11.00 | Coffee break |

| 11.00-13.00 | **Session 2**  
**Drug development and targeting purine and pyrimidine metabolism**  
**Chairs:** Suzanne Peyrottes & Frits Peters |
|---------------|----------------|
| **S2.1** 11.00-11.15 | Sean Rudd  
Targeting SAMHD1 to enhance nucleoside-based cancer therapies |
| **S2.2** 11.15-11.30 | Chris Mulder  
Sustained effectiveness, safety and therapeutic drug monitoring of thioguanine in a cohort of 274 Inflammatory Bowel Disease patients, intolerant for conventional therapies |
| **S2.3** 11.30-11.45 | Aránzazu Medieiro  
Effects Of Antiretroviral Therapy With Tenofovir And Other Antiretroviral Drugs On The Inflammatory State And Bone Remodeling On newly diagnosed HIV-Patients At Basal And 3 and 12 Months after starting Treatment |
| **S2.4** 11.45-12.00 | Jamshed Iqbal  
Inhibition of Membrane Bounded Ecto-5′-nucleotidase (CD73): A Novel Target for Treatment of Cervical Cancer |
| **S2.5** 12.00-12.15 | Christophe Mathé  
Design and synthesis of purine nucleot(s)ide analogues as CD73 inhibitors |
<table>
<thead>
<tr>
<th>Session</th>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2.6</td>
<td>12.15-12.30</td>
<td>Laurent Chaloin</td>
<td>Drug discovery and optimization targeting CD73 to restore antitumor immune response</td>
</tr>
<tr>
<td>S2.7</td>
<td>12.30-12.45</td>
<td>Tormod Karlsen Bjánés</td>
<td>Sonoporation and gemcitabine delivery in pancreatic cancer cell lines</td>
</tr>
<tr>
<td>S2.8</td>
<td>12.45-13.00</td>
<td>Simin Zhang</td>
<td>Targeting NUDT15 to improve the Antitumor Efficacies of Nucleoside Analogue Antimetabolites</td>
</tr>
<tr>
<td>13.00-14.30</td>
<td></td>
<td>Lunch and Poster session 1</td>
<td></td>
</tr>
</tbody>
</table>

**Session 3**: Purines and pyrimidines in cancer 1
Mounira Amor-Guérét & Jörgen Bierau

<table>
<thead>
<tr>
<th>Session</th>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>S3.1</td>
<td>14.30-14.55</td>
<td>Caius Radu</td>
<td>Mapping and targeting pyrimidine nucleotide biosynthesis and utilization in KRAS driven pancreatic cancer</td>
</tr>
<tr>
<td>S3.2</td>
<td>14.55-15.15</td>
<td>Clara Boces Pascual</td>
<td>Role of miR-17 family on human Concentrative Nucleoside Transporter 1 (hCNT1) modulation during carcinogenesis</td>
</tr>
<tr>
<td>S3.3</td>
<td>15.15-15.30</td>
<td>Simone Allegreni</td>
<td>cN-II in the metabolic and proliferative profile of human lung tumor cell line NIC-H292</td>
</tr>
<tr>
<td>S3.4</td>
<td>15.30-15.45</td>
<td>Adrien Jaramillo</td>
<td>Dexamethasone may decrease the efficacy of gemcitabine-cisplatin but not of cytarabine; role of deoxycytidine kinase</td>
</tr>
<tr>
<td>S3.5</td>
<td>15.45-16.00</td>
<td>Liya Wang</td>
<td>The expression and activity of thymidine kinase 1 and deoxycytidine kinase is modulated by hydrogen peroxide and nucleoside analogs</td>
</tr>
<tr>
<td>16.00-16.30</td>
<td></td>
<td>Coffee break</td>
<td></td>
</tr>
</tbody>
</table>

**Session 4**: Purine and pyrimidine pools
Chairs: Veronika Barešová & Claus Desler Madsen

<table>
<thead>
<tr>
<th>Session</th>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>S4.1</td>
<td>16.30-16.45</td>
<td>Magdalena A Zabielska</td>
<td>Cardiac mitochondrial nucleotide pool and its role in the post-ischemic adenosine production</td>
</tr>
<tr>
<td>S4.2</td>
<td>16.45-17.00</td>
<td>Anders Clausen</td>
<td>Nucleotide pools dictate the identity and frequency of ribonucleotide incorporation in mitochondrial DNA</td>
</tr>
<tr>
<td>S4.3</td>
<td>17.00-17.15</td>
<td>Barbara Kutryb-Zajac</td>
<td>Clinical correlations of extracellular nucleotide metabolism eco-enzymes in patients with calcific aortic valve disease</td>
</tr>
<tr>
<td>17.15-18.15</td>
<td></td>
<td>Anne Simmonds Memorial Lecture</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monika Loeffler</td>
<td>This lecture is sponsored by PUMPA</td>
</tr>
<tr>
<td>19.30</td>
<td></td>
<td>Departure for dinner</td>
<td></td>
</tr>
<tr>
<td>20.00-23.30</td>
<td></td>
<td>Symposium dinner on the rivers Rhône and Saône–Hermès boat</td>
<td></td>
</tr>
</tbody>
</table>

**Thursday June 13th 2019**
The Auditorium, IARC

**Session 5**: Enzymes in purine and pyrimidine metabolism 2
Chairs: Doreen Dobritzsch-Lohkamp & Laurent Chaloin

<table>
<thead>
<tr>
<th>Session</th>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>S5.1</td>
<td>9.00-9.25</td>
<td>Santiago Ramón-Maiques</td>
<td>CAD, a multienzymatic protein at the head of de novo pyrimidine biosynthesis</td>
</tr>
<tr>
<td>S5.2</td>
<td>9.25-9.40</td>
<td>Davide M Ferraris</td>
<td>Structural investigation of enzymes of Mycobacterium tuberculosis involved in the synthesis of purine and pyrimidine nucleotides</td>
</tr>
<tr>
<td>S5.3</td>
<td>9.40-9.55</td>
<td>Sekine Mai</td>
<td>Analysis of the purine metabolic effect of allopurinol and its derivative</td>
</tr>
<tr>
<td>S5.4</td>
<td>9.55-10.10</td>
<td>Chiara Rampazzo</td>
<td>SAMHD1 deficiency affects DNA replication fidelity and telomere homeostasis in human fibroblasts</td>
</tr>
<tr>
<td>S5.5</td>
<td>10.10-10.30</td>
<td>Miriam Yagüe Capilla</td>
<td>Deciphering the role of HD52, a mitochondrial nucleotidase essential for pyrimidine homeostasis in Trypanosoma brucei</td>
</tr>
<tr>
<td>S5.6</td>
<td>10.30-10.45</td>
<td>Ken Okamoto</td>
<td>Effect of xanthine oxidoreductase inhibitors on purine metabolism in mouse brain under hypoxic environment</td>
</tr>
</tbody>
</table>
**Session 6**

**Inborn errors of purine and pyrimidine metabolism 1**

*Chairs: Yolanda Cámara & Sylvain Latour*

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>11:35-12:00</td>
<td>Aida Mata-Ventosa</td>
<td>Deficiency of perforin and hCNT1, a novel inborn error of pyrimidine metabolism, associated with a rapidly developing lethal phenotype due to multi-organ failure</td>
</tr>
<tr>
<td>12:00-12:20</td>
<td>Jonathan Shintaku</td>
<td>Ribonucleotide Reductase Subunit RRM1 is Required for Mitochondrial DNA Maintenance via Regulation of dNTP and rNTP Pools</td>
</tr>
<tr>
<td>12:20-12:40</td>
<td>Norbert Minet</td>
<td>Characterization and complementation of cellular models of CTPS1 and CTPS2 deficiencies</td>
</tr>
<tr>
<td>12:40-13:00</td>
<td>Emmanuel Martin</td>
<td>A homozygous hypomorphic mutation is responsible for CTPS1 immunodeficiency : immunological and molecular characterization from a cohort study</td>
</tr>
</tbody>
</table>

**Session 7**

**Purines and pyrimidines in cancer 2**

*Chairs: Tormod Karlsen Bjånes & Takahiro Yamauchi*

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:30-14:50</td>
<td>Btissame El Hassouni</td>
<td>Acquired resistance to Fluorocyclopentenylcytosine (RX-3117) in non-small lung cancer cells is related to a decrease of active RX-3117 nucleotides</td>
</tr>
<tr>
<td>14:50-15:10</td>
<td>Claus Desler Madsen</td>
<td>The importance of nucleotide metabolism for successful immunotherapy for cancer</td>
</tr>
<tr>
<td>15:10-15:25</td>
<td>Octavía Cadassou</td>
<td>What roles for the 5’-nucleotidases cN-II and CD73 in the interplay between the cancer cell and its innate immune microenvironment?</td>
</tr>
<tr>
<td>15:25-15:40</td>
<td>Mihoko Morita</td>
<td>How the combination of 6-mercaptopurine with febuxostat affects xanthine oxidase activity in vitro</td>
</tr>
<tr>
<td>15:40-16:00</td>
<td>Fiona McKissock</td>
<td>Novel ProTide NUC-3373: a potent inhibitor of thymidylate synthase</td>
</tr>
</tbody>
</table>

17.00-18.30  Sightseeing in the old city of Lyon

19.30-21.00  PPS Board Meeting at CRCL lab, Faculté Rockefeller

**Friday June 14th 2019**

**Session 8**

**Inborn errors of purine and pyrimidine metabolism 2**

*Chairs: Sandra Pérez-Torras & André van Kuijlenburg*

<table>
<thead>
<tr>
<th>Time</th>
<th>Speaker</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.30-8.45</td>
<td>Michio Hirano</td>
<td>Deoxynucleoside Therapy for Mitochondrial DNA Depletion</td>
</tr>
<tr>
<td>8.45-9.00</td>
<td>Cora Blázquez-Bermejo</td>
<td>Age-related metabolic changes limit efficacy of deoxynucleoside-based therapy in TK2-deficient mice</td>
</tr>
<tr>
<td>9.00-9.15</td>
<td>Olga Součková</td>
<td>Preparation of standards for LC-MS/MS detection of various metabolites of the <em>de novo</em> purine synthesis and their analysis in urine samples of healthy controls and patients with unspecific neurological symptoms</td>
</tr>
<tr>
<td>9.15-9.35</td>
<td>Ferran Vila-Julìà</td>
<td>AAV-mediated gene therapy shows efficacy on the biochemical and neurological phenotype of the nucleoside-enhanced mouse model of MNGIE</td>
</tr>
<tr>
<td>9.35-9.50</td>
<td>Veronika Barešová</td>
<td>Pathological purinosome assembly in cell model systems with de novo purine synthesis and salvage pathway deficiencies can be repaired by complementation with wt-protein</td>
</tr>
<tr>
<td>Time</td>
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<tr>
<td>9.50-11.00</td>
<td>PPS General assembly</td>
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<tr>
<td>11.00-11.30</td>
<td>Coffee break</td>
<td></td>
</tr>
<tr>
<td>11.30-13.00</td>
<td><strong>Session 9</strong></td>
<td></td>
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<td></td>
<td><strong>Advances in gout and dysuricemia</strong></td>
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<td></td>
<td>Chairs: Ivan Šebesta &amp; Kimiyoshi Ichida</td>
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<tr>
<td>S9.1</td>
<td>11.30-11.45 Werner Löffler</td>
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<tr>
<td>S9.2</td>
<td>11.45-12.00 Rosa Torres Jiménez</td>
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<tr>
<td>S9.3</td>
<td>12.00-12.15 Kiyoko Kaneko</td>
<td></td>
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<tr>
<td>S9.4</td>
<td>12.15-12.30 Tappei Takada</td>
<td></td>
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<tr>
<td>S9.5</td>
<td>12.30-12.45 Akiyoshi Nakayama</td>
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<tr>
<td>S9.6</td>
<td>12.45-13.00 Akiyoshi Nakayama</td>
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<tr>
<td>13.00-14.30</td>
<td>Lunch and Poster session 3</td>
<td></td>
</tr>
<tr>
<td>14.30-16.00</td>
<td><strong>Session 10</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Purines and pyrimidines in cancer 3</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chairs: Maria Grazia Tozzi &amp; Caius Radu</td>
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</tr>
<tr>
<td>S10.1</td>
<td>14.30-14.55 Mounira Amor-Guéret</td>
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<tr>
<td>S10.2</td>
<td>14.55-15.15 Jörgen Bierau</td>
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<tr>
<td>S10.3</td>
<td>15.15-15.30 Godefridus J Peters</td>
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<tr>
<td>S10.4</td>
<td>15.30-15.45 Eduard Mas</td>
<td></td>
</tr>
<tr>
<td>S10.5</td>
<td>15.45-16.00 Muhammad-Zawwad Raza</td>
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</tr>
<tr>
<td>16.00</td>
<td>Coffee and goodbye</td>
<td></td>
</tr>
</tbody>
</table>

**Posters**

<table>
<thead>
<tr>
<th>Number</th>
<th>Presenter</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Cora Blázquez-Bermejo</td>
<td>Increased dNTP pools rescue mtDNA depletion in human POLG-deficient fibroblasts</td>
</tr>
<tr>
<td>P2</td>
<td>Alicja Bulinska</td>
<td>Cardiac adenine nucleotide pool and mitochondrial function in dyslipidemic mice</td>
</tr>
<tr>
<td>P3</td>
<td>Mélanie Donnette</td>
<td>Precision medicine in hematology-oncology: CDA as a predictive marker for azacytidine efficacy in LAM and MDS patients</td>
</tr>
<tr>
<td>P4</td>
<td>Mélanie Donnette</td>
<td>Precision medicine in hematology-oncology: CDA as a predictive marker for Cytarabine exposure in AML patients</td>
</tr>
<tr>
<td>P5</td>
<td>Lynette Fairbanks</td>
<td>An HPLC method for ADA2 in plasma</td>
</tr>
<tr>
<td>P6</td>
<td>Lynette Fairbanks</td>
<td>Phenotype / Genotype Findings in PRPS</td>
</tr>
<tr>
<td>P7</td>
<td>Jun Mei Hu Frisk</td>
<td>An alternative TMP kinase in human</td>
</tr>
<tr>
<td>P8</td>
<td>Takuji Hosoya</td>
<td>Establishment of high hypoxanthine phosphoribosyltransferase activity-uricase knockout mice as novel hyperuricemic model and effects of purine/nonpurine type xanthine oxidoreductase inhibitors</td>
</tr>
<tr>
<td>Page</td>
<td>Author</td>
<td>Title</td>
</tr>
<tr>
<td>------</td>
<td>-------------------------</td>
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</tr>
<tr>
<td>P9</td>
<td>Makoto Hosoyamada</td>
<td>Rescure of Xor knockout mouse with high HPRT activity by NAD⁺ supplementation</td>
</tr>
<tr>
<td>P10</td>
<td>Takeshi Ikenaga</td>
<td>Effect of phytic acid on postprandial serum uric acid level in healthy volunteers: a randomized, double-blind, crossover study</td>
</tr>
<tr>
<td>P11</td>
<td>Adrien Jaramillo</td>
<td>Expression of the nucleoside transporters hENT1 and HCNT1 in pediatric acute myeloid leukemia</td>
</tr>
<tr>
<td>P12</td>
<td>Jae-Bum Jun</td>
<td>Distribution of serum uric acid levels and prevalence of hypouricemia and hyperuricemia: A cross-sectional, nationwide health examinee cohort study in Korea</td>
</tr>
<tr>
<td>P13</td>
<td>Hiroshi Kano</td>
<td>Lactic acid bacteria show different patterns of incorporation of purine mononucleotides and nucleosides depending on the species</td>
</tr>
<tr>
<td>P14</td>
<td>Tormod Karlsen Bjānes</td>
<td>Role of intracellular cytidine deaminase activity for gemcitabine metabolism in three pancreatic cancer cell lines</td>
</tr>
<tr>
<td>P15</td>
<td>Masafumi Kurajoh</td>
<td>Association between plasma xanthine oxidoreductase activity and vascular endothelial dysfunction in subjects without hypertension or diabetes – MedCity21 health examination registry</td>
</tr>
<tr>
<td>P16</td>
<td>Ane Larrañaga-Vera</td>
<td>Pannexin1 KO osteoclasts are insensitive to Tenofovir induced differentiation</td>
</tr>
<tr>
<td>P17</td>
<td>Thuc M Le</td>
<td>Novel mass spectrometry approaches to investigate nucleotide biosynthesis and utilization in pancreatic cancer</td>
</tr>
<tr>
<td>P18</td>
<td>Carlos López Gómez</td>
<td>Response to dC+dT therapy in TK2 deficiency is influenced by nucleoside bioavailability and levels of Tk1 and Dck</td>
</tr>
<tr>
<td>P19</td>
<td>Aránzazu Medieiro</td>
<td>Tenofovir causes bone loss via decreased bone formation and increased bone resorption, which can be counteracted by adenosine A2A receptor in mice</td>
</tr>
<tr>
<td>P20</td>
<td>Yoko Nakajima</td>
<td>Development of HPRT activity assay method using dried blood spot by UPLC-MS/MS</td>
</tr>
<tr>
<td>P21</td>
<td>Rie Nishi</td>
<td>Venetoclax and alvocidib are cytotoxic to the nucleoside analog-resistant acute myeloid leukemia HL-60 variants with BCL-2 and MCL-1 overexpression in vitro</td>
</tr>
<tr>
<td>P22</td>
<td>Takeshi Nishino</td>
<td>The Role of Purine Salvage Pathway in Bioenergetic Supply for the Neurological Cells</td>
</tr>
<tr>
<td>P23</td>
<td>Nozomi Otsuki</td>
<td>Effect of female hormone and menopause on uric acid clearance</td>
</tr>
<tr>
<td>P24</td>
<td>Iwona Pelikant-Malecka</td>
<td>Effect of prolonged physical exercise on nicotinamide metabolism in marathon runners</td>
</tr>
<tr>
<td>P25</td>
<td>Catalina Perelló-Reus</td>
<td>Impact of FLT3 modulation on cytarabine metabolism</td>
</tr>
<tr>
<td>P26</td>
<td>Godefridus J Peters</td>
<td>Gene profiling of tumor cell lines and (patient derived) xenografts to optimize selective treatment with RX-3117 (fluorocyclopentenylcytosine)</td>
</tr>
<tr>
<td>P27</td>
<td>Suzanne Peyrottes</td>
<td>A ball milling strategy for the synthesis of dinucleotides</td>
</tr>
<tr>
<td>P28</td>
<td>Ivan Sebesta</td>
<td>Modified forearm ischemic test in hypouricemic patients</td>
</tr>
<tr>
<td>P29</td>
<td>Václava Škopová</td>
<td>Clinical manifestations and molecular aspects of phosphoribosylpyrophosphate synthetase superactivity in females</td>
</tr>
<tr>
<td>P30</td>
<td>Blanka Stibůrková</td>
<td>Clinical and functional characterization of a novel URAT1 dysfunctional variant in a pediatric patient with renal hypouricemia</td>
</tr>
<tr>
<td>P31</td>
<td>Fukue Takayanagi</td>
<td>Measurement of total purine content and free nucleosides, nucleotides and purine bases composition in Japanese anchovies (Engraulis japonicas) using high-performance liquid chromatography</td>
</tr>
<tr>
<td>P32</td>
<td>Marta Tomczyk</td>
<td>Nucleotides metabolism failure as a new clue for hunting the cause of Huntington’s disease related cardiomyopathies</td>
</tr>
<tr>
<td>P33</td>
<td>Pierre-Olivier Vidalain</td>
<td>Functional interactions between de novo pyrimidine biosynthesis, innate immunity and lipogenesis in hepatocytes</td>
</tr>
<tr>
<td>P34</td>
<td>Naruomi Yamada</td>
<td>Lactobacillus gasseri PA-3 incorporates purine mononucleotides and utilizes them for RNA/DNA synthesis</td>
</tr>
<tr>
<td>P35</td>
<td>Noriko Yamaoka</td>
<td>Effect of crude drug extracts on purine metabolism in HepG2 cells</td>
</tr>
<tr>
<td>P36</td>
<td>Shio Yoshida</td>
<td>Association between plasma xanthine oxidoreductase activity and blood pressure – MedCity21 health examination registry</td>
</tr>
</tbody>
</table>
PP19 ABSTRACT BOOK
Abstract S1.1
Targeting purine nucleotide metabolizing enzymes to achieve AMP-activated protein kinase activation for the treatment of type 2 diabetes

Mark H. Rider

a de Duve Institute and Université catholique de Louvain, Avenue Hippocrate 75, 1200-Brussels, BELGIUM

E-mail of corresponding author: mark.rider@uclouvain.be

Objectives: To investigate whether targeting AMP deaminase-1 (AMPD1), soluble 5’-nucleotidase 1A (NT5C1A) or soluble 5’-nucleotidase II (NT5C2) would be a viable strategy to achieve AMP-activated protein kinase (AMPK) activation for treating metabolic disorders such as type 2 diabetes (T2D).

Methods: In collaboration with the pharmaceutical company AstraZeneca, small-molecule pharmacological AMPD inhibitors were produced. In addition, whole body AMPD1, NT5C1A and NT5C2 knockout mice were generated.

Results: In contracting skeletal muscles, pharmacological AMPD inhibition potentiated increases in intracellular AMP concentration and AMPK activation but without affecting glucose uptake. In incubated mouse muscles from AMPD1 knockout mice, increased AMP levels induced by electrical stimulation were potentiated considerably, but enhanced AMPK activation was at best moderate. NT5C1A or NT5C2 enzyme knockout did not lead to enhanced AMP concentration in response to contraction in extensor digitorum longus (EDL) and soleus mouse muscles, with no potentiation either of increases in AMPK activity or glucose transport. Moreover, dual blockade of AMP metabolism in EDL using an AMPD inhibitor combined with NT5C1A deletion did not enhance rises in AMP or increase AMPK activation by electrical stimulation. By contrast on a high-fat diet (HFD), NT5C2 knockout mice displayed reduced body/fat weight gain, improved glucose tolerance, reduced plasma insulin, triglyceride and uric acid levels.

Conclusions: Pharmacological inhibition of AMPD1, NT5C1A or NT5C2 in skeletal muscle would not be a viable strategy for the treatment of T2D. However, NT5C2 deletion in mice protected against HFD-induced weight gain, adiposity, insulin resistance and associated hyperglycemia. Therefore, NT5C2 might be a viable drug target for the treatment of T2D and pharmacological NT5C2 inhibitors that have been developed as anti-cancer agents might also have anti-diabetic potential.

Relevant references:
- Plaideau et al. (2012) FASEB J 26, 2685-2694
- Plaideau et al. (2014) Chem. Biol 21, 1497-1510

KEYWORDS: AMPD1, NT5C1A, NT5C2, AMPK
Abstract S1.2

The purine intermediate AICAR impairs the mitotic spindle assembly checkpoint by affecting the *de novo* pyrimidine biosynthesis pathway

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Objectives: AICAR (or Acadesine) is a pharmacological precursor for the biosynthesis of purine nucleotides with anti-tumor properties. Interestingly, it has been shown to preferentially affect aneuploid cells relative to euploid cells. As aneuploidy and more particularly chromosome gain promotes genomic instability and contributes to tumorigenesis, we wanted to characterize the potential effects of AICAR on mitosis.

Methods: Immunofluorescence and videomicroscopy were used to monitor the progression of mitosis in HEK and MDCK epithelial cells. The effects of AICAR on biosynthesis pathways of purine and pyrimidine were performed by HPLC.

Results: Videomicroscopy experiments on MDCK cells expressing H2B-GFP, to follow the chromosomes behavior, showed potent mitotic phenotypes characterized by lack of chromosome alignment at the metaphase plate and loss of chromosomes during anaphase, resulting in micronuclei formation and cell death. Immunofluorescence revealed severe defects in microtubule-kinetochore interactions, a decrease of the spindle assembly checkpoint proteins localization at the kinetochores, such as BubR1 and MAD2. Western Blot and RT-qPCR confirmed the drop of BubR1 and MAD2 expressions. Metabolomic experiments revealed that AICAR induces a strong decrease of the pyrimidine UTP and CTP in these cells. Interestingly, other drugs affecting the pyrimidine biosynthesis pathway at different steps also led to the same mitotic phenotypes. Finally, we showed that addition of uridine completely restored UTP and CTP levels and rescued the mitotic phenotype.

Conclusions: Taken together, our results suggest that part of the toxic effect of AICAR is due to pyrimidine starvation by affecting mitotic processes and hence identified pyrimidine biosynthesis as a potential molecular target for future therapeutics in cancer.

KEYWORDS: de novo pyrimidine pathway, AICAR, mitosis, mitotic checkpoint
Abstract S1.3

Crystal structure and pH-dependent allosteric regulation of human β-ureidopropionase

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Objectives: β-Ureidopropionase (βUP) catalyzes the third step of the reductive pyrimidine catabolic pathway responsible for breakdown of uracil-, thymine- and pyrimidine-based antimetabolites such as 5-fluorouracil. Nitrilase-like βUPs use a tetrad of conserved residues (Cys233, Lys196, Glu119 and Glu207) for catalysis and occur in a variety of oligomeric states. Positive co-operativity toward the substrate N-carbamoyl-β-alanine and an oligomerization-dependent mechanism of substrate activation and product inhibition have been reported for the enzymes from some species, e.g. rat, but not others. We performed studies on human βUP (HsβUP) to investigate to what extent it shares characteristics of allosteric regulation with its orthologue from rat.

Methods: We determined the steady-state kinetics of native HsβUP and analyzed its oligomeric state in absence and presence of reaction substrate and product as well as structurally analogous inhibitors. Furthermore, we functionally characterized mutant HsβUP variants designed to exclusively exist in a defined oligomeric state (dimeric or higher), to further elucidate the link between enzyme activity, oligomerization state and mode of oligomer assembly, and determined the crystal structure of one variant.

Results: Similar to rat βUP, the activity of recombinant HsβUP regulated by substrate and product, but in a pH-dependent manner. Existing as a homodimer at pH 9, the enzyme increasingly associates to form octamers and larger oligomers with decreasing pH. Only at physiological pH is the enzyme responsive to effector binding, with N-carbamoyl-β-alanine causing association to more active higher molecular mass species, and β-alanine dissociation to inactive dimers. Disruption of dimer–dimer interfaces by site-directed mutagenesis generated dimeric, inactive enzyme variants. The crystal structure of the T299C variant refined to 2.08 Å resolution revealed high structural conservation between human and fruit fly βUP, and supports the hypothesis that enzyme activation by oligomer assembly involves ordering of loop regions forming the entrance to the active site at the dimer–dimer interface, effectively positioning the catalytically important Glu207 in the active site.

Conclusions: HsβUP is activated by its substrate NCβA and inhibited by β-alanine, which is associated with ligand-induced changes in oligomerization state, however, only within a relatively narrow window around physiological pH. Whether the pH responsiveness of βUP is of physiological relevance is currently unknown. Nevertheless, it suggests that the mechanism of effector binding-induced association and dissociation involves changes in protonation state, and thus opens up avenues for further investigations into the allosteric regulation of βUP. Incapability to form higher oligomers is synonymous with enzyme inactivation, which is in agreement with our hypothesis that upon substrate binding flexible loops at the active-site entrance become ordered, inducing oligomerization, which in turn stabilizes the catalytically active conformation with Glu207 inserted into the active site.

Relevant references:

KEYWORDS: reductive pyrimidine degradation, allosteric regulation, crystal structure, site-directed mutagenesis, amidohydrolase
Abstract S1.4

An unexpected link between ecto-5'-nuclotidase deficiency and impaired cardiac vitamin B12 metabolism

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Objectives: CD73 is known as enzyme of extracellular nucleotide degradation and adenosine production. Our earlier studies highlighted metabolic alterations in CD73 knock out mice hearts. Recent clinical reports and population studies suggested a potential link between cardiomyopathies and deteriorations in vitamin B12 metabolism. In this study we aimed to investigate the cardiac metabolism of vitamin B12 in CD73 deficient mice (CD73 KO) and their WT littermates.

Methods: We examined CD73 KO mice and their WT littermates. Vitamin B12, transcobalamin I (TCN I), transcobalamin II (TCN II), methymalonic acid (MMA), homocysteine (HCY) serum levels were measured. We investigated also cardiac vitamin B12 concentration and methylmalonyl-CoA mutase and methionine synthase activities. Cardiac levels of receptor for transcobalamin saturated with cobalamin (CD320), lysosomal cobalamin transporter (LMBD1), ATP binding cassette subfamily D member 4 (ABCD4) levels were measured. Blood morphology and mice behavioral analysis (elevated plus-maze test, open field test, locomotor activity, forelimb grip strength measurement) were also performed. Moreover, we investigated the transcobalamin II (TCN II) production and vitamin B12 cellular uptake in lung endothelial cells (LECs) isolated from CD73 KO and WT mice.

Results: Despite normal blood concentration, cardiac vitamin B12 level was decreased in CD73 KO (215.4 ± 5.4 ng/mg tissue in WT; 197.8 ± 4.6 ng/mg tissue in CD73 KO; p=0.02) which led to decreased activity of vitamin B12 dependent enzymes such as methylmalonyl CoA mutase (MMU) and methionine synthase (MS) (MMU: 21.1 ± 4.1 nmol/min/mg tissue in WT; 4.1 ± 0.4 nmol/min/mg tissue in CD73 KO, p=0.002 and MS: 29.97 ± 5.28 in WT; 13.71 ± 3.28 in CD73 KO, p=0.049). Disruption of this pathways lead to an elevated serum MMA (5.6 ± 0.4 ng/ml in WT; 33.2 ± 6.2 ng/ml in CD73 KO, p=0.002) and HCY (5.3 ± 0.4 µM/L in WT; 11.0 ± 0.5 µM/L in CD73 KO, p<0.0001) levels in CD73 KO mice. Furthermore, CD73 KO exhibited typical symptoms of vitamin B12 deficiency such as changes in blood morphology and impaired learning and memory capabilities. Impaired vitamin B12 cellular uptake in CD73 KO was accompanied by decreased levels of lysosomal transcobalamin transporters such as LMBD1 and ABCD4 (4.02 ± 0.11 nmol/min/mg tissue in WT; 3.60 ± 0.11 ng/mg tissue in CD73 KO; p=0.013 in LMBD1 levels and 4.38 ± 0.11 ng/mg tissue in WT; 3.93 ± 0.12 ng/mg tissue in CD73 KO; p=0.015 in ABCD4 levels). Moreover, lower production of TCN II by LECs isolated from CD73KO vs. WT was exhibited (92.54 ± 3.68 ng/ml in WT LECs; 80.46 ±1.28 ng/ml in CD73 KO LECs; p=0.03).

Conclusions: Decreased levels of vitamin B12 lysosomal transporters suggested that CD73 might be one of the protein which is necessary to transport vitamin B12 from lysosome to cytoplasm, nevertheless more studies are needed to clarify this interaction. Furthermore, inactivation of CD73 might be linked with reduced TCN II endothelial synthesis and leads to impaired cellular vitamin B12 uptake. Thus, we suggest that CD73 may play an important role in vitamin B12 intracellular transport and its lower activity may contribute to dysfunction of vitamin B12 dependent pathways even in normal vitamin B12 supply.

KEYWORDS: ecto-5'-nuclotidase, vitamin B12, transcobalamin II, heart, lungs endothelial cells
Reconstitution of human dihydroorotate dehydrogenase for neutron scattering studies

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Human dihydroorotate dehydrogenase (hDHODH) is an integral protein of the inner mitochondrial membrane (IMM) that catalyzes the fourth step of the de novo pyrimidine biosynthesis and is functionally connected to the respiratory chain via its co-substrate, ubiquinone Q\textsubscript{10}. This enzyme is also the target for anti-inflammatory drugs approved for the treatment of rheumatoid arthritis and multiple sclerosis [1]. Mutations in hDHODH have been identified as the cause of Miller syndrome, a rare autosomal recessive disorder resulting in abnormalities of the head, face and limbs. The N-terminus of hDHODH consists of a signal peptide for mitochondrial import, a transmembrane helix domain, followed by a microdomain consisting of two amphipathic alpha-helices which interact with the lipids of the IMM and have been proposed to form the binding site of ubiquinone Q\textsubscript{10} [2, 3]. However, the mechanism by which hDHODH interacts with ubiquinone is still largely unknown.

Objectives: We aim to investigate the mechanisms by which hDHODH interacts with the lipids of the IMM and with ubiquinone Q\textsubscript{10} in a non-crystalline, physiologically relevant membrane-bound state and how mutations found in Miller Syndrome (G19E, E52G and R135C) affect these interactions.

Methods:

- Protein production: Truncated hDHODH (lacking the mitochondrial signal peptide and transmembrane domain) is expressed in \textit{E. coli}, whereas full-length hDHODHs (both wild-type and Miller Syndrome variants) are expressed in both \textit{E. coli} and insect cells. All the proteins are purified by standard chromatographic methods. The characterization of the effects of the mutations on enzymatic activity ($V_{\text{max}}$, $K_m$, $k_{\text{cat}}$) is performed by means of an established chromogenic reduction assay [4].

- Protein-lipid interactions: To characterize how DHODH interacts with lipids of the IMM and with ubiquinone Q\textsubscript{10} in a non-crystalline, physiologically relevant membrane-bound state we use neutron reflectivity (NR). NR is a non-invasive surface scattering technique that can determine the composition and structure of lipid bilayers in the direction perpendicular to the membrane surface at 2-3 Å resolution, and determine the low-resolution structure-profile of embedded membrane proteins using selective deuterium labeling of either the protein or the lipids.

Results:

- **Truncated hDHODH interacts weakly with IMM mimics as indicated by NR.** We have expressed and purified truncated human DHODH from \textit{E. coli} to a specific activity of 105 Units per mg by means of immobilised metal affinity chromatography and gel filtration. The yield of enzyme per liter of culture was typically 3 mg. Quartz crystal microbalance (QCM-D) experiments indicated that the truncated enzyme had only minor affinity towards bilayers consisting only of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). In QCM-D measurements, the interaction of truncated hDHODH was found to be dependent on the presence of at least 10 mol% tetraoleoyl cardiolipin (TOCL). Neutron reflectometry measurements were performed at the Institut Laue-Langevin and at the ISIS Neutron and Muon Source in order to investigate the interaction of the truncated enzyme and bilayers mimicking the IMM (POPC, TOCL and Q\textsubscript{10}) deposited by means of vesicle fusion. NR data suggests that the interaction between the supported bilayers and the protein is weak and transient, both in the presence and absence of Q\textsubscript{10}, as indicated by the rather small changes observed in the reflectivity curves, and the removal of some of the protein by buffer rinsing.

- **Miller Syndrome mutants display varying levels of expression and enzymatic activity in transfected insect cells.** \textit{Spodoptera frugiperda} (S19) and \textit{Trichoplusia ni} (High Five) cells were infected with recombinant baculovirus encoding full-length hDHODH and three Miller Syndrome variants (G19E, E52G, R135C – respective mutations in regions presumably interacting with the IMM). For both cell lines, Western blot analysis of the crude cell lysates indicates that protein expression reaches a maximum 96 h after infection for all constructs except E52G, which peaks at 48 h p.i. Compared to the wild type enzyme, the G19E variant is expressed at significantly lower levels. In both cell lines, the E52G mutant is expressed at even lower levels. On the other hand, the R135C variant displays a similar expression to that of the wild-type, but it displays a much lower activity.

Conclusions: We have expressed and purified a truncated variant of hDHODH from \textit{E. coli} cells. NR measurements indicate that the truncated enzyme lacking the transmembrane domain interacts only weakly and transiently with IMM mimics, suggesting that the mitochondrial signal peptide and transmembrane domains might be a prerequisite for stable binding. We have also expressed full-length hDHODHs (wild-type and three different Miller Syndrome variants) in two insect cell lines. Future experiments will focus on the reconstitution of the full-length enzymes into supported lipid bilayers by means of co-adsorption from mixed detergent-lipid micelles [5].
Reference List:

KEYWORDS: membrane proteins, protein reconstitution, neutron reflectivity, ubiquinone, pyrimidine metabolism
Abstract S1.6

**DCTPP1 preserves genomic integrity through the modulation of dCTP and dTTP homeostasis**

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Objectives: The aim of this work was to gain insight into the role of DCTPP1 in the homeostasis of the dNTP pool and its implication in the preservation of genome integrity.

Methods: We investigated the metabolic function of DCTPP1 by using two different reverse genetics approaches: transiently down-regulating DCTPP1 expression by small interference RNA and using a stable DCTPP1-knockout cell line. The dNTP pool was determined by a DNA polymerase-based assay. Uracil content in the genome was quantified by a qPCR. Phosphorylation of histone H2AX was assessed by immunostaining and cells showing 5 or more foci γH2AX were considered positive. The spontaneous mutation frequency was determined using the gene hypoxanthine phosphoribosyltransferase gene (HPRT) as a marker for nuclear mutagenesis. Resistance to chloramphenicol as a result of mutations in the 16S rRNA gene was used to assess mitochondrial mutagenesis.

Results: The nucleotide pool and the dUTP/dTTP ratio are severely altered in DCTPP1-deficient cells, which exhibit an accumulation of uracil in genomic DNA, an activation of the DNA damage response and a hypermutator phenotype affecting both nuclear and mitochondrial genomes. Notably, DNA damage can be reverted by incubation with thymidine or dUTPase overexpression. Moreover, DCTPP1-deficient cells are highly sensitive to down-regulation of nucleoside salvage.

Conclusions: Our results shed new light on the mechanisms that regulate dNTP homeostasis. We have identified DCTPP1 as a crucial element in de novo synthesis of pyrimidines, providing a novel pathway for the conversion of dCTP into dTTP. In addition we have established that a defect in DCTPP1 causes disturbed nucleotide pools, replication stress and genomic instability.

Relevant references:

KEYWORDS: DCTPP1, DNA damage, genomic integrity, pyrimidine homeostasis, dNTP pool
Abstract S1.7

Extracellular metabolism of NAD+ and NMN on the surface of murine lung endothelial cells

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Objectives: Nicotinamide adenine dinucleotide (NAD+) plays a crucial role in cell energy metabolism and signaling pathways. Little is known about the metabolism and the role of NAD+ outside the cell. Our previous studies have shown that ecto-5'nucleotidase (CD73) is one of the enzymes involved in the catabolism of adenine nucleotides on the surface of the vascular endothelium. CD73 deletion in mice (CD73−/−) may lead to impaired NAD+ and nicotinamide (Nam) metabolism. In this study, we investigate the catabolic pathway of extracellular NAD+ and its derivatives on the surface of CD73−/− lung endothelial cells.

Methods: Murine lung endothelial cells were isolated from CD73−/− and WT mice and cultured in DMEM medium with D-Valine (4.5 g/l glucose (Immuniq), 10% FBS, 15 mg/500 ml ECGS, 2 mM L-glutamine and penicillin-streptomycin) in a 5% CO2 humidified atmosphere at 37°C. For the experiments, cells between two to five passage were used. Cells were seeded in 24-well cultures plates at a density of 2 x10^5 cells/well. Upon reaching 80-100% confluence, the incubation medium was removed and each well was washed three times with PBS pH 7.4. After removal of PBS, 0.5 ml HBBS buffer was added to each well. After 15 min incubation with the specific inhibitor for CD73, NAD+glycohydrolase (CD38), ecto-nicotinamide pyrophosphatase/phosphodiesterase (eNPP), and alkaline phosphatase (ALP), 5 µl 5mM of NAD+, nicotinamide mononucleotide (NMN), Nam, and nicotinamide riboside (NR) were added as a substrate. Samples in the amount of 50 µl were collected after 0, 30, 60, and 120 min of incubation at 37°C, then were centrifuged (14000g/15min/4°C). Metabolic changes were tracked by high-performance liquid chromatography (HPLC).

Results: NAD+ and NMN are catabolized on the surface of murine endothelial cells. However, Nam and NR were not metabolized. Initial experiments with specific inhibitors demonstrated that CD38 had the largest involvement in NAD+ degradation because ADPR and nicotinamide were the main product of its catabolism. The ADPR concentration decreased during the incubation, which may indicate the entry of this compound into the cell. CD73 had a diminished share in the metabolism of NAD+. In the case of NMN, metabolism by CD38 was not observed. On the cells isolated from CD73−/− mice, hydrolysis of NMN to NR was significantly reduced.

Conclusions: Cells isolated from mouse lungs are capable to metabolize extracellular NAD+ and NMN, in contrast to Nam and NR. Pathways mediated by CD38 are the most important role while CD73 are minor. This is different to human aortic valves and mouse vessels which highlights the specific role of NAD+ metabolism in lung vessels.

KEYWORDS: Nicotinamide Adenine Dinucleotide, lung endothelial cells, nucleotide metabolism, NAD+-glycohydrolase, ecto-5'nucleotidase
Targeting SAMHD1 to enhance nucleoside-based cancer therapies

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Objectives: Manipulation of DNA precursor pools to influence genome integrity has long been exploited in cancer therapy\(^1\), and a prime example of this is the use of nucleobase and nucleoside analogues\(^2\). Despite being cornerstones in the treatment of many common cancers, the efficacy of these therapies can vary greatly between patients within the same diagnostic group, and treatment failure can occur through lack of response or relapse. Our current understanding of the metabolism of these drugs cannot fully account for, or predict, patient response, and thus there is a clear need to identify additional factors involved in their metabolism, and to utilise these for the development of new strategies to improve cancer treatments\(^2\). Our research sets out to define the role of the deoxynucleoside triphosphate (dNTP) triphosphohydrolase SAMHD1 in the metabolism of nucleobase and nucleoside analogues, and determine whether this could be exploited therapeutically to enhance the efficacy of these therapies.

Methods: A multidisciplinary approach is employed in our research, combining in vitro biochemical and biophysical assays, high-throughput screening of small molecule libraries and subsequent chemical probe development, experiments in cancer cell models, in vivo xenograft cancer mouse models, and ex vivo experiments with primary patient material.

Results: We identified the active metabolite of the deoxycytidine analogue cytarabine (ara-C), a critical drug in the treatment of acute myeloid leukaemia (AML), as a SAMHD1 substrate. We demonstrated that removal of SAMHD1 in various AML models dramatically increases the efficacy of ara-C therapy, and in accordance, that ara-C-treated AML patients with low versus high SAMHD1 levels have a significant survival advantage\(^3\). Continuing from this study, we subsequently demonstrated that many other clinically used nucleoside analogues are subject to SAMHD1 control in cancer cells\(^4\), altogether highlighting SAMHD1 as a potential biomarker and therapeutic target\(^5\). In our current work, we seek to target SAMHD1 with small molecules to enhance the efficacy of nucleoside-based cancer therapies, in particular ara-C therapy in AML, and have conducted several high-throughput screening campaigns, yielding a collection of promising candidates currently under evaluation. With one of these molecules, identified from a phenotypic screen, we can sensitise AML cell and xenograft mouse models to ara-C therapy, in addition to patient material in ex vivo experiments.

Conclusions: SAMHD1 controls the therapeutic response of AML to ara-C and is thus a potential biomarker and promising therapeutic target, which can be exploited with small molecules.

Relevant references:
1. Rudd et al. (2016) Pathways controlling dNTP pools to maintain genome stability. DNA Repair.
5. Rudd et al. (2017) SAMHD1 is a barrier to antimetabolite-based cancer therapies. Molecular and Cellular Oncology.

KEYWORDS: Cancer, SAMHD1, cytarabine (ara-C), acute myeloid leukaemia (AML), precision medicine
Abstract S2.2
Sustained effectiveness, safety and therapeutic drug monitoring of thioguanine in a cohort of 274 Inflammatory Bowel Disease patients, intolerant for conventional therapies

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Objectives: Thioguanine has been considered as an alternative drug in patients with inflammatory bowel disease (IBD) who failed prior conventional immunomodulating therapy. In this study, we report on effectiveness, safety and therapeutic drug monitoring (TDM) data of a real-life cohort of patients with prolonged thioguanine maintenance therapy.

Methods: In this nationwide, multicentre study, medical records of thioguanine using IBD patients were retrospectively reviewed. Patient and drug characteristics together with effectiveness and safety profile of thioguanine therapy were registered. Response to therapy was defined as clinical effectiveness, without (re)initiation of corticosteroids, concurrent biological therapy or surgical intervention. All adverse events that occurred during the entire follow-up were listed and graded according to the common terminology criteria (CTC).

Results: In total, 274 patients (female 63%, Crohn’s disease in 68%) were included with median treatment duration of 51 months, 1567 patient-years of follow-up and median 20 mg/day thioguanine dosage. Thioguanine treatment was tolerated in 79%, clinical effectiveness at 6 months was documented in 66% and sustained clinical effectiveness during 12 months in 51% of patients. About 40% of patients developed adverse events, of which 5% were graded as severe. Adverse events comprised infection requiring hospitalization in 3 and skin cancer in 8 patients (two melanomas). Asymptomatic nodular regenerative hyperplasia of the liver occurred in two out of 52 patients with liver biopsies (3.8%), and portal hypertension in three patients whereof one potentially associated with thioguanine (0.4%). Clinical effectiveness was correlated with 6-thioguaninenucleotide threshold concentrations of > 682 pmol/8×10\textsuperscript{8} RBC (p<0.05).

Conclusions: Long-term thioguanine therapy of at least 12 months was effective in 51% and well tolerated as a maintenance treatment for IBD in about 70% of patients. Adverse events were common, but mainly mild (65%) or moderate (30%). 6-thioguaninenucleotide threshold concentration ≥ 700 pmol/8×10\textsuperscript{8} RBC is proposed as target level with higher odds for clinical effectiveness.

Relevant references:

KEYWORDS: Thiopurines; Thioguanine; Therapeutic Drug Monitoring; 6-thioguaninenucleotide
Objectives: Bone alterations have been observed in the course of HIV infection, with a notable decrease in bone mineral density (BMD) and fractures due to fragility. Anti-retroviral drugs may have a direct or indirect effect on bone cells, via alterations in RANK/RANKL, cytokines profile, mitochondrial function and changes in phosphate/Vitamin D metabolism. The aim of this study was to evaluate the deleterious effects in bone metabolism and systemic inflammation, produced by Tenofovir vs. other HIV treatment in naïve patients.

Methods: A cohort of 114 HIV-naïve patients were included in the study. Patients were separated by treatment: 1) Tenofovir Disoproxil Fumarate (TDF) (n=23), 2) Tenofovir Alafenamide (TAF) (n=22), 3) Abacavir/Dolutegravir/Lamivudin combo (ADL) (n=39), 4) Protease Inhibitors (PI) (n=12), and 5) patients who changed treatment during the study (n=18). Epidemiological, immunological, and metabolic parameters, as well as BMD were evaluated. Bone markers, proinflammatory and anti-inflammatory cytokines were analyzed in serum at basal and 3 and 12 months post-treatment by MILLIPLEX® MAP Luminex® Technology. The diagnosis of osteopenia/osteoporosis was made according to the WHO criteria.

Results: The mean age was 34.7 years (range 19-50 years). 91% was on CDC stage A. The median CD4 was 481 cell/µL (IQR=339.5), 10% had CD4 under 200 cell/µl, and 42% had CD4/CD8 under 0.4. 71% (71/143 p) had low Vitamin D levels, 4% low BMI (<18.5). Osteopenia (op) or osteoporosis (OP) was found in 53% and 11% respectively. In the serum we found differences at molecular level among different treatments (Tables 1 and 2). We observed that both TDF and TAF presented a resortive profile but not the other treatments starting 3 months after treatment initiation. All treatments reduce proinflammatory cytokines 3 months after treatment but no differences among treatments were found.

Conclusion: HIV-naïve patients under 50 years have a high prevalence of osteopenia/osteoporosis, and patients treated with Tenofovir had greater bone deterioration than other patients.

KEYWORDS: HIV, tenofovir, osteopenia, inflammation.
Table 1: Bone markers in pg/ml. Data is expressed as mean±S.E.M.

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<tr>
<td></td>
<td>12 m</td>
<td>1344±110</td>
<td>1344±110</td>
</tr>
<tr>
<td><strong>SOST</strong></td>
<td>Basal</td>
<td>1344±110</td>
<td>1344±110</td>
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<tr>
<td></td>
<td>3 m</td>
<td>1344±110</td>
<td>1344±110</td>
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<tr>
<td></td>
<td>12 m</td>
<td>1344±110</td>
<td>1344±110</td>
</tr>
<tr>
<td><strong>DDK</strong></td>
<td>Basal</td>
<td>1344±110</td>
<td>1344±110</td>
</tr>
<tr>
<td></td>
<td>3 m</td>
<td>1344±110</td>
<td>1344±110</td>
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<tr>
<td></td>
<td>12 m</td>
<td>1344±110</td>
<td>1344±110</td>
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Change from Baseline: 3 m = 1344±110, 12 m = 1344±110

Note: All values are expressed as mean±S.E.M. for each time point.
<table>
<thead>
<tr>
<th></th>
<th>IL2</th>
<th>INFg</th>
<th>IL4</th>
<th>IL10</th>
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<tbody>
<tr>
<td>Tto.</td>
<td>Basal</td>
<td>3 m</td>
<td>12 m</td>
<td>Basal</td>
</tr>
<tr>
<td>TDF (n=23)</td>
<td>2.3±0.44</td>
<td>2.24±0.38</td>
<td>2.39±0.35</td>
<td>32±10.45</td>
</tr>
<tr>
<td>TAF (n=22)</td>
<td>1.53±0.16</td>
<td>1.9±0.20</td>
<td>2.35±0.29</td>
<td>5.71±0.74</td>
</tr>
<tr>
<td>ADL (n=19)</td>
<td>2.5±0.33</td>
<td>2.1±0.26</td>
<td>2.5±0.28</td>
<td>35.6±9.72</td>
</tr>
<tr>
<td>PI (n=12)</td>
<td>2.11±0.19</td>
<td>1.94±0.33</td>
<td>2.97±0.5</td>
<td>5.7±0.72</td>
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<tr>
<td>Change of treatment (n=18)</td>
<td>1.91±0.18</td>
<td>2.14±0.23</td>
<td>2.71±0.41</td>
<td>7±0.71</td>
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<tr>
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<th>TNFa</th>
<th>IL6</th>
<th>IL8</th>
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<td>Tto.</td>
<td>Basal</td>
<td>3 m</td>
<td>12 m</td>
<td>Basal</td>
</tr>
<tr>
<td>TDF (n=23)</td>
<td>1±0.27</td>
<td>1.1±0.26</td>
<td>0.89±0.30</td>
<td>3.4±0.71</td>
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<td>TAF (n=22)</td>
<td>0.54±0.08</td>
<td>0.62±0.08</td>
<td>0.54±0.06</td>
<td>1.7±0.24</td>
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<tr>
<td>ADL (n=19)</td>
<td>1.24±0.25</td>
<td>0.91±0.14</td>
<td>0.80±0.15</td>
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<tr>
<td>PI (n=12)</td>
<td>0.59±0.07</td>
<td>0.72±0.14</td>
<td>0.67±0.09</td>
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</tr>
<tr>
<td>Change of treatment (n=18)</td>
<td>0.67±0.07</td>
<td>0.73±0.08</td>
<td>0.58±0.1</td>
<td>2.45±0.27</td>
</tr>
</tbody>
</table>

Table 2: Cytokines (pg/ml). Data is expressed as mean ± S.E.M.
Inhibition of Membrane Bounded Ecto-5′-nucleotidase (CD73): A Novel Target for Treatment of Cervical Cancer

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Objectives: Ecto-5′-nucleotidase (CD73) is a membrane bounded protein, belongs to metallophosphoesterases family, widely distributed among vertebrates, plants and even in bacteria. CD73 exhibits much diverse affinity for naturally occurring substrates. Primarily, CD73 catalyze the hydrolysis of AMP into a phosphate group and a corresponding ribo-nucleoside such as adenosine. In purinergic signaling cascade, this enzyme terminates the activities P2X and P2Y receptors and provide adenosine as an agonist for P1 receptors. Adenosine is known to be involved in various patho-physiological conditions such as anti-inflammatory, anti-nociceptive, vasodilatory, anti-diuretic and immunosuppressive activities. Ecto-5′-nucleotidase has been reported to play a tumor promoting function, thus its inhibition proved to be a potential therapeutic role against cancer especially gliomas, melanomas and breast cancer.

Methods: For this purpose, the inhibitory activities of synthesized compounds were explored by malachite green assay. Furthermore, the effect of inhibitors on expression level of desired enzyme was investigated by real time polymerase reaction and western blotting. Cell cycle and cell surface apoptosis analysis was carried out using flow cytometry. To determine mechanism of enzyme inhibition, enzyme kinetics experiments were performed. The physico-chemical properties of screened inhibitors were further examined and analyzed by molecular docking and dynamics studies.

Results: After evaluation of potential inhibitors of ecto-5′-NT by malachite green assay, the inhibitors were explored for their effects on the cancer progression and it was observed that ecto-5′-NT positively affects the cancer progression. Analysis of mRNA transcription of ecto-5′-NT in HeLa cell lines was confirmed by RT-PCR. Moreover, protein expression of the Ecto-5′NT/CD73 was done by Flow Cytometry to approve the presence on the cell surface. The results confirmed the data obtained from RT-PCR. At the end, the docking analysis of potential inhibitors was subjected to molecular dynamics and simulation studies to determine the key interactions.

Conclusions: It can be suggested that the ecto-5′-NT is a significant target for regulating HeLa cells progression, and thereby suggesting an innovative diagnostic target for beneficial intervention.

KEYWORDS: Purinergic signaling, ectonucleotidases, cervical cancer cells (HeLa), cell-cycle arrest.
CD73 is a member of 5'-nucleotidases that is overexpressed in several cancers.\textsuperscript{1} It is a cell surface anchored enzyme catalyzing the irreversible hydrolysis of extracellular adenosine-5'-monophosphate (AMP) into adenosine (Ado) and inorganic phosphate.\textsuperscript{2} Then, adenosine is able to interact with several adenosine receptors (A1, A2a, A2b and A3) expressed in tumor microenvironment, thus inducing an immunosuppressive effect leading to tumor cells growth and proliferation.\textsuperscript{3,4}

So far, many analogues of adenosine diphosphate (ADP) have been described as potent inhibitors. However, these compounds suffer from poor bioavailability and low metabolic stability.

Herein, we will present the synthesis and the inhibitory activities of new substrate analogues as potential CD73 inhibitors.

Relevant references:

KEYWORDS: adenosine, nucleotid, substrate analogues, inhibitors, CD73
Abstract S2.6

**Drug discovery and optimization targeting CD73 to restore anticancer immune response**

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\textsuperscript{b} Institut des Biomolécules Max-Mousseron (IBMM), CNRS, Univ. Montpellier, ENSCM, Montpellier, France.

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Objectives: Ecto-5'-nucleotidase also known as CD73 is a dimeric enzyme attached to the extracellular cell surface and regulates the pool of circulating AMP and adenosine (ADO). The AMP hydrolysis reaction is regulated in concert with the upstream CD39 that catalyzes the ATP conversion in AMP. CD73 is primarily expressed on both immune and cancer cells with overexpression often observed in tumor cells (for instance, breast cancers). This aberrantly high concentration of CD73 promotes an increase in circulating ADO and as a consequence, ADO induces a strong immunosuppressive response which favors tumor growth and metastasis. To overcome this problem and to restore the immune response, we applied a drug development program to block irreversibly the CD73 enzymatic activity by using bioinformatics approaches. The original idea was to block the enzyme dynamic and its conformational changes occurring during the reaction and therefore its enzymatic activity.

Methods: Using an innovative approach which included molecular dynamics simulations and virtual screening, we first explored the conformational space used by CD73 during active site formations prior AMP hydrolysis (requiring large concerted domain motions) to appreciate the functional dynamics of this enzyme. Several conformations taken from the molecular dynamics simulations were used to identify new druggable binding sites different to that of the substrate. An undisclosed allosteric binding site located at the dimerization interface was targeted to find new and selective inhibitors against CD73. This step was achieved by virtual screening using commercially available chemical libraries. Hit compounds were further evaluated by inhibition kinetics assays using the purified recombinant enzyme and lead molecules were optimized by chemoinformatics approaches such as 3D-pharmacophore to improve their efficacy.

Results: From dynamics simulations, a new allosteric cavity was identified at the dimerization interface and used as target site to discover new and selective CD73 inhibitors by screening 320,000 chemical compounds. Among the dozen of active hits, two compounds were highly promising with an inhibition constant below the micromolar range and both presented a non-competitive inhibition mode. This result was important to corroborate the initial hypothesis of an allosteric inhibition mechanism. Using 3D-pharmacophore models based on the most active compounds, we extended our screening to other chemical libraries such NCI, CN-CNRS and MolPort database in order to find optimized compounds (based on the physicochemical properties of first hits) with a final goal being to end up with more potent drug candidates showing high selective and bioavailability.

Conclusions: Two families of lead compounds have been identified and inhibit CD73 activity by interfering with its functional dynamics. The inhibitors are targeting a different site to that of the substrate binding site which should improve their selectivity (no off-target effects). The leads have been optimized by chemoinformatics and will be synthetized and evaluated in model cell lines to determine their potency to restore anticancer immune response.

Relevant references:

KEYWORDS: drug design; virtual screening; molecular dynamics; cancer therapy; allosteric inhibitors
Sonoporation and gemcitabine delivery in pancreatic cancer cell lines

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Corresponding author: tormod.karlsen.bjanes@helse-bergen.no

Objectives: The dense desmoplastic stroma in pancreatic ductal adenocarcinoma (PDAC) is a limiting factor for local drug delivery and contributes to the poor prognosis in PDAC patients. Sonoporation combines ultrasound and microbubbles to increase the permeability of biological barriers. Gemcitabine combined with sonoporation has previously been shown to inhibit PDAC tumour growth in mice and displayed promising effect in a phase I clinical trial (1). The aim of this study was to assess the effect of sonoporation on cellular gemcitabine delivery in an in vitro PDAC model system.

Methods: The PDAC cell lines BxPC-3, MIA PaCa-2 and PANC-1, were cultured in hypoxic bioreactors (Petaka G3™ LOT) to mimic in vivo intratumoral conditions. Cells were incubated for 60 minutes with gemcitabine (dFdC) either with or without the membrane transport-inhibitor dilazep, and exposed to clinically applicable ultrasound intensities and Sonazoid® microbubbles. Intracellular concentration of the active triphosphate metabolite dFdCTP, and extracellular concentration of the inactive deaminated metabolite dFdU, were measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) (2,3). Concentrations of dFdCTP and dFdU in each cell line treated with gemcitabine alone were used as controls for the adjuvant experimental interventions, dilazep and sonoporation.

Results and Discussion: In gemcitabine-treated BxPC-3 without dilazep, extracellular dFdU increased up to 150 % of control following sonoporation, suggesting that cell membranes had been permeabilized. No change in dFdU concentrations was seen in MIA PaCa-2 and PANC-1; both of which are known to have lower deamination enzyme activity of gemcitabine to dFdU. Intracellular dFdCTP did not increase significantly following sonoporation in any of the three cell lines treated with gemcitabine without dilazep, possibly indicating that metabolic activation of gemcitabine was already saturated. In gemcitabine- and dilazep-treated BxPC-3, extracellular dFdU was reduced to 10 % of control, which increased to 40 % following sonoporation, indicating a partial restoration of membrane transport with sonoporation. In MIA PaCa-2 and PANC-1, dFdU concentrations were already low in controls, and no change could be detected with dilazep. Intracellular dFdCTP was reduced to 12, 32 and 14 % of controls in gemcitabine- and dilazep-treated BxPC-3, MIA PaCa-2 and PANC-1, respectively, which was considered to reflect suppressed uptake of gemcitabine. In cells with reduced gemcitabine uptake, sonoporation induced a significant increase in dFdCTP up to 54 and 29 % of controls in MIA PaCa-2 and PANC-1, respectively, whereas in BxPC-3 no change in dFdCTP was seen. The latter may be explained by a high intracellular turnover to dFdU that may have masked an increased gemcitabine uptake.

Conclusions: Our findings indicate that sonoporation increased gemcitabine transmembrane transport in all three cell lines, but that preexisting transporters and intracellular enzyme activities are the predominant determinants of gemcitabine uptake and metabolism. Our findings encourage further studies into the use of additional interventional tools to support the effect of sonoporation.

References:

KEYWORDS: gemcitabine, pancreatic cancer, membrane transport, sonoporation
Targeting NUDT15 to Improve the Anticancer Efficacies of Nucleoside Analogue Antimetabolites

Simin Zhang, Thomas Hellday et al.

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Objectives: Previously, Nudix hydrolase 15 (NUDT15) has been shown to exhibit pyrophosphohydrolase activity against the tri-phosphorylated cytotoxic metabolites of thiopurines, and thereby limiting their anti-cancer treatment outcomes\(^1-^3\). Here, we aimed to improve the therapeutic efficacies of these NUDT15 nucleoside analogue substrates through targeting the enzymatic activity of NUDT15 using in-house developed, small molecule inhibitors\(^4\).

Methods: We have developed a library of novel small-molecule NUDT15 inhibitors, and further characterized their in vitro and in vivo efficacies in inhibiting NUDT15 enzymatic activities and in potentiating thiopurine efficacies. Specifically, our inhibitor development and characterization funnel progressively involved 1) biochemical high-throughput screening (HTS) utilizing enzyme-coupled malachite green assays; 2) in-cell target engagement; 3) resazurin cell viability assay-based HTS for evaluating potential synergy with thiopurine; and lastly, 4) in vivo AML/ALL xenograft animal models.

Results: Our in-house developed NUDT15i with nanomolar biochemical potency substantially promoted intracellular accumulation and incorporation of thiopurine active metabolites, and consequently potentiated the thiopurine-induced killing of AML/ALL cell lines by ~10-fold.

Conclusions: Altogether, our data demonstrate that targeting NUDT15 with small molecule inhibitors is a promising strategy to increase the efficacy of NUDT15 antimetabolite substrates, which could potentially improve current cancer therapeutic regimens.

Relevant references:

KEYWORDS: Nucleoside analogue, NUDIX enzyme, small molecule inhibitor, ALL/AML
Abstract S3.1

Mapping and targeting pyrimidine nucleotide biosynthesis and utilization in KRAS driven pancreatic cancer

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Rationale and Objectives: Pancreatic ductal adenocarcinoma (PDAC), an aggressive malignancy with increasing incidence and the lowest survival of any human cancer, urgently requires better therapies. Mutant KRAS, PDAC’s principal oncogenic driver, orchestrates extensive metabolic adaptations required to sustain the increased biosynthetic demands of continual unregulated proliferation. Amongst these, increased nucleotide biosynthesis may be especially important since it supports key processes essential for tumor growth and survival including, but not limited to, RNA and DNA synthesis. PDAC cells are traditionally thought to generate their pyrimidine ribonucleotides primarily via the de novo pathway. However, the disappointing clinical experience in PDAC and other cancers with inhibitors of de novo enzymes suggest a more complex picture. The failure of de novo pathway inhibitors to control PDAC growth in patients may be explained by the existence of alternate pyrimidine biosynthetic pathways capable of compensating for insufficient de novo nucleotide biosynthesis. Indeed, our data show that PDAC cells adapt to de novo pathway inhibition by upregulating two adaptive mechanisms: a nucleoside salvage pathway, which captures extracellular pyrimidines and converts them to nucleotides, and a nucleotide recycling pathway likely fueled by products of ribosomal RNA turnover. Our long-term goal is to determine how KRAS inhibition impacts these convergent pyrimidine biosynthetic pathways, and whether these effects can be amplified by combination therapies. The recent development of potent KRAS G12C inhibitors such as ARS-1620, AMG 510 and MRTX849 coupled with our highly refined and precise methods to measure nucleotide metabolism provides an unprecedented opportunity to explore the interrelationships between pyrimidine biosynthetic pathways and KRAS signaling in PDAC and use this information to design new combination therapies for future translational evaluation. In this presentation I will describe initial findings from our work to achieve the following objectives: (i) to quantify the impact of KRAS inhibition on de novo and salvage pyrimidine biosynthesis; (ii) to map the molecular steps in the lysosomal nucleotide recycling pathway upregulated in PDAC cells by KRAS inhibition; and (iii) to design new combination therapies that amplify the effects of KRAS inhibition on pyrimidine biosynthesis via the de novo, salvage and recycling pathways.

Methods: We have generated novel human and murine models for KRAS G12C driven PDAC and characterized their responses to pharmacological KRAS inhibition using an integrated proteomic/phosphoproteomic/metabolomic mass spectrometry platform.

Results: Preliminary data obtained in multiple KRAS G12C PDAC models show that direct KRAS inhibition downregulates pyrimidine biosynthesis via both de novo and salvage pathways. However, these effects are incomplete due to residual MYC and PI3K activity in the face of KRAS inhibition and may also be counteracted by the upregulation of nucleotide recycling from increased lysosomal RNA turnover.

Conclusions: Direct pharmacological inhibition of mutant KRAS leads to a semi-quinocesent state, as clonal regrowth emerges when PDAC cells are released from the drug. Importantly, nucleotide pools are significantly reduced as are key enzymes involved in the de novo and salvage pathways. In contrast, autophagy and ribophagy are increased suggesting that recycling of nucleotides may be an essential mechanism to maintain cellular viability. These data support our central hypothesis that the ability of PDAC cells to avoid catastrophic nucleotide insufficiency following KRAS inhibition is an important resistance mechanism that helps them maintain their viability and clonogenic potential. In summary, we suggest that an improved understanding of how PDAC cells reprogram their nucleotide biosynthesis and utilization following KRAS inhibition will guide the development of new combination therapies that may potentiate this critical metabolic process in tumor cells, without affecting the nucleotide supply of normal tissues.

Relevant references:


KEYWORDS: pancreatic ductal adenocarcinoma, KRAS, pyrimidine nucleotide biosynthesis, lysosome, RNA turnover.
Objectives: miRNAs are small non-coding RNAs which can regulate the expression of their target genes. miR-17 family is altered in cancer and it is described as oncomiR, so there is evidence that miR-17 family is important during carcinogenesis. Human Concentrative Nucleoside Transporter 1 (hCNT1) is important in anticancer therapies, not only for their ability to translocate nucleoside-derivatives drugs, but also for their role as a transceptor being important on cell physiology. It has been demonstrated that hCNTs are decreased in some cancers. Here, we address the possibility that hCNT1 loss of expression can be related with the aberrant expression of miR-17 family in cancer. The aim of the project was to assess if miR-17 family regulates hCNT1, and if this regulation plays a role on carcinogenesis.

Methods: Different bioinformatics databases were used to determine candidates of miR-17 family to bind the 3’UTR of hCNT1. RT-PCR was performed to analyze the hCNT1, hCNT2 and candidate miRNA expression in paired clinical samples of CRC, PDAC, HCC and their surrounding non-malignant tissue. miRNA target validation of hCNT1 was determined by luciferase activity assays of 3’-UTR. The relationship between miR-17 family and hCNT1 expression was tested by miRNA family modulation in 2D (monolayer) and 3D (spheroids) cell cultures. Spheroids growth was analyzed following the same miRNA experimental conditions.

Results: Analysis of paired clinical samples showed a decrease of hCNT1 expression in colorectal cancer (CRC), pancreatic ductal adenocarcinoma (PDAC) and hepatocarcinoma (HCC) and an increase of some members of miR-17 family. hCNT1 was described for the first time as a direct target of some members of miR-17 family by luciferase assay. miRNA mimics or silencing modulate hCNT1 expression in 2D and 3D cell culture of CRC and PDAC, also affecting spheroid growth. The analysis of miR-17 family in clinical samples demonstrated a significant negative correlation with hCNT1 reinforcing in vitro results.

Conclusions: This study elucidates the relation between miR-17 family and hCNT1 loss during carcinogenic process. The fact that hCNT1 is regulated by specific members of miR-17 oncomiR family reinforces the role of hCNT1 loss during oncogenic process.

Relevant references:

KEYWORDS: miRNA, concentrative nucleoside transporter 1, carcinogenesis
Abstract S3.3

cN-II in the metabolic and proliferative profile of human lung tumor cell line NIC-H292

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Objectives: Intracellular purine homeostasis is maintained by a purine cycle in which the regulated member is a cytosolic 5'-nucleotidase II (cN-II) which generates nucleosides from IMP and GMP (1). cN-II activity is dependent on energy charge, and on a number of phosphorylated compounds. Its expression is particularly high in proliferating cells, indeed high cN-II activity or expression in hematological malignancy has been associated to poor prognosis and resistance to chemotherapeutic drugs (2-4). Furthermore, in a human lung carcinoma cell line A549, the decrease of cN-II expression is followed by a reduction of cell proliferation, mobility and fermentative metabolism (5). We intend to clarify the molecular mechanisms connecting cN-II expression and metabolic regulation.

Methods: We utilized a human lung mucoepidermoid carcinoma cell line (NCI-H292), partially silenced for the enzyme. We measured nucleotide content, proliferation, reduced glutathione, migration and viability, activity of enzymes involved in glycolysis and Krebs cycle and lactate production. Finally, we performed immunoblot analysis of AKT, AMPK, p53 and p21.

Results: Our results demonstrate that high cN-II expression is associated with a glycolytic, highly proliferating phenotype, while partial silencing is associated with a reduction of proliferation and migration ability, and an increase of oxidative performances. Immunoblotting analysis demonstrated that cN-II silencing is associated with a decrease of AKT phosphorylation, an increase of AMPK activation and p53 and p21 expression. We are now attempting to verify if there is a causal relationship between p53 overexpression and all the effects related to cN-II silencing.

Conclusion: Work is in progress to demonstrate, by the use of specific inhibitors, whether the metabolic and proliferative alterations caused in NIC-H292 cell line by cN-II silencing are caused by p53 activation. Presently, the molecular mechanism correlating cN-II expression and p53 activation can be only hypothesized.

Relevant references:

KEYWORDS: lung tumor, cN-II, p53, p21, cell proliferation
Abstract S3.4
Dexamethasone may decrease the efficacy of gemcitabine-cisplatin but not of cytarabine; role of deoxycytidine kinase

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Corticosteroids are frequently used in the treatment of cancer, either to increase the anticancer effect (leukemia) or to protect against gastro-intestinal toxicity (dexamethasone for platinum regimens). Earlier we demonstrated that dexamethasone (DEX) decreased gemcitabine sensitivity of wild-type SW1573 non-small lung cancer cells 23-fold, but not in MRP1 overexpressing cells; however in these cells the combination of verapamil (an inhibitor of MRP1) and DEX decreased the effect of gemcitabine. The effect of DEX was associated with a decreased activity of deoxycytidine kinase (dCK) and thymidine kinase 2 (TK2) which play a role in activation of both gemcitabine and cytarabine (ara-C). Therefore we investigated the effect of DEX on the in vivo sensitivity of acute myeloid leukemia (AML) to ara-C in the Brown Norway Myeloid Leukemia (BNML) rat model for AML, and of gemcitabine-cisplatin sensitivity of the Lewis Lung Carcinoma (LLC) in the mouse in relation to the effects on dCK.

The antileukemic effect was evaluated as survival of the rats and by FACS analysis of circulating leukemic cells, and the antitumor activity as the ratio of the tumor size of treated/untreated mice. Enzyme activity was measured in rat liver and spleen (consisting of BNML cells), 24 hr after treatment with ara-C or DEX with radioactive deoxycytidine (CdR) as a substrate.

Treatment with ara-C increased life-span of BNML with 210%, which was not affected by DEX. However, DEX decreased the sensitivity to the gemcitabine-DEX combination and to the gemcitabine-cisplatin combination-DEX (T/C increased from 0.11 to 0.35) in LLC. However, DEX decreased weight loss. In the liver of BNML bearing rats DEX increased dCK activity 120%, but ara-C decreased dCK activity slightly (93%), but in the combination of ara-C and DEX the effect on dCK was neutralized. In leukemic spleen cells DEX increased dCK activity 140%, but DEX and ara-C did not affect dCK activity. In LLC gemcitabine (with or without DEX) did not affect the dCK activity, but cisplatin with DEX increased the activity to about 225%; in the triple combination this effect was 158%. However, in the liver of LLC mice, both gemcitabine and cisplatin increased dCK to 162% and 195%, respectively, but DEX itself decreased dCK 23%, in the triple combination the effect was 130%.

In conclusion; in the rat model for AML the corticosteroid DEX did not affect the antileukemic effect of ara-C, nor the dCK activity. However, in the mouse model DEX decreased sensitivity to gemcitabine and gemcitabine-cisplatin possibly because DEX decreased dCK activity in the triple combination. However, DEX protected against toxicity.

KEYWORDS: deoxycytidine kinase, cytarabine, gemcitabine, acute myeloblastic leukemia, non-small cell lung cancer
The expression and activity of thymidine kinase 1 and deoxycytidine kinase is modulated by hydrogen peroxide and nucleoside analogs

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Objectives: Thymidine kinase 1 (TK1) and deoxycytidine kinase (dCK) are responsible for the activation of thymidine analogs such as 5FdU and TFT and cytidine analogs e.g. dFdC and araC used in antiviral and anticancer therapies. Many anticancer drugs cause oxidative stress, for example, doxorubicin treatment led to significant elevation of oxidative stress marker e.g. increased ROS levels and lipid peroxidation. Nucleoside analogs are also known to produce ROS and oxidative stress, e.g. AraC treatment causes substantial increases in ROS production both in non-proliferating cells and leukemic cells. Our earlier studies have shown that oxidative stress induced down regulation of both thymidine kinase 2 and deoxyguanosine, however, it is not know if oxidative stress affects TK1 and dCK expression and activities. In this study we investigate the effects of hydrogen peroxide, nucleoside analogs and chemotherapeutic agent on the expression and activity of TK1 and dCK.

Methods: The human osteosarcoma cell line (U2OS) was used in this study. The activity and protein levels of TK1 and dCK were measured in cell extracts by using radiochemical methods and western blot analysis after treatment with hydrogen peroxide, nucleoside analogs or doxorubicin.

Results: Treatment with hydrogen peroxide resulted in induction of TK1 protein synthesis. However, the large increase in TK1 protein level did not correlated with TK1 activity, suggesting that the induced TK1 protein was inactive. The level of dCK protein, however, was not affected by hydrogen peroxide concentration up to 4 mM and decreased only at high concentrations (10 mM). Treatment with TFT or 5FdU resulted in down regulation of both TK1 and dCK. However, addition of araC and dFdC resulted in increased dCK protein level but decreased dCK activity, indicating that part of the induced dCK protein was inactive. However, both TK1 protein and activity were upregulated after araC and dFdC treatments. Doxorubicin treatment led to upregulation of the TK1 but downregulation of dCK concentrations as shown previously.

Conclusions: Both TK1 and dCK expression and activity is apparently affected by oxidative stress and the presence of nucleoside analogs. These results demonstrate the importance of characterizing the expression and activity of TK1 and dCK during chemotherapy with thymidine and deoxycytidine analog in order to enhance their efficacy. The mechanism behind these changes are most likely both dependent on cell cycle as well as post-translational processes.

References:

KEYWORDS: Thymidine kinase 1, deoxycytidine kinase 1, nucleoside analogs, oxidative stress, doxorubicin
Abstract 4.1

Cardiac mitochondrial nucleotide pool and its role in the post-ischemic adenosine production

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Objectives: Myocardial adenosine release and other purine production decrease in the course of repeated ischemic events. However, whether the mechanism of this relates purely to altered energy metabolism or other changes such as depletion of nucleotide pool has not been clarified. In this study we evaluated the relationship between repeated ischemic events and intracellular high energy phosphate concentrations in mitochondria and mitochondria function in control and nucleotide pool replenished hearts before, during and after repeated ischemic intervals.

Methods: An isovolumic preparation of the rat heart perfused at constant pressure was used. Hearts were subjected to one of three protocols: in ischemic group (I) hearts were subjected to 1 min of global (37 °C) ischemia after 40 min of perfusion, 10 min of global (37 °C) ischemia after 50 min of perfusion and to 1 min ischemia after 85 min of perfusion. In ischemic + adenosine group (I+Ado) a similar protocol was used but with an infusion of 30 µM adenosine during the first 15 min of reperfusion after 10 min ischemia. In the control group without 10 min ischemia (C), hearts were subjected three times to 1 min ischemia at 40, 60 and 85 min of perfusion. At the end of experiments, hearts were freeze-clamped or heart apexes were cut off, mitochondria isolated and analyzed for the activity and ATP with related metabolite concentration by HPLC.

Results: Mitochondrial ATP, ADP and total purine nucleotides (TAN) concentration were decreased in I group when compared to the C, I+Ado and initial concentration heart samples. ATP, ADP and TAN were respectively: 0.56±0.1\textsuperscript{*}, 1±0.2\textsuperscript{#} and 2.61±0.2\textsuperscript{*} mol/dry weight in the I group; 1.1±0.1, 2.48±0.2 and 4.84±0.7 mol/dry weight in the initial concentration group; 0.91±0.1, 2.17±0.3 and 3.92±0.9 mol/dry weight in the C group and 0.81±0.1, 1.51±0.2 and 3.17±0.3\textsuperscript{#} mol/dry weight in the I+Ado (mean±SEM, (n=5-9). \textsuperscript{*}: p<0.05 vs. initial concentration. \textsuperscript{#}: p<0.05 vs. no 10 min ischemia, \textsuperscript{&}: p<0.05 vs. 10 min ischemia). No such effects were observed in concentrations of AMP, NAD and ADPR. Oxygen consumption rate in the complex I of isolated mitochondria was increased in the I+Ado group in comparison to the initial concentration, C and I groups of rat hearts.

Conclusions: These results shows that although changes in energy metabolism are key factor which regulate adenosine and other purine production in the heart even small change in nucleotide pool and its redistribution between mitochondria and cytosol considerably affects purine production without changes in energy metabolism. This may have important implications for pharmacological regulation of the endogenous adenosine production.

Acknowledgements: MN – 01-0349/08/261

KEYWORDS: adenosine, ischemia, mitochondria, ATP, nucleotide catabolites
Abstract 4.2

Nucleotide pools dictate the identity and frequency of ribonucleotide incorporation in mitochondrial DNA

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Objectives: Previous work has demonstrated the presence of ribonucleotides in human mitochondrial DNA (mtDNA). In the present study we use a genome-wide approach to precisely map the location of these ribonucleotides in DNA isolated from fibroblasts, HeLa cells and from cells derived from patients with defects in mitochondrial nucleotide metabolism.

Methods: Ribonucleotides were mapped genome-wide using HydEn-seq (Clausen AR et al, Nat. Struct. Mol. Biol. 2015; 22:185-91). We also used in vitro assays to determine ribonucleotide incorporation by DNA polymerase γ.

Results: We find that ribonucleotides are distributed evenly between the heavy- and light-strand of mtDNA. The relative levels of incorporated ribonucleotides reflect that DNA polymerase γ discriminates the four ribonucleotides differentially during DNA synthesis. The observed pattern is also dependent on the mitochondrial deoxyribonucleotide (dNTP) pools and disease-causing mutations that change these pools alter both the absolute and relative levels of incorporated ribonucleotides. Our analyses strongly suggest that DNA polymerase γ-dependent incorporation is the main source of ribonucleotides in mtDNA and argues against the existence of a mitochondrial ribonucleotide excision repair pathway in human cells. Furthermore, we clearly demonstrate that when dNTP pools are limiting, ribonucleotides serve as a source of building blocks to maintain DNA replication.

Conclusions: Increased levels of embedded ribonucleotides in patient cells with disturbed nucleotide pools may contribute to a pathogenic mechanism that affects mtDNA stability and impair new rounds of mtDNA replication.

Relevant references:

KEYWORDS: ribonucleotides, DNA, DNA polymerase γ, nucleotide metabolism
Clinical correlations of extracellular nucleotide metabolism ecto-enzymes in patients with calcific aortic valve disease

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Objectives: Extracellular adenine nucleotide metabolism contributes to endothelial injury, chronic inflammation and tissue mineralization by controlling nucleotide and adenosine concentrations and its further purinergic effects. In valves, disturbances in purinergic signaling may lead to calcification. In our previous study, we described nucleotide-converting ecto-enzyme pattern on the surface of aortic valve during calcific aortic valve disease (CAVD) that consisted of decreased extracellular nucleotide hydrolysis and adenosine production as well as increased degradation of adenosine in stenotic valves. The aim of this current work was to correlate the rates of nucleotide and adenosine degradation in aortic valves with the parameters of CAVD severity and clinical data.

Methods: Human aortic valves from patients undergoing aortic valve replacement due to CAVD (n=101) were subjected to the assessment of ecto-enzyme activities by functional assays using RP-HPLC. Since previous studies demonstrated marked differences in extracellular nucleotide metabolism on the aortic surface (fibrosa) of stenotic valve vs. nonstenotic, ecto-enzyme activities estimated on the fibrosa surface of stenotic valves were further correlated by Spearman correlation method with echocardiographic and biochemical parameters of CAVD severity and clinical data. In order to determine the mechanisms regulating the activity of ecto-enzymes in aortic valves, we used valvular cells isolated from human aortic valves.

Results: The rates of extracellular nucleotide degradation on the fibrosa surface of stenotic aortic valve negatively correlated with echocardiographic parameters of CAVD severity (maximum velocity across aortic valve vs. ATP hydrolysis: r=-0.28, p<0.05; vs. AMP hydrolysis: r=-0.36, p<0.05) and valvular concentration of Ca²⁺ (AMP hydrolysis vs. Ca²⁺: r=-0.26, p<0.05). Importantly, these correlations were more pronounced in patients with tricuspid aortic valve. Among biochemical parameters, the strongest correlations were observed between the rate of ATP hydrolysis and prothrombin time (r=0.33, p<0.05) as well as INR (r=0.37, p<0.01), while AMP hydrolysis negatively correlated with plasma concentration of total (r=-0.27, p<0.05) and LDL cholesterol (r=-0.36, p<0.05). Interestingly, these negative correlations between AMP hydrolysis on the valve surface and lipid parameters were also more pronounced in patients with tricuspid valve (vs. total cholesterol: r=-0.39, p<0.01; vs. LDL: r=-0.48, p<0.01) and maintained highly significant in patients treated with statins (vs. total cholesterol: r=-0.36, p<0.05; vs. LDL: r=-0.38, p<0.05). Simultaneously, in the group of statin-treated patients, correlations of AMP hydrolysis with CAVD severity parameters were abolished (AMP hydrolysis vs. maximum velocity: r=-0.10, p>0.05 and vs. valvular Ca²⁺: r=-0.18, p>0.05). When patients undergoing lipid-lowering therapy were excluded from the study group, the relationships between AMP hydrolysis and lipid parameters were weaker (AMP hydrolysis vs. total cholesterol: r=-0.15, p>0.05; vs. LDL: and r=-0.30, p>0.05), while a strong positive correlation of adenosine deamination with valvular Ca²⁺ concentration appeared (r=0.34, p<0.05). These results suggested that the rates of AMP hydrolysis and adenosine deamination on the surface of stenotic aortic valves could be modulated by statins. Indeed, we demonstrated that atorvastatin up-regulated cell-surface AMP hydrolysis and down-regulated the rate of adenosine deamination in endothelial cells in vitro. Using specific inhibitors of individual ecto-enzymes, we showed that the main enzyme responsible for AMP hydrolysis on aortic valve was ecto-5’nucleotidase (e5’NT), while for adenosine deamination it was ecto-adenosine deaminase 1 (eADA1). The mechanisms for regulation of e5’NT and eADA1 activity on the surface of valvular cells may include the effects of statins on passive relaxation of cell cytoskeleton and decreased transcytosis of membrane proteins or PI3K/Akt pathway activation that were thoroughly investigated in this work.

Conclusions: This work highlights the modulation of aortic valve extracellular nucleotide metabolism in CAVD, particularly in patients with no anatomical malformations that prone to stenosis. In these patients, alterations in the rates of extracellular nucleotide hydrolysis and adenosine production correlated with CAVD severity. Moreover, the decreased activities of nucleotide-converting ecto-enzymes correlated with a prothrombotic and hyperlipidemic phenotype. We also indicated that lipid-lowering therapy may be beneficial in these patients by affecting extracellular nucleotide and adenosine metabolism on aortic valves supporting an anti-inflammatory milieu that could augment valve calcification.

KEYWORDS: calcific aortic valve disease, ecto-5’nucleotidase, adenosine deaminase, statins, tricuspid aortic valve
Abstract

The Pathway to Pyrimidines: its essential focus on DHODH, the mitochondrial enzyme coupled to the respiratory chain

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This review of our research is dedicated to H. Anne Simmonds whose contribution to the identification and study of inborn errors of metabolism - especially those involving the renal and immune system - were exemplary and outstanding. New Zealand-born Anne was a dedicated European, and a founding member of the ESSPPMM (now Purine and Pyrimidine Society). Together with 18 European countries we were involved in her EC BMH4 project ‘European Structure for Coordination of Research and Diagnosis of Inherited Purine and Pyrimidine Disorders’, which resulted in continuing collaborations [1]. The project supported PhD studies in Marburg including the development of analytical methods to identify intermediates of pyrimidine de novo synthesis, and thus our research programme on dihydroorotate dehydrogenase (DHODH).

For many years, our group in Marburg and collaborators have undertaken extensive studies on the respiratory-chain-coupled enzyme DHODH [2] including its purification from mitochondria, gene cloning, expression and characterization of the recombinant human, rodent, insect, plant and fungal protein [3,4]. The potential of this enzyme as an important target in drug discovery and development to reduce aberrant immunological reactions [5], and to combat proliferation of parasitic protozoa, malignant cells, and fungi was recognized rather late, but is still a topical issue. Anne’s earliest work in pyrimidine pathway concerned the identification of a patient with defective UMP synthase (the enzyme catalysing steps 5 and 6), and its successful treatment with uridine [6]. Defects in the earlier four steps were thought to be lethal, hence they were candidates for the control of proliferation and growth. During Anne’s studies of the metabolic products in human blood (erythrocytes and plasma) she never encountered a patient with an inborn defect in CAD (steps 1-3) or DHODH (step 4). Defects in DHODH were only discovered by the first successful attempt to use exome sequencing to identify the cause of a gene defect, as the cause of the very rare Miller Syndrome [6]. Anne would have appreciated modern work, building on her own, that allows us to focus on these steps in the Pathway to Pyrimidines. The effects of inborn errors and the potential of anti-pyrimidine drugs for the battle against proliferating cells and parasites are described in a popular German Textbook of Biochemistry [7].

KEYWORDS: pyrimidine de novo synthesis - mitochondria - oxygen - inborn errors -- drugs

Abstract S5.1

**CAD, a multienzymatic protein at the head of de novo pyrimidine biosynthesis**

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Objectives: CAD is a 1.5 MDa particle formed by hexameric association of a 250 kDa protein that carries the enzymatic activities for the first three steps in the *de novo* biosynthesis of pyrimidine nucleotides: glutamine-dependent Carbamoyl phosphate synthetase (CPS), Aspartate transcarbamoylase (ATC) and Dihydroorotase (DHO). This metabolic pathway is essential for cell growth and proliferation and is conserved in all living organisms. However, the fusion of the first three enzymatic activities of the pathway into a single multienzymatic protein only occurs in animals. In prokaryotes, by contrast, these activities are encoded as distinct monofunctional enzymes that function independently or forming more or less transient complexes. Whereas the structural information about these enzymes in bacteria is abundant, the large size and instability of CAD only allowed a fragmented characterization of its structure. We aim to decipher the architecture of CAD and to understand its catalytic and regulatory mechanisms.

Methods: To this end, we combine protein engineering, X-ray crystallography and single-particle cryo-electron microscopy (EM), together with biochemical, functional and cellular assays.

Results: We determined the crystal structures of the DHO and ATC domains of human CAD, and demonstrated that these domains self-assemble as "dimers of trimers", making the central scaffold of the CAD particle. We further characterized these enzymatic activities in detail, identifying key structural elements for substrate binding and catalysis. We also produced and isolated the full-length CAD protein from different organisms and obtained a 3D reconstruction of the CPS domain by cryo-EM, defining its mode of interaction with the DHO domain.

Conclusions: Piece by piece, the structural information about the functional domains of CAD allows us to build a model of the hexameric 1.5 MDa particle that initiates and controls the *de novo* biosynthesis of pyrimidine nucleotides. This knowledge should guide in the design of specific inhibitors with potential chemotherapeutic use, and will be of paramount importance for helping in the correct diagnosis and treatment of patients with potential CAD deficits.

Relevant references:

KEYWORDS: Glutaminase, Carbamoyl phosphate synthetase, Aspartate transcarbamoylase, Dihydroorotase, Pyrimidine metabolism
Abstract S5.2

Structural investigation of enzymes of *Mycobacterium tuberculosis* involved in the synthesis of purine and pyrimidine nucleotides

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The intracellular pathogen *Mycobacterium tuberculosis* (MTB) is the main cause of human tuberculosis (TB), the ninth cause of death worldwide and the leading cause from a single infectious agent. TB still remains today one of the most deadliest human infectious diseases, and there is a pressing need for the research of new, effective antitubercular drugs.

The *de novo* and salvage synthesis of purine and pyrimidine nucleotides, the key precursors of DNA and RNA, represents a source of promising targets for the development of new anti-tubercular drugs. In this perspective, our work aimed at investigating the crystal structures of key enzymes of MTB involved in the synthesis of purine and pyrimidine.

We focused our attention on the structural elucidation of the OPRT enzyme (*pyrE*, Rv0382c), which converts orotic acid into orotate monophosphate. The crystal structure of OPRT revealed an unprecedented and serendipitous presence, in the active site, of a metallorganic molecule, giving promise to the development of new antitubercular metallorganic agents.

The *de novo* pathway for guanine-containing nucleotides as well as the salvage pathways of purine nucleotides converge to the synthesis of a common intermediate, inosine 5'-monophosphate (IMP). The enzyme IMPDH (*guaB2*, Rv3411c) converts IMP to xanthine 5'-monophosphate (XMP) and has been established as a promising antitubercular target. In the context of phenotypic screening, a promising molecule (VCC234718) targeting IMPDH could induce MTB mortality in macrophages and mouse lung xenografts, while retaining low toxicity on mammalian cells. Notably, the resistant MTB strain developed a spontaneous mutation (Y487C) on the *guaB2* gene, thus identifying a key IMPDH variant conferring MTB resistance to the active molecule. IMPDH has been recently the target for the development of novel inhibitory compounds with promising efficacy regarding their antibacterial efficacy.

A key metabolite involved in the nucleotide biosynthetic pathway is phosphoribosyl pyrophosphate (PRPP). The MTB enzyme PrsA (Rv1017c) is the sole responsible for the Mg\(^{2+}\)-dependent conversion of ribose 5-phosphate (R5P) to phosphoribosyl pyrophosphate (PRPP) using ATP. As we failed in obtaining MTB PrsA crystals, we crystallized *M. smegmatis* ortholog (MsPrsA) in the ligand-free form. Structure comparison between human and MsPrsA suggested structural differences that could be exploited for the design of specific MTB inhibitors.

KEYWORDS: *Mycobacterium tuberculosis*, protein structure, drug design
Abstract 5.3

Analysis of the purine metabolic effect of allopurinol and its derivative

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Objectives: Allopurinol, a xanthine oxidoreductase (XOR) inhibitor, is used as an anti-gout agent. Most of allopurinol is converted to oxypurinol in vivo by XOR or aldehyde oxidase (AO). The XOR inhibition mechanism is quite complicated. Allopurinol itself serves as a substrate to form oxypurinol as a reaction product. Allopurinol reduces Mo (VI) of Moco, the electrons are transferred to FAD via two iron-sulfur centers, finally moves to NAD+ or oxygen. Oxypurinol inhibits by forming covalent binding with transiently-formed Mo (IV) in this reaction. Therefore, the inhibition is time-dependent. On the other hand, Allopurinol also acts as a substrate of hypoxanthine phosphoribosyltransferase (HPRT) which is a purine salvage enzyme, to form allopurinol-ribotide. This work aimed to compare the XOR inhibitory effect of allopurinol and oxypurinol and to examine HPRT Inhibition of allopurinol and its derivatives, oxypurinol, allopurinol-riboside, and allopurinol-ribotide in detail and analyze their effects on purine metabolism.

Methods: The inhibitory effects of allopurinol and oxypurinol were compared using XDH and XO which utilizes NAD+ and molecular oxygen as the electron acceptor, respectively. HPRT was purified from human erythrocytes and reacted with allopurinol. Produced allopurinol-ribotide was identified and quantified by mass spectroscopic analysis. The enzymatic reaction was measured by monitoring IMP concentrations by HPLC.

Results: The XOR inhibitory activity of oxypurinol was found to be significantly lower than that of allopurinol under physiological conditions. That is, in the presence of xanthine, NAD+ and NADH, both inhibitory effects of allopurinol and oxypurinol increased with time-dependent. However, inhibition of oxypurinol took a considerably long time. On the other hand, the inhibitory effect of each allopurinol derivatives on HPRT showed that allopurinol and oxypurinol showed competitive inhibition, and allopurinol-ribotide showed non-competitive inhibition. Allopurinol-riboside had no inhibitory effect.

Conclusions: The XOR inhibitory effect of oxypurinol was weaker than that of allopurinol, and it was suggested that the inhibitory effect of oxypurinol itself in vivo is not sufficient. Regarding the increase of inhibition in the presence of NAD+, we assumed that this is due to the potential of the reaction center rises and the transfer of electrons becomes easy. On the other hand, HPRT inhibition of allopurinol and derivatives was considered to be present even with the clinical administration dose of allopurinol. We report that XOR inhibitors are useful for neurodegenerative disease model mice\textsuperscript{1}. However, there was no pathological delaying effect in allopurinol. In this study, it is suggested that allopurinol and its derivatives act as inhibitors of HPRT and decrease the pathological delaying effect by reducing the purine salvage of the tissue.

Relevant references:


KEYWORDS: Allopurinol, Oxypurinol, HPRT, xanthine oxidoreductase inhibitor
Abstract S5.4
SAMHD1 deficiency affects DNA replication fidelity and telomere homeostasis in human fibroblasts

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Objectives: In mammalian cells, the dNTP triphosphohydrolase SAMHD1 influences the sizes and balance of the four dNTP pools both in cycling and in quiescent cells. In order to investigate in normal human cells the effects of an endogenous lack of SAMHD1 on cell proliferation and genome stability, we used primary skin fibroblasts isolated from four unrelated Aicardi Goutieres syndrome (AGS) patients. We examined the state of the dNTP pools, cell-cycle progression, DNA replication dynamics and fidelity. Notably, in AGS fibroblasts dGTP is over-represented in the pool, a condition that may affect telomere homeostasis. Telomere stability was evaluated by metaphase Quantitative Fluorescence In situ Hybridization (mQ-FISH).

Methods: Our experimental system consists of proliferating and quiescent cultures of non-transformed or immortalized human skin fibroblasts isolated from Aicardi Goutieres syndrome patients.

Results: In both cycling and resting AGS fibroblasts dNTP pools were increased and imbalanced. The largest expansion occurred for dGTP, the preferred substrate of SAMHD1. AGS fibroblast lines reacted to their abnormal dNTP pool composition either by entering senescence prematurely or by upregulating genes involved in DNA replication. The proliferating AGS cells exhibited a normal DNA replication dynamics. We compared de novo mutations in AGS and WT cells by genomic next generation sequencing. Somatic variant analyses highlighted a clear-cut mutator effect in AGS cells, demonstrating that the perturbation of dNTP pools in SAMHD1 deficiency is \textit{per se} mutagenic. Another possible target of dNTP imbalances might be telomere homeostasis. Indeed, in yeast, telomere length positively correlates with dGTP level. On this basis, we explore the consequences of the high dGTP pool of SAMHD1-deficient fibroblasts on telomere stability.

Conclusions: SAMHD-1 mutated fibroblasts are characterized by large and imbalanced dNTP pools that affect their genome stability.

KEYWORDS: SAMHD1, dNTP catabolism, genome stability, Aicardi Goutieres Syndrome
Deciphering the role of HD52, a mitochondrial nucleotidase essential for pyrimidine homeostasis in *Trypanosoma brucei*

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**Objectives:** We aimed to elucidate the role of TbHD52, a nucleotidase that belongs to the SAMHD1 family, in dTTP biosynthesis and pyrimidine homeostasis in *Trypanosoma brucei*.

**Methods:** Intracellular localization of TbHD52 was determined by immunofluorescence studies. TbHD52 knock-out cell lines were generated upon thymidine supplementation to further establish the impact of TbHD52 on cell viability, cell cycle progression and pyrimidine homeostasis. Several cell lines overexpressing enzymes involved in pyrimidine metabolism, such as hDCTD or TbCDA, in a *TbHD52* dKO background were also generated for a more comprehensive study. Additionally, metabolomics studies were performed in order to identify global disturbances, specifically in dNTP homeostasis, in the absence of TbHD52.

**Results:** TbHD52 is an exclusive mitochondrial nucleotidase essential for *Trypanosoma brucei* viability, as knock-out cells are pyrimidine auxotrophs. Additionally, our findings show that the lack of HD52 can be counteracted by the expression of enzymes involved in dUMP formation. In the absence of exogenous thymidine or deoxyuridine supplementation, *TbHD52* dKO cells show strong defects in cell cycle progression and nuclei and kinetoplast segregation. Furthermore, the metabolomics profile was severely perturbed, affecting profoundly cytosine- and thymidine-derived metabolites.

**Conclusions:** TbHD52 is an essential enzyme in *Trypanosoma brucei* and our results suggest that it plays a key role providing mitochondrial deoxycytidine and thymidine for dTTP biosynthesis via TK salvage. Thus, our findings firmly support that TbHD52 is a valuable drug target against African trypanosomiasis.

**Relevant references:**
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**KEYWORDS:** *Trypanosoma brucei*, SAMHD1, pyrimidines, nucleotidase
Effect of xanthine oxidoreductase inhibitors on purine metabolism in mouse brain under hypoxic environment

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Background: Xanthine oxidoreductase (XOR) inhibitor lowers blood uric acid level, while raises hypoxanthine concentration. Since hypoxanthine serves as a substrate for the purine salvage pathway, XOR inhibitors can be expected to have an effect of tilting ATP regeneration of cells from de novo synthesis pathway to purine salvage pathway. De novo synthetic pathway requires several ATPs, but since ATP is not used in the purine salvage pathway, cells can regenerate ATP more efficiently by utilizing the purine salvage pathway. Administration of febuxostat, an XOR inhibitor, to amyotrophic lateral sclerosis (ALS) model mice delayed the onset of ALS and had an effect of prolonging survival (Kato et al, 2017). We believe that ATP could be efficiently regenerated by the purine salvage pathway, so that the denatured protein was decomposed ATP-dependent by proteasome or the like before aggregation in nerve cells.

Objectives: To promote purine metabolism accompanying the maintenance of adenylate energy charge (EC) by giving hypoxic stress to mice, comparing and examining intermediate metabolites with or without XOR inhibitor.

Methods: Leave the mouse in a tent with hypoxia (5% or 10% O\textsubscript{2}) to give hypoxic stress. Mouse brain metabolism is stopped promptly by microwave irradiation. Purine and oxypurines are identified and quantified by HPLC analysis of the extract of the excised frontal lobe. The XOR inhibitor is administered orally 30 minutes before hypoxic stress.

Results: As a result of analysis of oxypurines using HPLC, values around EC 0.8 were obtained in the microwave irradiated mouse brain, and it was confirmed that the ATP metabolism stops instantaneously in this experimental operation. Furthermore, in low oxygen stress mice at 5% O\textsubscript{2} for 3 minutes, EC decreased to 0.7, and an increase in AMP, IMP and hypoxanthine, intermediate metabolites of purine degradation was also confirmed. In mice treated with febuxostat, no decrease in EC and no increase in AMP and IMP were observed even when hypoxic stress was applied. In the allopurinol-administered group, such effects were not observed.

Conclusions: Administration of febuxostat leads to accumulation of hypoxanthine, a substrate of the purine salvage pathway. This suggests to be advantageous for maintenance of the energy charge during hypoxic stress and the efficiency of ATP regeneration.


KEYWORDS: Xanthine oxidoreductase, Xanthine oxidoreductase inhibitor, purine salvage pathway, hypoxia
Abstract S5.7
Ecto-5'-nucleotidase (CD73) deficiency in mice leads to age dependent impaired L-arginine metabolism and endothelial dysfunction


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Objectives: Changes in the ecto-5'-nucleotidase (CD73) activity – an extracellular nucleotide catabolic enzyme activity may alter inflammation and function of endothelium. We investigated the effect of CD73 deletion in mice on the endothelial function and L-arginine metabolism in different age groups.

Methods: 1-, 3-, 6- and 12-month old, male Wild Type (WT) and CD73−/− mice were used. Serum levels of amino acids, Interleukin 6 (IL-6), Intercellular Adhesion Molecule 1 (ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1) and endothelial Nitric Oxide Synthase (eNOS) were measured. Serum and aortic nitrate/nitrite, as well as aortic arginase and NOS activity in endothelial cells (EC) were also evaluated. Blood samples were used for the analysis of adenine nucleotide concentrations. Results are presented as mean ± SEM.

Results: CD73 deletion led to increase in IL-6, ICAM-1 and VCAM-1 concentration compared to WT. All CD73−/− mice age groups were characterized by reduced L-Arginine and eNOS level. Significantly lower NOS activity was noticed in EC isolated from CD73−/− mice lungs (266.0 ± 85.7 RFU/mg of protein) in comparison to WT (534.8 ± 77.8 RFU/mg of protein). The L-Arginine/ADMA ratio in the CD73−/− decreased in age-dependent manner in comparison to WT. The nitrate/nitrite ratio was reduced in serum (33.9 ± 8.0 vs. 11.5 ± 2.4) and in aortas (1.18 ± 0.05 vs. 0.93 ± 0.04) of 6-month-old CD73−/− mice as compared to WT. Blood (erythrocyte) Adenosine-5'-triphosphate (ATP) and Adenosine-5'-diphosphate (ADP) levels were reduced in favour to higher blood Adenosine-5'-monophosphate (AMP) concentration in CD73−/− mice in comparison to WT.

Conclusions: Deletion of ecto-5'-nucleotidase activity leads endothelial dysfunction in mice that progressed with age and was associated with impaired L-arginine metabolism.

KEYWORDS: adenosine, endothelium, L-arginine metabolism, nitric oxide, ecto-5'-nucleotidase
Deficiency of perforin and hCNT1, a novel inborn error of pyrimidine metabolism, associated with a rapidly developing lethal phenotype due to multi-organ failure

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Objectives: Elucidation of the genetic basis of a clinical case. A patient was presented with fever, hepatosplenomegaly, persistent lactate acidosis, severely disturbed liver enzymes, uridine-cytidineuria and ultimately multorgan failure.

Methods: Whole genome sequencing was performed to further assess the genome of the patient for additional variants. Genes involved in uridine and cytidine metabolism were sequenced. SLC28A1 CDS was cloned into pcDNA3.1 vector. Site-directed mutagenesis were performed to obtain the presented variants c.1528C > T (p.R510C) and c.1682G > A (p.R561Q), together with the double mutant p.R510C;R561Q. The analysis of hCNT1 and the variants was assessed by transient transfection into HEK293 cells, which do not express CNTs. The activity of both variants, mimicking the trans disposition of c.1528C > T (p.R510C) and c.1682G > A (p.R561Q) in the patient, significantly impaired hCNT1 biological function. Whole genome sequencing identified two pathogenic variants c.50delT; p.(Leu17Argfs*34) andc.853_855del; p.(Lys285del) in the PRF1 gene, indicating that our patient was also suffering from Familial Hemophagocytic Lymphohistiocytosis type 2.

Conclusions: We report the first clinical case of pyrimidineuria associated with a genetic defect in a member of the SLC28 family (hCNT1). The presence of two co-existing monogenic defects might have resulted in a blended clinical and biochemical phenotype of the patient.

Relevant references:

\begin{itemize}
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KEYWORDS: hCNT1, Uridine-cytidineuria, Pyrimidine metabolism, PRF1
Ribonucleotide Reductase Subunit RRM1 is Required for Mitochondrial DNA Maintenance via Regulation of dNTP and rNTP Pools

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Objectives: We have identified the first patients with mitochondrial disease due to mutations in \textit{RRM1}, which encodes the catalytic subunit of ribonucleotide reductase. This critical component of \textit{de novo} nucleotide synthesis converts ribonucleotides into deoxyribonucleotides and is therefore essential for DNA replication. Here, we investigated patients’ \textit{RRM1} variants and the molecular mechanisms of disease pathogenesis.

Methods: Muscle biopsies were examined by histology and Southern blot for signs of mitochondrial disease. \textit{RRM1} variants were evaluated with Multiple Sequence Alignment, PROVEAN, and PyMOL. \textit{In vitro} experiments were performed with cultured primary fibroblasts from patients and healthy controls.

Results: Patients from three families presented with ptosis, ophthalmoplegia, and other manifestations indicative of mitochondrial disease. Muscle biopsies revealed cytochrome c oxidase deficient fibers and mtDNA multiple deletions. Although whole exome sequencing was negative for known disease variants, \textit{RRM1} variants were identified in each patient based on (1) its role in nucleotide synthesis, (2) the predicted functional impact of each patient’s \textit{RRM1} variant, and (3) the segregation of \textit{RRM1} alleles within each family. Importantly, ribonucleotide reductase activity and mtDNA content were significantly diminished in all patient fibroblasts. Remarkably, the recessive variants of two families modify the same amino acid residue (p.381Arg>His and p.381Arg>Cys). We identified evolutionarily conserved interactions between Arg381 and the \textit{RRM1} specificity site that are impaired in these variants. The third variant is dominant and resides in the \textit{RRM1} catalytic site. We also found abnormal rNTP and dNTP pools in patient fibroblasts, coupled with substantially elevated ribonucleotide content incorporated in mtDNA, suggesting a mechanism for mtDNA instability and impaired mtDNA maintenance.

Conclusions: Through whole exome sequencing, structural modeling of variants, and molecular analyses of patient fibroblasts, we identified mutations in \textit{RRM1} responsible for a new form of mtDNA maintenance disorder. Imbalanced rNTP and dNTP pools, and mtDNA incorporation of ribonucleotides appear to contribute to the pathomechanism of this novel disease.

KEYWORDS: mtDNA depletion syndrome, ribonucleotide reductase
Abstract S6.3

Characterization and complementation of cellular models of CTPS1 and CTPS2 deficiencies

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Objectives: CTP is the limiting nucleotide in cells required for nucleic acids and phospholipids synthesis. CTP can be produced via a \textit{de novo} pathway which depends on CTP synthetase activity (CTPS) and a salvage pathway that utilizes cytidine produced from nucleic acids degradation. CTPS activity is responsible of the catalytic conversion of UTP to CTP. In human, two genes, \textit{CTPS1} and \textit{CTPS2} (CTP synthetase 1 and 2) are involved in the \textit{de novo} synthesis of CTP. We previously showed that CTPS1 is strongly upregulated in activated T lymphocytes in response to TCR stimulation and that CTPS1 deficiency is associated with a poor proliferation of activated T lymphocytes leading to a severe immunodeficiency in patients. The aim of this study is the establishment and characterization of cellular models of CTPS1 and CTPS2 deficiency to elucidate the respective roles of these enzymes.

Methods: CTPS1 and/or CTPS2-deficient cell lines were established from the Jurkat T leukemia cells (Jurkat cells) and the human embryonic kidney 293 cells (HEK cells) using the CRISPR-Cas9 technology. The impact of the inactivation of \textit{CTPS1} and \textit{CTPS2} on cell proliferation and viability was assessed using the IncuCyte live cell analysis system and cytometry. CTPS activity was measured from cell extracts by MS. Complementation of the cells with GFP-tagged and wild-type CTPS1 and CTPS2 was done using transfection and lentiviral transduction and assessed by western blot and FACS analysis.

Results: We found that HEK cells expressed CTPS1 and CTPS2 while Jurkat cells only expressed CTPS1. Inactivation of \textit{CTPS1} in Jurkat resulted in a complete abrogation of cell proliferation followed by apoptosis in absence of supplementation of the cells with cytidine that allows CTP production via the salvage pathway. Inactivation of \textit{CTPS1} or \textit{CTPS2} in HEK cells have respectively a moderate or no effect on cell proliferation and cells survive in absence of cytidine supplementation. Inactivation of both \textit{CTPS1} and \textit{CTPS2} in HEK resulted in a complete abrogation of proliferation, while the cells survived and proliferation could be restored by cytidine supplementation. Defective proliferation in these models correlated with a decrease or absence of CTPS activity. Complementation of these cell lines with wild-type or GFP-tagged CTPS1 or CTPS2 restored cell proliferation and survival.

Conclusions: In conclusion, we report two CTPS1/CTPS2-deficient cell models showing the overlapping role of both CTPS1 and CTPS2 in cell growth as CTPS2 can compensate for CTPS1 deficiency. These models represent useful tools to further elucidate the structure/function of CTPS1 and CTPS2.

Relevant references:

KEYWORDS: CTPS, proliferation, CTPS deficiency models
A homozygous hypomorphic mutation is responsible for CTPS1 immunodeficiency: immunological and molecular characterization from a cohort study

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Background / Objectives: Immune cell metabolism has an active role in controlling functions of immune cells and shaping of the immune response. T cell lymphocytes have an extremely active metabolism that is required to facilitate growth, rapid cellular proliferation, and the production of large quantities of effector molecules. Among the different existing metabolic pathways, purine and pyrimidine metabolisms have a critical role in the de novo synthesis of nucleic acids and lipids. These pathways are necessary to increase the nucleotide pools needed for efficient proliferation of activated T lymphocytes. We previously demonstrated the particular importance of pyrimidine synthesis pathway in T cell proliferation by the first identification of CTP synthase 1 (CTPS1) deficiency in patients suffering from recurrent viral infections. CTPS1 catalyzes the rate-limiting step for the de novo synthesis of CTP, a limiting precursor of the metabolism of nucleic acids. The aim of this study is to further characterize the molecular and immunological impact of the IVS18-1 G to C CTPS1 mutation that is responsible of CTPS1 deficiency based on a cohort of patients.

Methods:
- Multiparametric flow cytometry analysis was used to identify cell populations in control and patient bloods.
- Functional studies of T lymphocytes from controls and patients were performed including degranulation, proliferation, cytokine secretions.
- CTPS activity was done using enzymatic assay coupled with mass spectrometry analysis to quantify CTP production in control and patient cells.
- CTPS1-deficient Jurkat cells were complemented with the mutant CTPS1 form (expressed in patients).

Results: In the present work, we reported four new siblings, all presenting various viral infections caused by previously described CTPS1 mutation. Exhaustive immune phenotype was performed in 7 CTPS1- deficient patients showing absence of mucosal associated T cells and invariant NKT cells, low numbers of memory B cells and NK cells in peripheral blood. Proliferation and IL-2 secretion of T cells in response to antigen were drastically decreased in all patients. Finally, we showed that the mutation IVS18-1 G>C causing CTPS1 deficiency is hypomorphic resulting in a residual expression of mutant CTPS1 protein associated with very low CTPS activity in cells of patients. The mutant CTPS1 was not able to restore proliferation of CTPS1-deficient Jurkat cells.

Conclusions: In the context of rare disease diagnosis, this study defines detailed immunological parameters associated with CTPS1 deficiency that will help to its diagnosis. The availability of new tools allowed us to go further in the understanding of the role of the CTPS1 mutation. We demonstrate that the mutation behaves as a hypomorphic mutation associated with protein instability leading to weak CTPS1 expression but conserved activity.

References:

KEYWORDS: CTPS Synthase, immunodeficiency, lymphocyte, proliferation
Acquired resistance to Fluorocyclopentenylcytosine (RX-3117) in non-small lung cancer cells is related to a decrease of active RX-3117 nucleotides

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Objectives: Cytidine analogs are being used for years for the treatment of hematological malignancies and various solid cancers such as lung and pancreatic cancer. However, resistance remains a perpetual limitation warranting the development of novel analogs. Recently, the cytidine analog fluorocyclopentenylcytosine (RX-3117) was synthesized by addition of a Fluor and double bond on the ribose moiety, leading to activity in gemcitabine resistant models and becoming a poor substrate for inactivation enzyme cytidine deaminase (CDA). RX-3117 is currently being tested in phase 2 trials as monotherapy for patients with advanced bladder cancer and combined with Abraxane for metastatic pancreatic cancer treatment. Hence, the resistance mechanisms, both intrinsic and acquired, of RX-3117 need to be elucidated.

Methods: RX-3117 resistance was induced by exposing non-small cell lung cancer cell lines A549, SW1573 and H460 to increasing concentrations of RX-3117 and the resistance induction was monitored by the SRB assay. Investigation of the transporters and (de-)activation enzymes was examined by activity assays, RT-PCR, immunocytochemistry and western blot. Intracellular concentration of RX-3117 metabolites was measured by LC/MS-MS. Lastly, next generation sequencing (NGS) followed by differential expression analysis was performed on the RX-3117 resistant and parental cell lines.

Results: Resistance to RX-3117 varied from 63 to 78 fold. RX-3117 induced resistance was stable in the A549 and SW1573 variants, whereas H460 resistant cell lines required RX-3117 supplemented medium to maintain resistance. Cross-resistance to the analogs ethynylcytidine, azacytidine and cyclopentenylcytosine was observed. In the stable resistant variants, a decrease in active RX-3117 nucleotides was found. Although intrinsic resistance revealed a deficient transport and decreased uridine-cytidine kinase 2, no loss of nucleoside transporters (SLC29A1, SLC28A1), or the rate-limiting enzyme UCK2 and potential activation kinases UMP-kinase and CMP-kinase was found. Conversely, the deactivation enzymes 5'-nucleotidase, MTH1 (NUDT1-nudix hydrolase 1) and DCTPP1 showed an increased protein expression in the RX-3117 resistant cells. NGS showed a decreased expression of cell cycle related genes.

Conclusions: RX-3117 is an orally administered cytidine analog with a good bioavailability that shows efficacy in gemcitabine resistant models. In this study, we determined that the underlying acquired resistance mechanism might be explained by a decreased accumulation of active nucleotides, possibly due to an increased degradation.

KEYWORDS: Fluorocyclopentenylcytosine (RX-3117), resistance, NSCLC, cytidine analog
Objective: Immunotherapy for cancer is an approach against cancer that, despite its current success, still has an enormous potential for treatment of even more types of cancers in the future. One approach for immunotherapy is to harvest T cells residing within a tumour, grow them and reintroduce them into the host to recognize and kill cancer cells. The success of this approach is dependent on the ability of the harvested T cells to be activated. When immunotherapy for cancer is not successful, it is often the result of tumour environment having modified the metabolism of the resident T cells so they can no longer be activated.

In this project we hypothesize that one of the targets of the tumour environment is the capability of T cells to perform de novo pyrimidine synthesis through the dihydro orotate dehydrogenase (DHODH). We present our preliminary data that suggest that functional energy metabolism and functional nucleotide synthesis is important regulators of T cell activation. We hope that the perspectives for this study allows the development of specific markers for selection of best suited T cells to be used for immunotherapy for cancer.

Methods: Cells were treated with brequinar to monitor changes of levels of dNTP and cellular response to the inhibition. Activation of T cells has been quantified after inhibition of the pyrimidine pathway. T cells harvested from cancers will have

Results: Inhibition of DHODH affects cellular dNTP levels and induce a LC3 independent autophagy.

KEYWORDS: Up to 5: Immunotherapy, biogenesis, cancer.
Abstract S7.3
What roles for the 5′-nucleotidases cN-II and CD73 in the interplay between the cancer cell and its innate immune microenvironment?

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Objectives: In tumors, the immune system plays a central role in the control of cancer cell proliferation and is also responsible for immunoediting. These processes involve innate and adaptive immune cells. cN-II and CD73 that are two 5′-nucleotidases respectively able to dephosphorylate intracellular and extracellular nucleoside monophosphates into corresponding nucleosides, and thus generate adenosine among others. Considering adenosine's roles as an immunomodulator in a tumor context, it is possible that cN-II and CD73 expressed by tumor cells are involved in processes that modulate innate immune cells in the tumor microenvironment.

Methods: We abolished cN-II and/or CD73 expressions in the breast cancer cell line MDA-MB-231, using the CRISPR/Cas9 technique, and evaluated the impact of cN-II and CD73 in the secretion of cytokines that are known to orientate innate immune cells phenotype. We also assessed NK cells and macrophages survival and function in co-incubation with cancer cells expressing or not cN-II and/or CD73.

Results: cN-II and CD73 expressions in the tumor do not seem to impact NK cells survival, but we observed that they could enhance their antibody-driven cytotoxic activity. We also observed that cN-II deficiency was associated with a higher sensitivity to NK spontaneous cytotoxicity. Preliminary results showed that cN-II expression on cancer cells might also improve phagocytosis completion by THP-1-derived macrophages. The obtained results will allow to define whether cN-II and CD73 expressions in tumors can impact the immune infiltrate and thus modify their escape and aggressiveness.

Conclusions: At a time when the scientific community aims to Reduce, Replace and Refine animal studies, these in vitro assessments constitute original preliminary results before studying the interaction between cancer cells and innate immune cells in vivo. Indeed, they aim to obtain as much results from in vitro immune studies as possible in order to define the most relevant parameters to study in rodents.

KEYWORDS: cN-II; CD73; cancer; adenosine; immunity
Abstract S7.4

How the combination of 6-mercaptopurine with febuxostat affects xanthine oxidase activity in vitro

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Objectives: Febuxostat is a non-purine analog xanthine oxidase (XOD) inhibitor which was approved in Japan in 2011. While it became widely used, the combination of 6-mercaptopurine (6MP) with febuxostat is not a combination attention but a combination prohibition. When concomitant with 6MP and allopurinol, it is well known that allopurinol increases the plasma level of 6MP by inhibiting XOD that catabolize 6MP and moreover allopurinol is metabolized to oxypurinol, which has greater and longer activity of XOD inhibition than allopurinol. As a result, in patients receiving 6MP, the concomitant allopurinol will require a reduction in dose to approximately one-third to one-fourth of the usual dose of 6MP. On the other hand, febuxostat has a strong inhibitory activity of XOD, but it is not metabolized to oxypurinol, so it is unknown how much 6MP metabolism is affected. The objective is to evaluate the pharmacological examination of the interaction between XOD inhibitor and 6MP at the cellular level.

Methods: For determination of cell growth, XTT assay was performed using CCRF-CEM cell line. For drug interaction, the combination index was calculated based on the method of Chou and Talalay, and the values were determined by using the computer software CalcuSyn. To evaluate XOD activity, xanthine oxidase fluorometric assay was performed.

Results: The 50% inhibition of cell growth (IC\textsubscript{50}) values showed 0.9 μM for 6MP. Febuxostat and allopurinol did not inhibit the cell growth at the concentration up to 100 μM. The IC\textsubscript{50} of 6MP, in the presence of 4 μM febuxostat was 0.67 μM. The IC\textsubscript{50} of 6MP with 10 μM allopurinol was 0.92 μ. The combination index (CI) clearly showed the synergism with the values of 0.114 for concomitant use of 6MP with febuxostat, and 0.119 for 6MP with allopurinol. The XOD activity of control was 84 μU/ml. The XOD activity under treatment with febuxostat was 74.7 μU/ml, 67.8 μU/ml and 45.8 μU/ml, for 2hr, 6hr and 24hr, respectively. The XOD activity under treatment with allopurinol was 71.0 μU/ml, 60.8 μU/ml and 55.0 μU/ml, for 2hr, 6hr and 24hr, respectively. At 6hr, allopurinol showed stronger XOD inhibition than febuxostat, which would suggest the effect of oxypurinol. At 24hr, febuxostat had stronger and longer activity of XOD inhibition than allopurinol.

Conclusions: The present study indicated clear synergism for the combination of 6MP with febuxostat for the cell growth inhibition of CEM. The XOD activity was decreased with the time by febuxostat or allopurinol, which would lead to the enhancement of 6MP's cytotoxicity.

KEYWORDS: Drug interaction, 6MP, Febuxostat, combination index, XOD activity
Deoxynucleoside Therapy for Mitochondrial DNA Depletion

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Objectives: Encoded by the nuclear DNA gene TK2, thymidine kinase 2 is a mitochondrial protein required for synthesis of pyrimidine deoxynucleoside triphosphate (dNTP) building blocks for mitochondrial DNA (mtDNA) replication. Autosomal recessive TK2 mutations cause depletion and multiple deletions of mtDNA that manifest predominantly as a myopathy usually beginning in childhood and progressing relentlessly. Pre-clinical studies in a Tk2 H126N knock-in mouse model demonstrated efficacy of deoxynucleoside monophosphate (dNMP) and deoxynucleoside therapies.

Methods: To assess safety and efficacy of dNMP and deoxynucleoside treatments for TK2 deficiency treated under a compassionate use program. We administered dNMP and deoxynucleoside to 16 TK2-deficient patients.

Results: In 5 patients with early onset and severe disease, survival and motor functions improved significantly compared to historical untreated patients. In 11 childhood- and adult-onset patients, all clinical measures stabilized or improved. Three of 8 patients who were non-ambulatory at baseline gained ability to walk on therapy; 4 of 5 patients who required enteric nutrition were able to discontinue feeding tube use; and 2 of 10 patients who required mechanical ventilation became able to breath independently. In motor functional scales, improvements were observed in the: 6-minute walk test performance in 7 of 8 subjects, Egen Klassification in 2 of 3, and North Star Ambulatory Assessment in all 5 tested. The only side-effect observed in 8 of the 16 patients was dose-dependent diarrhea, which did not require withdrawal of treatment. Among 12 other TK2 patients treated with dT+dC, two adults developed elevated liver enzymes that normalized following discontinuation of therapy.

Conclusions: Our compassionate use program indicates favorable side-effect profiles and clinical efficacy of dNMP and deoxynucleoside therapies for TK2 deficiency.

KEYWORDS: TK2, treatment, mitochondria
Abstract S8.2
Age-related metabolic changes limit efficacy of deoxynucleoside-based therapy in TK2-deficient mice

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Objectives: Thymidine kinase 2 (TK2) catalyses the first rate-limiting phosphorylation of the pyrimidine deoxynucleosides (dNs), thymidine (dThd) and deoxycytidine (dCtd) within mitochondria. TK2 deficiency reduces availability of dTMP and dCMP, precursors for dTTP and dCTP synthesis, and ultimately leads to mtDNA depletion or accumulation of multiple deletions. In patients, Tk2 mutations typically manifest as a rapidly progressive myopathy with infantile onset, leading to respiratory insufficiency and encephalopathy in the most severe clinical presentations. dThd-dCtd administration has proven to effectively delay disease progression and expand lifespan in a knockin murine model of the disease. Despite the promising pre-clinical results, dN-treated mice still die prematurely. We have performed a deep analysis of dNTP metabolism in treated Tk2KO mice to identify elements that may be lessening the therapeutic potential of dNs.

Methods: We daily administered Tk2KO mice from postnatal day 4 with an oral gavage of dThd-dCtd (each at a dose of 400 mg/kg/day), dTMP-dCMP (each at an equimolar dose of 620 mg/kg/day), dThd alone (400 mg/kg/day) or dCtd alone (400 mg/kg/day). We monitored body weight and survival on a daily basis. On dThd-dCtd (or dThd alone) treated mice, we also studied different parameters at 12 or 29 days of age. We determined mtDNA copy number in target tissues by qPCR, as well as metabolite levels in mouse plasma and tissues by LC-MS/MS. In addition, we analysed the expression and enzyme activity of catabolic and anabolic enzymes related to dNs metabolism.

Results: Daily oral administration of dThd-dCtd or dTMP-dCMP extended average lifespan of Tk2KO mice from 16 to 34 or 32 days of age respectively, dThd-dCtd treatment rescued mtDNA copy number in skeletal muscle of 12-day-old mice (mtDNA/nDNA copy number mean±SD: Tk2WT-PBS 284.7±114.1; Tk2KO-PBS 177.4±37.8; Tk2KO-dThd-dCtd 288.4±111.0), but not in brain. However, the treatment was ineffective in all analysed tissues at 29-days of age. On the other hand, dThd and dCtd bioavailability markedly decreased during mouse development. We found that after treatment, plasma concentrations of dThd and dCtd were notably lower in older mice (µM mean±SD of 12-day-old mice; dThd: Tk2WT-dThd-dCtd 207.9±29.3; dCtd: Tk2WT-dThd-dCtd 90.9±11.2 and of 29-day-old mice; dThd: Tk2WT-dThd-dCtd 40.6±17.0; dCtd: Tk2WT-dThd-dCtd 7.5±9.8). Consistently, levels of the therapeutic dNs were significantly higher in tissues from younger animals.

The activity of the catabolic enzymes, thymidine phosphorylase (TP) and cytidine deaminase (CDA) was markedly increased with age in small intestine (nmol product/h*mg protein mean±SD of TP; 12-day-old Tk2WT-PBS 37.9±6.9; 29-day-old Tk2WT-PBS 661.9±59.7 and CDA; 12-day-old Tk2WT-PBS 110.3±11.7; 29-day-old Tk2WT-PBS 253.8±59.4), a decisive tissue for orally administered metabolites. Conversely, the activity of thymidine kinase 1 and deoxycytidine kinase, the anabolic enzymes responsible for dThd and dCtd activation, decreased in target tissues during mouse development.

Of particular note, we found that administration of dThd alone had the same impact on survival to that of dThd+dCtd, whereas dCtd alone had no influence on lifespan. dThd reached higher plasma levels when administered alone (µM mean±SD of 12-day-old mice; dThd: Tk2WT-dThd 486.4±92.0), and this greater bioavailability translated, at an early age, into superior mtDNA repopulation in some tissues of Tk2KO mice. However, we observed that this preferential response was highly dependent on the tissue involved, indicating that there are major differences in dNTP-homeostasis between tissues.

Conclusions: We found that dThd+dCtd treatment recruits alternative cytosolic salvage pathways for dNTP synthesis, suggesting that this therapy would be of benefit for any Tk2 mutation. During the first weeks after birth, mice experience marked ontogenetic changes in dN metabolism that could limit the therapeutic efficacy of dThd+dCtd to the early developmental stages. These age-related changes and the tissue-specific metabolic regulation warrant further research to optimize dN therapy and should be incorporated in the design of a clinical trial.

References:

Keywords: mitochondria, TK2, deoxynucleoside therapy, mtDNA depletion, encephalomyopathy
Abstract S8.3

Preparation of standards for LC-MS/MS detection of various metabolites of the de novo purine synthesis and their analysis in urine samples of healthy controls and patients with unspecific neurological symptoms

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Objectives: Up to this date, there are two described autosomal recessive disorders in the de novo purine synthesis (DNPS) – ADSL deficiency and AICArbosiduria. Three forms of ADSL deficiency are recognized. Most severe fatal neonatal form is described by ratios of SAdo/SAICAr metabolites less than 1 in bodily fluids. Severe childhood form – type I with heavy psychomotor retardation and frequently early death has a ratio of SAdo/SAICAr close to 1. The moderate and mild form - type II is typical for psychomotor retardation and autism with ratio of SAdo/SAICAr more than 2. SAICAr is considered to be toxic for nervous system whereas SAdo should be a protective compound, which can diminish the toxic effect of SAICAr. AICArbosiduria is another severe metabolic disorder leading to severe neurological problems. It is accompanied by the high accumulation of AICAr in body fluids. Higher concentrations of SAICAr and SAdo are also presented compared to healthy controls. No other genetically determined defects in DNPS are known thus far, although such defects are presumable. These defects are anticipated to be manifested by nonspecific neurological symptoms and by DNPS intermediates accumulation in body fluids.

Methods: Mass spectrometric fragmentation of newly-synthesized DNPS intermediates using LC-MS/MS was performed and an analytical method for diagnosis of DNPS disorders was developed (1). The method was applied in the analysis of previously prepared CRISPR-Cas9 genome-edited HeLa cells deficient in individual steps of DNPS and the salvage pathway (CR-cells) (2). Identity of newly-synthesized intermediates forming under pathological conditions of known and theoretical defects of DNPS was confirmed by comparing fragmentation patterns of synthesized metabolites of DNPS with those produced by CR-cells. Complementation study of CR-cells was performed with vectors encoding BFP-labelled wild-type (wt) proteins (3). LC-MS/MS analysis of DNPS intermediates in urine of 40 healthy controls and 1040 patients with unspecific neurological symptoms was carry out.

Results: Mass spectrometric fragmentation of synthesized DNPS intermediates was possible in the third to sixth stages, and the spectra were compared with in silico spectra present in free online databases. CR-cells assessed the accumulation of the substrate(s) for the defective enzyme in cell lysates and growth media except in the cells with defective GART enzyme. After complementation study of CR-cells with vectors encoding wt proteins no accumulated substrate was detected. The physiological ranges of DNPS intermediates were established in urine samples of healthy controls and one patient with high level of formylglycinamide ribonucleoside in urine was detected.

Conclusions: LC-MS/MS technique is important tool to explore, determine and quantify known and unknown rare metabolic disorders of DNPS.

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Relevant references:

KEYWORDS: de novo purine synthesis disorders, LC-MS/MS, standards, ADSL deficiency, AICArbosiduria
AAV-mediated gene therapy shows efficacy on the biochemical and neurological phenotype of the nucleoside-enhanced mouse model of MNGIE

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Objectives: To study the effect of gene therapy using different liver-targeted AAV vectors on the enhanced biochemical and neurological phenotype of a murine model of mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), stressed by chronic oral administration of thymidine (dThd) and deoxyuridine (dUrd).

Methods: Tymp/Upp1 double KO mice (dKO) were fed with dThd and dUrd in drinking water (166 mg/L each) from weaning until the end of the study (22 months). Eight-weeks-old dKO mice were treated with different doses of liver-targeted AAVs (AAV8) expressing the human TYP coding sequence (htCTYMP) under the control of three liver-specific promoters (TBG, AAT, HLP). Plasma dThd and dUrd concentrations were measured at different ages. Nucleoside and mitochondrial deoxynucleotide levels were also measured in different tissues at the end of the study. Thymidine phosphorylase activity and vector copy number were quantified in liver. The neurological phenotype of the model, enhanced by the chronic exposure to dThd and dUrd, was studied by assessing the motor function with the Rotarod test in 25-week-old mice, and by brain magnetic resonance imaging (MRI) in 84-week-old mice.

Results: Chronic nucleoside exposure in dKO mice resulted in plasma dThd and dUrd levels of up to 30 times the normal concentration of non-exposed WT mice. AAV-treatment provided elevated thymidine phosphorylase activity in liver and limited the nucleoside systemic levels in exposed dKO mice. Nucleoside pharmacokinetics studies showed that dThd and dUrd exposure was reduced by the AAV vectors in a dose-dependent manner. Thymidine triphosphate was increased in liver and brain mitochondria, and deoxyctydine triphosphate was reduced in liver mitochondria of exposed dKO mice. Treatment with AAV prevented these imbalances. MRI showed enlarged third and lateral brain ventricles in exposed mice, which was also prevented by the treatment with AAV vectors. Rotarod test showed motor impairment in exposed dKO mice, which was partially prevented by AAV treatment. Among all promoters tested, the AAT promoter showed the greatest efficacy in all variables included in this study.

Conclusions: Our results confirm that, as previously reported, chronic oral administration of dThd and dUrd to the dKO murine model of MNGIE enhances its biochemical and neurological phenotype, which constitutes an improved tool to test the efficacy of experimental therapies for MNGIE. We also confirm that AAV-mediated gene therapy improves the biochemical phenotype of the model and, for the first time, that gene therapy using these vectors improves the neurological phenotype in the enhanced model. Among all tested promoters, AAT showed the best results, supporting the notion that this should be the candidate promoter for clinical use.

Relevant references:

KEYWORDS: MNGIE, thymidine phosphorylase, adeno-associated vector, gene therapy, nucleosides
Abstract S8.5

**Pathological purinosome assembly in cell model systems with de novo purine synthesis and salvage pathway deficiencies can be repaired by complementation with wt-protein**

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Objectives: The enzymes involved in the de novo purine synthesis (DNPS), form a dynamic multienzyme complex called the purinosome (1). The purinosome has been observed in a broad spectrum of cells. Previously, we prepared cells deficient in particular DNPS steps (CR-DNPS cells), which were purinosome-free cells (2) and in the salvage pathway, which resulted in the purinosome formation regardless of purine level (CR-HGPRT cells) (3).

Methods: To restore or disrupt purinosome formation, we transiently transfected CR-DNPS and CR-HGPRT cells with vectors encoding BFP-labelled wild-type (wt) proteins. After the transfection followed the immunofluorescence staining of the endogenous proteins PPAT and GART, to detect purinosomes in CR-DNPS cells and in cells deficient in the salvage pathway, (CR-HGPRT cells).

Results: 24 hours post transfection we observed the normalization of purinosome formation, similar to control cells. The knock-out cells also ceased to accumulate the substrate(s) of the defective enzyme. The CR-DNPS cell line transfected with a DNA plasmid encoding an enzyme with zero activity served as a negative control for the purinosome formation. No purinosome formation was observed in these cells regardless of the purine level in the growth medium.

Conclusions: The complementation with wt protein resulted in the stabilization of the internal milieu of cells with defect in the DNPS or salvage pathway.

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Relevant references:


KEYWORDS: purinosome, inborn errors of DNPS, CRISPR/Cas9, complementation
Refractory gout (RG) has been increasingly recognized to be a major problem in clinical care recently. Patients diagnosed with RG have been assumed to be non-adherent, or underdosed, to the greater part. In a minority, pathophysiological mechanisms have been discussed: decreased drug absorption from the gut, genetic modifications of xanthine oxidase (XO) or aldehyde oxidase (AO), as well as increased renal loss of oxipurinol (OX) in the case of allopurinol (AL) treatment.

With multiple clinical and epidemiological studies during recent years, it was a common phenomenon that the numbers of patients diagnosed with RG were variable, irrespective of the study population chosen. None of the studies differentiated non-adherence from impaired response to drug treatment with urine results rarely reported. A definition of adherence has been proposed in the case of AL treatment (OX in serum, >20 µmol/l), which would seem to confirm a minimum dose of about 50 mg/d being taken by the patients. Guidelines for treating gout published by national or international rheumatology societies constitute a major problem with respect to RG, since these do provide very little, if any, information on how to evaluate patients with RG.

Coinciding with the development of the XO inhibitor, febuxostate, a moderate increase in the number of publications on RG was observed, with a sharp rise following after its approval for use in patients with gout. However, the number of samples sent to specialised departments for evaluation of RG like, for example, the Purine Lab of Guy’s and St. Thomas Hospital, London (GB), has drastically reduced. Apart from 3 patients described before the millenium, authors of this contribution, working in the field since the end of the 1970s, were unable to find additional patients with RG. It was demonstrated recently that intensive training and supervision of patients with gout resulted in extremely low numbers of patients possibly showing impaired response to UALDs. It should be remembered that AL, through its metabolism and excretion, is an ideal instrument for differentiating non-adherence from true impaired response.

In conclusion, we think that, apart from very rare patients, needing confirmation of such a diagnosis by metabolic ward studies, RG does not exist, and the high numbers reported in recent years are a myth of the 21st century.
Objectives: Gout is the most common arthritis and it is associated to urate monosodium crystals (UMS) deposits in articulations and soft tissue. The UMS crystals deposit initiates an inflammatory response; mediated by NLRP3 inflammasome, with the release of interleukin 1 β. Toll-like receptor 4 (TLR4) is involved in this response. Although serum urate level is a strong predictor of incident gout, only about half of those with serum urate concentrations ≥10mg/dL develop clinically evident gout over 15 years (1). Therefore, it has been postulated that another factors including genetic or immunity related factors seems to be necessary to the apparition of the acute gout flare beside hyperuricemia. The association of TLR4 single nucleotide polymorphism (SNP) rs2149356 and gout risk is controversial (2, 3) with different results according to different populations.

Methods: We have analyzed rs2149356 polymorphism of TLR4 gene in DNA extracted from 125 gouty patients and 300 health controls by automated DNA sequencing. Patients with primary gout were recruited from the outpatient clinic of the Metabolic-Vascular Unit, Division of Internal Medicine at La Paz University Hospital, Madrid. Primary gout was diagnosed according to the American Rheumatic Association criteria. The control group included DNA and plasma from 300 subjects from the Spanish National DNA Bank. All studies were conducted according to local regulations and the Declaration of Helsinki and were approved by the Institutional Research and Ethics Review Committees of La Paz University Hospital.

Results: 125 patients with primary gout completed our study. Mean age 58.9 ± 12.9 years, 120 males and 5 females, with a mean serum urate concentration of 8.1 ± 1.6 mg/dL and a mean body mass index (BMI) 29.05 ± 3.85 kg/m2. In 13.6%, manifestations were polyarticular, 5.6% presented with tophi and 14.4% with renal stones. In 26.4%, there were a familiar history of gout. 70.4% of gouty patients suffered hypertension, 57.6% hyperlipidaemia and 57.6% diabetes mellitus type 2. Genotypic frequencies for the study SNP complied with the Hardy-Weinberg equilibrium (p > 0.05) in control subjects. Allele frequency distribution in control samples were CC: 0.467 (140); CA 0.437 (131); and AA 0.097 (29).Allele distribution in gouty patients were CC: 0.512 (64); CA: 0.392 (49); and AA: 0.096 (12). No significant association was found between TRL4 rs2149356 polymorphism and risk of gout in the analyzed population.

Conclusions: Allele frequency for rs2149356 in our population was similar to other population of European ancestry, and in these populations; the polymorphism was not related to gouty risk.

Relevant references:

KEYWORDS: Gout, hyperuricemia, TLR4, SNP
Abstract S9.3

**Determination of total purine and purine base content of 82 foodstuffs to help nutritional therapy for gout and hyperuricemia**

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**Objectives:** In Japanese Guideline for the therapy of gout and hyperuricemia, the lifestyle guidance that are nutritional therapy, restriction of alcoholic beverage intake, and adequate exercise are recommended for the patients. Special attention on food intake should be paid in the management of gout and hyperuricemia, and intake of purines under 400mg per day is considered to be good as nutritional therapy to avoid serum uric acid elevation. We determined and reported total purine and purine base content of 270 foodstuffs for facilitating nutritional therapy for gout and hyperuricemia\(^{1}\). In this study, we report and add total purine and purine bases in 82 foodstuffs of which purine content has been determined after our previous work\(^{1}\) and has not yet been reported elsewhere.

**Methods:** 82 foodstuffs (noodle, bread, red pea, chick bean, walnut, 23 Japanese vegetables, pickle, 11 seasonings, 8 fishes, dairy food, 4 supplements, and other food) were purchased at supermarkets or retail stores. Each foodstuff was divided into several pieces. Three pieces of a foodstuff that had similar weight were homogenized and were frozen before lyophilization. After lyophilization, they were hydrolyzed with 70% perchloric acid at 95°C with stirring for 60min. By this process, the nucleic acids, nucleotides, and nucleosides were converted to corresponding each bases, adenine, guanine, hypoxanthine and xanthine. These four purine bases were quantitatively determined with the use of high-performance liquid chromatography (HPLC) and with peak-shift method according to our previous method\(^{2}\). Total purine content was calculated by the sum of each purine bases.

**Results:** Contents of purine bases was as follows; noodle: 6.8-12.1 mg/100g, bread: 4.4 mg/100g, red pea: 25.4 mg/100g, chick bean: 26.1 mg/100g, walnut: 19.6 mg/100g, Japanese vegetables: 0.9-47.1 mg/100g, pickle: 2.6-6.4 mg/100g, seasonings: 0.7-847.1 mg/100g, fishes: 19.0-385.4 mg/100g, dairy food: 0.0-1.4 mg/100g, and supplements: 96.5-605.6 mg/100g. Foodstuffs that contained a very large amount of purine (more than 300mg/100g) were two seasonings, two fishes, two fish milt, and three supplements. Food that contained next largest amount of purine (200-300mg/100g) were sweet prawns and fermented mackerel. Fish milt of blowfish (fugu) and that of cod both contained much purines, 375.4 and 559.8 mg/100g, respectively. When eating fish milt, their quantity should be reduced to 20-30 g at one time (total purine amount become 75-168 mg).

**Conclusions:** In Japan, for a gouty or hyperuricemic patient, it is recommended that the amount of dietary purines is less than 400 mg per day. Regarding nutritional therapy for gout or hyperuricemia, purine-rich food (more than 300mg/100g or 200-300mg/100g) should be eaten in less amount. Good dietary habits with a good balance of nutrients are commended.


**KEYWORDS:** gout, hyperuricemia, nutritional therapy, purine-rich food, determination of purine
Abstract S9.4

**Urate transporter ABCG2 is a physiological exporter of uremic toxin indoxyl sulfate and a crucial factor influencing CKD progression**

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Objectives: Chronic kidney disease (CKD) patients accumulate uremic toxins in the body, potentially require dialysis, and can eventually develop cardiovascular disease. CKD incidence has increased worldwide, and preventing CKD progression is one of the most important goals in clinical treatment. In this study, we conducted a series of in vitro and in vivo experiments and employed a metabolomics approach to investigate CKD.

Methods and Results: Our results demonstrated that ATP-binding cassette transporter subfamily G member 2 (ABCG2), which controls urate homeostasis in our body, is a major transporter of the uremic toxin indoxyl sulfate. ABCG2 regulates the pathophysiological excretion of indoxyl sulfate and strongly affects CKD survival rates.

Conclusions: Our study is the first to report ABCG2 as a physiological exporter of indoxyl sulfate and identify ABCG2 as a crucial factor influencing CKD progression. The above findings provided valuable knowledge on the complex regulatory mechanisms that regulate the transport of uremic toxins in our body and serve as a basis for preventive and individualized treatment of CKD.


KEYWORDS: ABCG2, CKD, urate, uremic toxin, transporter
Abstract S9.5

Multiple novel loci are associated with serum uric acid levels in Japanese population: a genome-wide meta-analysis

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Objectives: Gout is known well as a common non-infectious arthritis and resulted from elevated serum uric acid (SUA) levels. In the present study, we investigated loci which influence SUA by a genome-wide meta-analysis from Japanese population.

Methods: A genome-wide meta-analysis were performed with Japanese participants of three cohorts (J-MICC Study, KING Study, and the BioBank Japan). The trans-ethnic meta-analysis across the present study and the Global Urate Genetics Consortium (GUGC)-based study were also performed to carry out fine-mapping analysis.

Results: From 121,745 Japanese subjects, 8,948 variants at 36 genomic loci (P<5×10^{-8}), including eight novel loci, were identified to have association with SUA. Among of them, missense variants of \textit{SESN2} and \textit{PNPLA3} were predicted to be damaging to these proteins’ functions; another five loci, that is, \textit{TMEM18}, \textit{TM4SF4}, \textit{MXD3-LMAN2}, \textit{PSORS1C1-PSORS1C2}, and \textit{HNF4A}, are related to cell metabolism, proliferation, or oxidative stress. The remaining locus \textit{LINC01578} is unknown. Another 132 correlated genes whose expression levels are associated with SUA-increasing alleles were identified, and these genes are enriched for the UniProt transport term, suggesting the importance of transport-related genes in SUA regulation. Moreover, trans-ethnic meta-analysis across our own meta-analysis and the GUGC has revealed 15 more novel loci associated with SUA. Finally, we identified 62 genomic loci associated with SUA-increasing, including 23 novel loci in the present study.

Conclusions: Our novel insight into genetic background on SUA regulation will be a clue to provide pathogenesis, treatment, and prevention of hyperuricemia and gout.

KEYWORDS: gout; hyperuricemia; serum uric acid (SUA) level, genome-wide meta-analysis, Japanese
Abstract S9.6

Introduction of “Clinical Practice Guideline for Renal Hypouricemia”

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Objectives: Renal hypouricemia (RHUC) is a disease caused by dysfunctional variants of renal urate reabsorption transporter genes, including \textit{SLC22A12/URAT1} and \textit{SLC2A9/GLUT9}. Characteristically, RHUC shows low serum uric acid level and is known to be relatively common in Japanese, Jewish, and Roma population. However, diagnostic guidance and guidelines for RHUC have been lacking, partly due to the low evidence level of studies on RHUC. We therefore decided to develop clinical practice guideline (CPG) for RHUC to resolve these problems.

Methods: CPG for RHUC was developed following the “MINDS Manual for Guideline Development” methodology, which is prioritizes evidence-based medicine and developed in Japan. Two clinical questions and recommendations were developed from a systematic review of the literature. Also, textbook descriptions were added with expert consensus level.

Results: This CPG contains almost all of the clinical foci of RHUC: epidemiology, pathophysiology, diagnostic guidance, clinical examinations, differential diagnosis, and complications, including exercise-induced acute kidney injury (EIAKI, also known as ALPE: Acute renal failure with severe loin pain and Patchy renal ischemia after anaerobic Exercise) and urolithiasis. The guidance shown in this CPG makes it easy to diagnose RHUC from simple blood and urine tests. A CPG summary as well as a clinical algorithm to assist healthcare providers with a quick reference and notes from an athlete for both physicians and patients are included.

Conclusions: One of the goals of this CPG is to clarify the criteria for diagnosing RHUC, and another aim is to work towards a consensus on clinical decision-making. We hope that this CPG will help making clinical decisions by healthcare providers and patients, and that it will promote further researches on RHUC.


KEYWORDS: Renal hypouricemia (RHUC); Clinical practice guideline (CPG); Evidence-based medicine (EBM); Exercise-induced acute kidney injury (EIAKI); Acute renal failure with severe loin pain and patchy renal ischemia after anaerobic exercise (ALPE)
Abstract S10.1

**Cytidine deaminase deficiency: from genetic instability to cancer in the general population**

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The supply of deoxyribonucleotides (dNTPs) and the regulation of their pools are of great importance for successful DNA replication and repair, and thus for preventing cancer. Our project focus on the consequences of a defect in cytidine deaminase (CDA), an enzyme of the pyrimidine salvage pathway catalyzing the hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine (dU), respectively.

Objectives: Our objectives were (1) to investigate the effect of CDA deficiency on genome stability, (2) to characterize the molecular mechanisms underlying the tolerance of CDA-deficient cells to endogenous DNA damage and replication stress, and (3) to explore the potential relationship between CDA deficiency and cancer.

Methods: Classical biology-based methods, as well as microarray analyses, genome-wide RNAi screen, imaging, immunohistochemistry, \textit{in silico} analyses, etc…

Results: We found that the intracellular accumulation of dC/dCTP resulting from CDA deficiency leads to the reduction of basal activity of poly(ADP-ribose) polymerase 1 (PARP-1), a multifunctional enzyme involved in many cellular processes, including the response to DNA damage. The resulting low levels of PARP-1 activity disturb Chk1 activation and decrease the efficiency of downstream checkpoints, leading to the accumulation, during mitosis, of unreplicated DNA at some “difficult-to-replicate” loci in the genome, such as centromeres, fragile sites, leading to an excess of ultrafine anaphase bridge (UFB) formation. We also explored how CDA-deficient cells survive despite a constitutive genetic instability. Finally, we reported that CDA expression is downregulated in about 60% of tumors, mostly due to DNA methylation and identified CDA deficiency as a new predictive marker of susceptibility to anti-tumor drugs. Results on the potential mechanism leading to the reduction of basal PARP-1 activity in CDA-deficient cells and, thus, linking pyrimidine and NAD salvage pathways, will also be presented.

Conclusions: Our results demonstrate that the pyrimidine pool disequilibrium resulting from CDA deficiency generates a genetic instability that might potentially promote cancer development.

Relevant references:

**KEYWORDS:** Cytidine deaminase, pyrimidine pool disequilibrium, PARP-1, genetic instability, cancer
Evolution of dihydropyrimidine dehydrogenase (DPD) diagnostics in a single center in a time-period of eight years

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Background and Objectives: Treatment with fluoropyrimidines, the main chemotherapeutic agents used in many types of cancer, is not well tolerated in a subgroup of patients. The enzyme responsible for degradation of fluoropyrimidines is dihydropyrimidine dehydrogenase (DPD), the first and rate-limiting enzyme of the pyrimidine degradation pathway. DPD dysfunction leads to an increased exposure of active metabolites, which can result in severe or even fatal toxicity. Fluoropyrimidine treatment can be optimized based on DPD activity.

Methods: We provide an overview of eight years DPD diagnostics (n=1194). In this time-frame the test has evolved from a single enzyme measurement using Ultra-High Performance Liquid chromatography (UHPLC) in peripheral blood mononuclear cells (PBMCs) to a combined enzymatic and genetic test of four variants in the \textit{DPYD} gene (\textit{DPYD*2A}, \textit{DPYD*13}, c.2846A>T and 1129-5923C>G).

Results: In the group tested for four variants (n=814), patients with either one variant have a lower enzyme activity than the overall patient group. The majority of patients with the \textit{DPYD*2A} variant (83%) consistently showed a decreased enzyme activity. Only seventeen (22%) of the 77 patients with a low enzyme activity (tested for four variants) carried a variant. Complete \textit{DPYD} sequencing in a subgroup with low enzyme activity and without \textit{DPYD*2A} variant (n=47) revealed 10 genetic variants, of which four have not been described previously.

Conclusions: We did not observe a strong link between \textit{DPYD} genotype and enzyme activity. There is no doubt that DPD status should be determined before treatment with fluoropyrimidines to save patients’ lives and prevent unnecessary side effects. Our study in combination with literature shows that there is a discrepancy between the DPD enzyme activity and the presence of clinically relevant SNPs. Therefore at this moment a combination of a genetic and enzymatic test is preferable for diagnostic testing.

KEYWORDS: Dihydropyrimidine dehydrogenase, pharmacogenetics, 5-fluorouracil, fluoropyrimidines
Abstract S10.3
Resistance to differentiation affects pyrimidine analogs sensitivity and ribo- and deoxyribonucleotide pools in HL60 cells

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Objectives: For decades induction of differentiation has been considered as a target for cancer therapy. Loss of differentiation is a cause of several hematological malignancies. Differentiation is controlled by hypomethylation of DNA, since this will control the expression of a number of tumor suppressor genes. This (partially) explains the efficacy of several hypomethylating agents (e.g. 5-aza-2' deoxycytidine, DAC). HL60 myeloid leukemia cells are considered as one of the best models to study differentiation, which can be induced by several agents, such as retinoic acid (RA). We earlier reported resistance to differentiation induction in a variant of HL60, HL60-R (resistant to RA; 23% differentiation) compared to the sensitive variant HL60-S (sensitive to RA; 78% differentiation) and. Preliminary experiments indicated that there were pronounced differences in sensitivity to several pyrimidine analogs. In order to understand the mechanism of these differences we measured cellular ribonucleotide (NTP: ATP, GTP, CTP and UTP) and deoxyribonucleotide (dTNT: dATP, dGTP, dCTP and TTP) pools.

Methods: Differentiation was quantified with Nitroblue tetrazolium (NBT) staining. The sensitivity to pyrimidine analogs was measured by an MTT test after 72 hr of exposure. The concentrations of NTP and dNTP were measured using a validated anion exchange HPLC with a gradient of increasing NaCl and phosphate concentrations. For measurement of the dNTP the ribonucleotides were degraded by periodate.

Results: HL60-R cells were resistant to the pyrimidine de novo synthesis inhibitors PALA (2.5-fold), Brequinar (DUP-785, 3 fold), 5-fluorouracil (7-fold), hydroxyurea (2.5-fold), but more sensitive to cytarabine (ara-C; 0.19-fold), DAC (0.6) and even to thymidine (0.04). The NTP pools in both HL60 variants showed a normal pattern with ATP being the highest (2600-2800 pmol/10^6 cells) and CTP being lowest. However, several significant differences were observed between both cell lines; UTP pools were 2-fold higher in the HL60S cells (p<0.002), while CTP and GTP pools were 30% higher (p<0.01) compared to HL60-R cells. For the dNTP pools, larger differences were observed, with dATP (48 pmol/10^6 cells) being highest in HL60-R cells, but dATP was 4-fold lower in HL60-S cells. However, TTP pools were highest in HL60-S cells (27 pmol/10^6 cells), but were even higher in HL60-R cells (38 pmol/10^6 cells). Also dGTP pools were higher in HL60-R cells (3-fold) compared to HL60-S cells. RA decreased the pools of UTP and CTP in both cell lines, but more pronounced in HL60-S cells; the effect on ATP was less. Since changes in the NTP and dNTP pools may play a role in differentiation and drug sensitivity we also investigated the effect of modulators of NTP and dNTP pools. Supplementation by 100 μM uridine increased the pools of both UTP and CTP, but did not affect ATP and GTP pools, while the effects on dNTP in HL60S cells were marginal, but in HL60-R cells all dNTP pools decreased by about 25%. In contrast 100 μM thymidine increased TTP pools as expected 4-5 fold in both cell lines, and that of dGTP 4-5-fold in HL60-R, but not in HL60-S. The aspartate-transcarbamylase inhibitor PALA decreased UTP and CTP pools.

Conclusion: The pronounced differences in NTP and dNTP pools between HL60-S and HL60-R possibly play a role in the induction of differentiation and in sensitivity to the pyrimidine analogs. Normalization of the pools in HL60-R might be a tool in resensitizing these HL60-R cells to differentiation.

KEYWORDS: differentiation, pyrimidine de novo inhibitors, nucleotides, deoxynucleotides, leukemia
Abstract S10.4
Role of Nucleoside Transporters in Epigenetically-Modified Natural Nucleosides’ Bioavailability and Mechanism of Action

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Objectives: To study the interaction of the epigenetically-modified natural nucleosides 5-hydroxymethyl-2’-deoxycytidine (5hmCdC) and 5-formyl-2’-deoxycytidine (5fdCdC) and their cytotoxic deaminated metabolites, 5-hydroxymethyl-2’-deoxyuridine (5hmCdU) and 5-formyl-2’-deoxyuridine (5fdCdU), with nucleoside transporters encoded by the SLC28 and SLC29 gene families. To search for appropriate transporter expression profiles to exploit the therapeutic potential of these epigenetic nucleosides in the clinical setting based on their cytotoxic activity dependent on cytidine deaminase (CDA) expression.

Methods: All experiments were performed in the cholangiocarcinoma-derived TFK-1 cell line, which endogenously expresses CDA, and HEK293 cell lines stably expressing SLC28 (hCNT1, hCNT2 or hCNT3) or SLC22A1 (hOCT1) transporters. HEK293-pcDNA5 mock cell line was used as a control. All HEK293 and TFK-1 cell lines endogenously express the SLC29A1 (hENT1) transporter. CDA expression was evaluated by Western Blotting in all cell lines. Interactions of epigenetic nucleosides with transporters were evaluated by cis-inhibition transport assays in initial velocity conditions using tritiated uridine as a model substrate for nucleoside transporters or tritiated 1-methyl-4-phenylpirydinium (MPP+) for hOCT1. Cell toxicity induced by epigenetic nucleoside and membrane transporter combination was determined by performing MTT assays after a 72-hour drug incubation. CDA or each concentrative nucleoside transporter (hCNT1, 2 or 3) were transiently expressed in HEK293 or TFK-1 cells, respectively.

Results: 5hmCdC and 5fdCdC inhibit uridine uptake by pyrimidine-selective concentrative nucleoside transporters hCNT1 and hCNT3. Determination of kinetic interaction constants for each compound towards hCNT1 and hCNT3 showed that these nucleosides were better recognized by hCNT3 than hCNT1 and cytidine derivatives exhibited better interaction with both transporters than 5hmCdU. Additionally, the presence of a formyl group in position 5 of the nucleoside favours the interaction whereas the presence of a hydroxymethyl prevents it. Only two compound-transporter combinations, 5hmCdC with hCNT1 and 5fdCdC with hENT1, were found to be specific at a functional level in cytotoxicity assays. Moreover, 5fdCdC seems to be transported by both hCNT1 and hCNT2 too.

Conclusions: Pyrimidine-like epigenetically-modified natural nucleosides, 5hmCdC and 5fdCdC, are recognized by pyrimidine-selective concentrative transporters hCNT1 and hCNT3, but not by organic cation transporter hOCT1. Comparisons with uridine uptake cis-inhibition transport assays and cell viability experiments suggest that molecular interaction does not necessarily correlate with nucleoside translocation inside cells. Thus, 5fdCdC seems to be transported by hENT1, hCNT1 and hCNT2 but not by hCNT3. In this case, we would be facing the first cytidine-derived nucleoside which would be translocated by hCNT1 and purine-preferring transporter hCNT2, but not by the broad-selective transporter hCNT3. At a structural level, the 5-substituent appears to be determinant for the interaction between epigenetic nucleosides and nucleoside transporters.

Relevant references:

KEYWORDS: nucleosides; transporters; epigenetic nucleosides; nucleoside metabolism; cancer therapy
Abstract S10.5

5′-nucleotidases are involved in the biology of human lung cancer cell lines

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Objectives: Cytosolic-5′-nucleotidase II (cN-II) and ecto-5′-nucleotidase (CD73) are enzymes involved in the nucleotide metabolism dephosphorylating intracellular and extracellular purine nucleotide monophosphates, respectively. Both enzymes have been shown to be involved in cancer by modifying anticancer drug activity, cancer cell biology and immune modulation. The objective of this study is to increase the knowledge about the role of cN-II and CD73 in cancer cell biology.

Methods: We developed lung cancer cell models (NCI-H292) with a complete knockout of either or both enzymes using the CRISPR/Cas9 technique. These cell models are used to study cell proliferation and migration, cancer drug sensitivity, cellular behaviour and apoptosis using CFSE staining, IncuCyte relative confluence and wound healing assay, MTT assay, xCELLigence RTCA and caspase-3/7 apoptotic assay. SCID CB17 mice are also used to study their in vivo tumor growth.

Results: Our results show that there is no significant difference in proliferation between different cell models exposed or not to different concentrations of either adenosine or AMP under normoxic and hypoxic conditions. On the other hand, using xCELLigence RTCA technique, we have observed that CD73-deficient cells have higher cell index as compared to CD73 expressing cells under basal conditions during the first 72 hours of culture. However, adenosine globally decreases the cell index of all NCI-H292 cells, unlike AMP, which influences the cell index of cN-II-deficient cells but not CD73-deficient cells. Under hypoxic condition, cN-II-deficient cells showed higher cell index than the other phenotypes in control conditions, whereas cells lacking CD73 showed higher sensitivity to adenosine or AMP. As compared to other phenotypes, CD73 deficient cells are also more sensitive towards mitomycin and fludarabine. These cells are also more prone to adapt to hypoxic conditions and exhibit slower migration rate than their corresponding control cells. However, the effect of adenosine and AMP on migration rate is higher on cN-II-deficient cells. Mice experiments showed no difference in in vivo tumor growth using these cell models. However, the mice bearing cN-II deficient cells are more sensitive towards mitomycin.

Conclusions: Overall, we show that both CD73 and cN-II are important players in the cell biology of lung cancer cells and in their response to purines. Upcoming experiments should help us understand the molecular mechanisms underlying these observed differences.

KEYWORDS: 5′ nucleotidases, CD73, cN-II, hypoxia
Increased dNTP pools rescue mtDNA depletion in human POLG-deficient fibroblasts

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The mitochondrial polymerase gamma is a heterotrimer constituted by one catalytic subunit (POLG) and two accessory subunits (POLG2), and it is responsible for mitochondrial DNA (mtDNA) synthesis. Defective POLG-activity leads to mtDNA depletion and deletion syndrome with a wide spectrum of clinical phenotypes, ranging from severe infantile to mild adult-onset manifestations.

Deoxyribonucleoside triphosphate (dNTP) availability is an important factor for mtDNA maintenance. Enhancing dNTP synthesis by supplementation with precursors in the form of deoxyribonucleosides (dNs) has been proven effective in different disease models of mtDNA depletion due to defective nucleotide metabolism. Here, we have studied mtDNA copy number recovery rates after EtBr (ethidium bromide)-forced depletion in quiescent skin fibroblasts derived from patients harbouring mutations in different domains of POLG. Following EtBr treatment, all POLG-deficient cells experienced higher mtDNA depletion than fibroblasts from healthy controls, evidencing a defective replication process. We monitored mtDNA recovery after EtBr withdrawal in the presence or absence of all four dNs plus EHNA (specific inhibitor of deoxyadenosine (dAdo) degradation). Activation of dN salvage pathway by supplementation with dNs+EHNA supplementation lead to increased mitochondrial dNTPs levels. Under these conditions, control cells spontaneously recovered initial mtDNA copy number with or without dNs+EHNA supplementation while POLG-deficient cells recovered mtDNA levels only when exposed to the treatment, independently of the specific mutation causing the defect. Preservation of dAdo and concomitant increase in mitochondrial dATP levels are proved necessary for recovery. Importantly, the treatment did not alter POLG fidelity as no increase in multiple deletions or point mutations was detected as checked by Next-Generation Sequencing.

This work evidences that mutations affecting the mtDNA replication machinery may benefit from dNs administration by increasing mitochondrial dNTP availability, and suggests that such a therapy could be effective for treating POLG-deficiency and other conditions in which mtDNA maintenance is challenged.

Relevant references:

KEYWORDS: mitochondria, mtDNA replication, polymerase gamma, therapy, deoxyribonucleosides
Abstract P2

Cardiac adenine nucleotide pool and mitochondrial function in dyslipidemic mice

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Objectives: Dyslipidemia is one of the most common human disorders. One of the consequences of hyperlipidemia is atherosclerosis, which is a major cause of mortality in the Western World. Many clinical findings proved that this pathology is associated with mitochondria dysfunction. Mice are the most currently used model in dyslipidemic research. Hypercholesterolemia occurs as a result of genetic modifications in those animals. Previous studies revealed that apolipoprotein E and LDL receptor knockout mice (ApoE/LDLR--/--) were characterized by improved exercise capacity that may result from the increased mitochondria activity. The aim of this study was to examine the effect of dyslipidemia modifications in mice on nucleotide metabolism and mitochondria function in the heart.

Methods: Mitochondria function and nucleotides concentrations were measured in the heart of 3 month-old C57BL/6J and ApoE/LDLR (--/--) mice. The analysis of mitochondria function was performed using the Seahorse XFp metabolic flux analyzer, by recording the oxygen consumption rate (OCR). Concentrations of nucleotides were evaluated in freeze-cramped hearts using HPLC.

Results: We found a significant increased ATP and total adenine nucleotide pool in ApoE/LDLR (--/--) mice heart, while concentration of guanine and nicotinamide adenine dinucleotides were at similar levels. In turn, we did not observe differences in OCR between isolated cardiac mitochondria from ApoE/LDLR(--/--) and WT hearts mice.

Conclusions: Increased cardiac adenine nucleotides concentrations may contribute to better function of hearts. Details of the link between mitochondria function and nucleotide metabolism in hyperlipidemia require further studies.

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KEYWORDS: hyperlipidemia, mitochondria, nucleotides, hearts, mouse
Abstract P3

Precision medicine in hematology-oncology: CDA as a predictive marker for Cytarabine exposure in AML patients

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Objectives: Cytarabine (Ara-C) remains the backbone of the vast majority of protocols for treatment of acute myeloid leukemia (AML). We have previously demonstrated that clinical outcome with cytarabine was markedly influenced by genetic polymorphisms affecting CDA, the enzyme responsible for its detoxification to Ara-U in the liver. Interestingly, AML patients with CDA PM phenotype exhibited both high risk of severe/lethal toxicities upon cytarabine treatment, but a trend towards longer progression-free and overall survival as well. We can hypothesize that PM patients had probably higher circulating cytarabine plasma levels and lower circulating plasma levels of inactive Ara-U. To confirm this, we have monitoring drug and main metabolite level. The monitoring cytarabine concentrations in plasma required highly sensitive bioanalytical methods especially during induction phase with low dose cytarabine. We have developed a new LC-MS/MS method that meets these requirements.

Methods: Ara-C and Ara-U concentrations were determined in plasma samples using a new LC-MS/MS method. Blood samples were withdrawn from 7 patients treated for AML with 200mg/m2 Ara-C at as part of a study approved by the institutional review board of the Conception Hospital (Marseille, France) registered as # 2017-A00070-53. Patients were phenotyped for CDA status prior to starting the infusion following a spectrophotometric method previously described and categorized as Poor Metabolizer (PM) or Extensive Metabolizer following CDA activity. Patients were sampled at the end of the administration, then 5 min, 10 min, 1H, 2H and 6H after the end of the infusion.

Results: 4 patients were CDA deficient (i.e., CDA ≤ 2 U/mg, aka PM), and 3 patients were CDA no-deficient (i.e., CDA > 2 U/mg, aka EM). For Ara-C, AUCs were 3312 ± 326 ng/ml.min and 1502 ± 497 ng/ml.min, for PM and EM patients, respectively. The difference was statistically different (p>0.024, t test). For Ara-U, AUCs were 7.3.10^5 ± 2.1.10^5 ng/ml.min and 4.8.10^5 ± 0.8.10^5 ng/ml.min, for PM and EM patients, respectively. The difference was not statistically different (p>0.05, t test). Metabolization ratio between AUC of Ara-C and AUC of its metabolite Ara-U was calculated. The mean metabolization ratio was 255 ± 103 and 460 ± 218 for PM and EM patients, respectively. The 1.8-fold difference was not statistically different (p>0.05, t test).

Conclusions: This method was successfully applied to determine the pharmacokinetic profile of Ara-C and Ara-U in 7 patients and evidenced marked differences in both drug levels and metabolic ratio depending on patient’s CDA status. CDA status could be further used as a covariate to tailor durg dosage so as to ensure an optimal efficacy/toxicity balance in patients with AML. Evaluation of Ara-C pharmacokinetics as part of a prospective clinical trial is currently ongoing

Relevant references:
- CDA as a predictive marker for life-threatening toxicities in patients with AML treated with cytarabine. Fanciullino et al. 2018
- Cytidine deaminase residual activity in serum is a predictive marker of early severe toxicities in adults after gemcitabine-based chemotherapies, Ciccolini et al. 2010

KEYWORDS: Cytarabine, Cytidine deaminase (CDA), pharmacokinetic, LC-MS/MS, Acute Myeloid Leukemia
Precision medicine in hematology-oncology: CDA as a predictive marker for azacytidine efficacy in LAM and MDS patients

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Objectives: Azacytidine (Vidaza®) is a mainstay to treat acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). Little is known about predictive factors for response with azacytidine. Azacytidine is detoxified in the liver by a single enzyme, cytidine deaminase (CDA). CDA is an ubiquitous enzyme coded by a highly polymorphic gene, with subsequent great variabilities in resulting activities. Patients phenotypes can then be categorized as poor metabolizer (PM), extensive metabolizer (EM) or ultra-rapid metabolizer (UM). Previously we demonstrated that PM patients treated with cytarabine were at risk to experience life threatening toxicities but performed better in terms of survival. To what extent CDA could be a predictive marker of efficacy azacytidine has never been reported before.

Methods: Patient plasmas were collected from the hematology unit (n=46, F17 M29, 77.9+/\textpm10.7 years old). CDA activity was measured in serum using the spectrophotometry method as a surrogate for liver activity. Patients were classified next based on their status of CDA activity in two groups, CDA PM (CDA \leq 2U/mg) and CDA EM (CDA > 2U/mg). The prognosis was evaluated following IPSS and ELN 2017 scoring, and response was evaluated following IWG (2006) scoring.

Results: 50\% of patients exhibited low CDA activity and were classified as PM. Clinical benefit was higher in PM patients than in EM patients (45\% VS. 35\%) with longer survival (14 months VS. 12 months). Total number of administered courses was higher in PM patients than in EM patients (13 courses per PM patients VS. 7 courses per EM patients, p<0.05).

Conclusions: Preliminary results suggest that CDA PM patients perform better in terms of response, survival and overall number of courses than EM patients. Data regarding tolerance are being currently processed. CDA could be next used in a dosing algorithm to tailor azacytidine dosing, i.e. by increasing dosing in EM patients so as to maximize efficacy.

Relevant references:
- \textit{CDA as a predictive marker for life-threatening toxicities in patients with AML treated with cytarabine.} R. Fanciullino et al.2018
- \textit{Lethal toxicity after administration of azacytidine: implication of the cytidine deaminase-deficiency syndrome.} R. Fanciullino et al.2015

KEYWORDS: Azacytidine, Cytidine Deaminase CDA, predictive marker, blood disease, response
Abstract P5

An HPLC method for ADA2 in plasma

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Objectives: The ADA enzyme exists in 2 forms, ADA1 and ADA2. ADA1 is encoded by the ADA gene and is found in all cells. A deficiency of this enzyme results in severe combined immunodeficiency. ADA2 is encoded by the CECR1 gene and the enzyme is found in extracellular liquid (plasma). Two reports in the same NEJM March issue in 2014 found an association between ADA2 mutations and polyarteritis nodosa vasculopathy in 19 patients (1) and early onset stroke and vasculopathy in 9 patients (2). ADA2 activity in plasma is commonly measured by ELISA, here we describe a HPLC method with inhibition of ADA1 by EHNA, adapted from the Zavialov et al (3).

Methods: Samples sent for routine blood counts from GP surgery were collected after analysis, and spun down and the plasma removed, and stored at -20 °C until analysis. Expired blood transfusion plasma was used as a control material. 25 µl of plasma was incubated with 10 mmAR in 75 mM phosphate buffer (pH 6.5) + 0.125mM EHNA in a total volume of 200ul, for 3 hrs at 37 °C. A 1/36 dilution of washed red cells was also incubated with and without EHNA, as a control for inhibition of ADA1 activity. The assay was stopped with the addition of 50 µl of 40% TCA, and after spinning at 12000rpm in a microfuge, the supernatant transferred to clean tube. This was washed twice with watersaturated diethylether to remove the TCA, the 5ul injected onto waters Alliance HT system with PDA detection with a Hyperclone5u, ODS(C18) 150x4.60mm column, running an isocratic buffer (40 mM ammonium acetate+ 5 mM TBA, pH 2.75) at 1 ml a min. Hypoxanthine and Inosine were both measured as product peaks.

Results: There is some variation in the activity due to age, slightly lower activity in children less than 1 year, but control range for all ages (n=380) 6.9 to 59.7 IU/L. The assay of red cells with and without EHNA, is a useful control to indicate the EHNA is inhibiting ADA 1. 4 patients with ADA2 deficiency had an activity less than 0.1 IU/L.

Relevant references:


KEYWORDS: ADA2
Abstract P6

**Phenotype / Genotype Findings in PRPS**

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Objectives: To assess the functional activity of PRPS in red cells, from 4 different kindred’s with known variants in the PRPS gene. Patient 1 had a family history of PRPS superactivity and was diagnosed at birth. Patient 2 had a duplication picked on gene analysis, Patient 3 had variants picked on gene analysis.

Methods: EDTA blood was spun down and the plasma and buffy coat removed. The red cells were washed twice with saline. A red cell nucleotide and plasma extracts were prepared (1:1) washed red cells: 10%TCA and (1:2) plasma: 10%TCA. Aliquots of 100ul were frozen at -70°C until the assay was performed. The PRPS assay was performed at 125mM and 8 mmPi with and without GDP.

Results: All three patients has a raised plasma uric acid, patient 1 had a low red cell NAD and low activity, patient 2 a normal NAD, but slightly raised activity, and patient 3 a high NAD and a normal activity.

Conclusions: The results show a variation in red cell NAD and in the enzyme assay, patient 1 – although having the high plasma UA of purine overproduction, has low activity in red cells due to an unstable enzyme, this is reflected in the low red cell NAD. Patient 2, has a raised UA, and an increased activity of PRPS (almost 2x control), which showed inhibition by GDP. Patient 3, had a raised NAD, a normal PRPS activity, but no feedback inhibition by GDP leading to the purine overproduction.

This set of analyses gives further insight into the effect of the mutations found in these patients.

KEYWORDS: PRPS, NAD
Abstract P7

An alternative TMP kinase in human

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Objectives: Formation of dTDP from dTMP is an essential step for dTTP synthesis in both de novo and salvage pathways. The reaction is catalyzed by the only known enzyme thymidylate kinase (TMPK, EC2.7.4.9)1,2. While, small hairpin RNA against TMPK in colon cancer cells doesn’t affect the viability of the cells3. By carefully study different fractions of fibroblast cells, we found that an alternative TMP kinase exists. We are targeting to identify the new TMPK.

Methods: Cancer cell line: hTERTimmortalized BJ cells, were maintained in DMEM media with 10% FBS and 0.5% PEST. Fractions from nucleus, cytosolic, mitochondria, and membrane of mitochondria were isolate from freshly harvest BJ cells. Monoclonal antibody against TMPK was used in different protein fractions in Western blot; radiolabeled TMP was used as substrate and formation of radiolabeled dTDP/dTTP was measured in enzyme reactions. Proteins from membrane fraction of mitochondrial were further isolated to gain pure enzyme. Different isolation strategies were applied.

Results: Western blot showed strong signal from antibody against TMPK in cytosolic and nuclear fractions, no signal from membrane fraction of mitochondrial fraction. While, the enzyme activity showed strong signals from the cytosolic, nuclear, mitochondrial and membrane of mitochondria protein fractions. By combining the western blot and enzyme activity, it gave rise to hypothesis that another enzyme with similar TMP kinase activity exists in human. By monitoring long-term growth of different cell culture, we found that cancer cell line have constantly high enzyme activity on the membrane fraction of mitochondrial protein, while normal cells lines have variation of enzyme activity on the membrane of mitochondial. This may suggest cancer cells utilize this unknown TMP kinase to provide continuously its nucleotide synthesis in mitochondria. While, the unknown TMP kinas is not stable, and sensitive to increased salt concentration, which makes the isolation steps a huge challenge.

Conclusions: Except to known enzyme TMPK, another enzyme with TMP kinase activity exists in human.

Relevant references:

KEYWORDS: TMP kinase, dTTP synthesis, Mitochondria, cancer metabolism, nucleotides synthesis
Abstract P8

Establishment of high hypoxanthine phosphoribosyltransferase activity-uricase knockout mice as novel hyperuricemic model and effects of purine/nonpurine type xanthine oxidoreductase inhibitors

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Objectives: The species differences of purine metabolism of mice from that of human are known to be not only conserved uricase (Uox), but also low hypoxanthine phosphoribosyltransferase (HPRT) activity. The aim of this study is the establishment of high HPRT activity-Uox knockout (KO) mice as mouse model for the study of purine metabolism in human and the investigation of the pharmacological effects of purine-type xanthine oxidoreductase inhibitor (XOI), allopurinol and non-purine-type XO, topiroxostat on high HPRT activity-Uox KO mice.

Methods: High HPRT activity-Uox KO mice were established by mating B6-ChrXCMiem mice with Uox-KO mice. Allopurinol 30 mg/kg or topiroxostat 1 mg/kg were administered to the model mice for 7 days by feeding diet. Oxypurines (urate (UA), hypoxanthine (HX) and xanthine (XA)) and creatinine (Cr) in plasma and urine were measured by HPLC. Moreover, we demonstrated that inhibitory effects of oral administration of allopurinol 30 or 100 mg/kg and topiroxostat 1 mg/kg on erythrocyte HPRT activity of high HPRT activity-Uox KO mice.

Results: These model mice showed high plasma UA value, and HPRT activity was about 34 times higher than that of the low activity model mice. Plasma UA value and urinary UA/Cr ratio significantly decreased in allopurinol group and topiroxostat group. Although the plasma UA-lowering effect was equivalent in allopurinol group and topiroxostat group, the urinary HX/Cr ratio and XA/Cr ratio in topiroxostat group were significantly lower than those in allopurinol group. Moreover, urinary oxypurine/Cr ratio demonstrated a significant lowering effect in topiroxostat group, but not in allopurinol group. Allopurinol inhibited dose-dependently erythrocyte HPRT activity of this model mice, while topiroxostat not.

Conclusions: We established high HPRT activity-Uox KO mice as novel hyperuricemic animal models with human-like purine metabolism. In addition to topiroxostat has a potent plasma UA-lowering effect without affecting the salvage pathway unlike allopurinol, efficiently resulted in decreased total oxypurine excretion. These model animals may be useful tool and contributed to purine metabolic research in the future.

KEYWORDS: Hyperuricemia, Urate, hypoxanthine phosphoribosyltransferase (HPRT), XOR inhibitor
Rescue of Xor knockout mouse with high HPRT activity by NAD⁺ supplementation

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Objectives: Although xanthinuria type I is nonfatal and asymptomatic in humans, xanthine oxidoreductase knockout (XorKO) mice are reported to die until 8 weeks-old by renal failure. Among species differences between human and mice, the activities of hypoxanthine phosphoribosyl transferase (HPRT) of human and wild mice are higher than that of laboratory mice. The aim of this study is to elongate the life span of the XorKO mice with high HPRT activity.

Methods: High HPRT activity XorKO mice were established by crossbreeding with a consomic C57BL/6 mice whose Hprt allele is derived from a wild mouse, Mus musculus molossinus. Urinary substances of XorKO mice were separated by HPLC.

Results: In a low HPRT activity XorKO mouse, which died at 7 weeks of age, the excretion of xanthine (XA) decreased with increasing excretion of hypoxanthine (HX) without changing the total amount of urinary oxypurine excretion. A high HPRT activity XorKO mouse, which died at 23 weeks of age, the excretion of HX also increased. The change of urinary oxypurine excretion from xanthine to hypoxanthine might be a cause of death of XorKO mice, suggesting the inhibition of IMP dehydrogenase, a NAD⁺-dependent enzyme. Because hypoxanthine was reported to inhibit the production of nicotinamide mononucleotide from nicotinamide, the accumulation of hypoxanthine in XorKO mice may induce the inhibition of NAD⁺ synthesis. Supplementation of 1% L-tryptophan in feed, another precursor of NAD⁺ synthesis, recovered body weight gain in high HPRT activity XorKO mouse.

Conclusions: XorKO mice seem to be fall into a vicious cycle, hypoxanthine accumulation leads to NAD⁺ deficiency that aggravate the first. The body weight gain was recovered by NAD⁺ supplementation. Longer supplementation study is necessary to whether high HPRT activity XorKO mice will be able to bleed.

Relevant references:

KEYWORDS: xanthine oxidoreductase, hypoxanthine phosphoribosyl transferase, nicotinamide adenine dinucleotide, tryptophan
Abstract P10

Effect of phytic acid on postprandial serum uric acid level in healthy volunteers: a randomized, double-blind, crossover study

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Objectives: Phytic acid, a constituent of various plants, has been related to health benefits. Phytic acid has been shown to inhibit purine nucleotide metabolism in vitro and suppress elevation of plasma uric acid levels after purine administration in animal models. This study investigated the effect of phytic acid on postprandial serum uric acid (SUA) levels in humans.

Methods: This randomized, double-blind, crossover design study included 48 healthy subjects with normal fasting SUA levels. Subjects consumed a control drink and a phytic acid drink with purine-rich food. Serum and urine uric acid levels were measured for 360 min after purine loading.

Results: Phytic acid lowered the incremental areas under the curve (0-360 min) and the incremental maximum concentration of SUA after purine loading ($p < 0.05$). Phytic acid tended to lower cumulative urinary uric acid excretion (0-360 min) after purine loading ($p < 0.10$).

Conclusions: Phytic acid suppressed postprandial SUA in this clinical study. Altogether, our findings suggest that phytic acid may play a beneficial role in controlling postprandial SUA.

Relevant references:

KEYWORDS: Phytic acid; Serum uric acid; Purine; Hyperuricemia
Expression of the nucleoside transporters hENT1 and HCNT1 in pediatric acute myeloid leukemia

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Deoxynucleoside analogs (NAs) form a major group of antimetabolite cytotoxic drugs in current clinical use. Cytarabine (ara-C) for instance is the most effective agent in the treatment of acute myeloid leukemia (AML) and gemcitabine is used extensively for the treatment of solid tumors. A family of nucleoside transporters (human equilibrative nucleoside transporters (hENTs) and concentrative nucleoside transporters (hCNTs) mediate cellular uptake of NAs by facilitated diffusion or concentrative uptake. NAs differ with respect to their preferential transporters, hENT1 for instance is responsible for 80% of the influx of ara-C. In addition, hENT1 mRNA expression has been related to in vitro drug resistance and outcome in AML, implicating reduced hENT1 mRNA expression as a major resistance mechanism to ara-C in AML patients.

In order to determine hENT1 and hCNT1 protein expression in pediatric AML we have set up an immunocytochemical method, using rabbit-anti human hENT1 and hCNT1 antibodies and applied this method in 50 AML patient samples. Immunocytochemical staining was performed on cryo-preserved cytospins. The human leukemic cell line HL60 was used as a positive control. Negative controls were performed by omitting the first antibody. hENT1 and hCNT1 protein expression were evaluated by two independent investigators by scoring the intensity of the staining:

- (negative), -/+ (weak), + (positive) or ++ (strong).

HL60 cells consistently stained positive for both hENT1 and hCNT1 in the cytoplasm. Negative controls did not stain. Seven samples were non-evaluable due to poor morphology and one sample was lost during the procedure. HENT1 and hCNT1 were located at the membrane and in the cytoplasm in all AML patient samples. hENT1 staining was weak in 9/43 (21%) samples, positive in 26/43 (60%) samples and strong in 8/43 (19%) samples, and appeared to be slightly granular. hCNT1 staining was weak in 2/42 (5%) samples, positive in 35/42 (83%) samples and strong in 5/42 (12%) samples. In 8/42 (19%) AML patient samples hCNT1 was located predominantly at the cellular membrane. hENT1 and hCNT1 expression were not associated with age or WBC at diagnosis. Both hENT1 and hCNT1 expression appeared to be higher in (myelo-)monocytic (FAB M4/5) AML blasts compared to poorly differentiated/ myeloblastic (FAB M0-3) AML blasts. For ara-C we observed a tendency in the relation between the expression patterns of hENT1 with ex vivo drug sensitivity (measured by a 4-day MTT-assay), but not for hCNT1 and not to the other deoxynucleoside analogs cladribine, gemcitabine, fludarabine and decitabine.

In conclusion, the nucleoside transporters hENT1 and hCNT1, which are crucial for the transport of clinically important deoxynucleoside analogs (e.g. cytarabine), are broadly expressed in AML.

KEYWORDS: equilibrative nucleoside transporter, concentrative nucleoside transporter, cytarabine, gemcitabine, acute myeloblastic leukemia
Abstract P12

**Distribution of serum uric acid levels and prevalence of hypouricemia and hyperuricemia: A cross-sectional, nationwide health examinee cohort study in Korea**

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Objectives: The purpose of this study was to investigate the distribution of serum uric acid (SUA) level and to estimate the prevalence rate of hypo- and hyperuricemia in Korean population.

Methods: We conducted a cross-sectional study using data from a government funded health examinee cohort for Korean genome and epidemiology study. A total of 172,970 participants (58,981 male, 113,989 female) aged 40-79 years were included. Hypouricemia and hyperuricemia was defined as SUA level ≤ 2.0mg/dL and >7 mg/dL. We used the 2010 Population and Housing Census to estimate the prevalence of standardization by sex and age.

Results: The mean SUA levels were significantly higher in male than female (5.71 ± 1.27 mg/dL vs. 4.21 ± 0.96 mg/dL, \(p < 0.001\)). The mean SUA levels by age group divided by 10 years were increased with age in female, but not for male. The prevalence of hypouricemia was 0.4% (0.1% in male and 0.6% in female). The prevalence of hyperuricemia was 13.3% in male, 0.8% in female and overall 5.1% among total subjects. Standardized prevalence with age-, sex adjusted of hypo- and hyperuricemia was 3.64 per 1,000 persons (95% CI 3.27-4.07 per 1,000 persons) and 70.54 per 1,000 persons (95% CI 68.84-72.30 per 1,000 persons) respectively.

Conclusions: This survey is the most extensive study of SUA levels conducted in Korea. The standardized prevalence of hypouricemia and hyperuricemia from our study can be compared with those of other population and will be the basis for the study of association with other diseases.

Relevant references:

KEYWORDS: uric acid, hypouricemia, hyperuricemia

Comment; Some of the contents of this abstract were presented at the Korean College of Rheumatology in 2017.
Lactic acid bacteria show different patterns of incorporation of purine mononucleotides and nucleosides depending on the species

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OBJECTIVES: Many studies of metabolism in lactic acid bacteria use \textit{Lactococcus lactis} species. It is commonly accepted that lactic acid bacteria do not directly incorporate purine mononucleotides but intake them after decomposing into purine nucleosides by 5'-nucleotidases expressed on the bacterial cell surface. We have previously shown that \textit{Lactobacillus gasseri} PA-3, a kind of lactic acid bacteria, incorporated purine mononucleotides\textsuperscript{(1)} and it is likely that the intake of purine mononucleotides depends on the species or strain of lactic acid bacteria. In this study, we compared the direct incorporation of purine mononucleotides among \textit{L. gasseri}, \textit{L. lactis} and other species of lactic acid bacteria.

METHODS: We used each ten strains of \textit{L. gasseri} and \textit{L. lactis} and several strains of \textit{Pediococcus}. Each bacterial strain was incubated in the presence of \textsuperscript{32}P-AMP for 15 minutes at 37 °C. The bacterial cells were collected by filtration and washed with saline, and their radioactivity was measured by liquid scintillation counting. These bacterial strains were also incubated in the presence of \textsuperscript{14}C-adenosine for 60 minutes at 37 °C and the incorporation of adenosine was analyzed by liquid scintillation counting.

RESULTS: All investigated strains of \textit{L. gasseri} incorporated \textsuperscript{32}P-AMP while few strains of \textit{L. lactis} (two over ten) incorporated it. In bacterial strains of \textit{Pediococcus}, the incorporation of \textsuperscript{32}P-AMP depended on the strain or species of that genus of bacteria. In contrast, strains of \textit{L. lactis} displayed greater incorporation of \textsuperscript{14}C-adenosine than those of \textit{L. gasseri}.

CONCLUSIONS: Most strains of \textit{L. lactis} do not directly incorporate purine mononucleotides as the common view. However, some species of lactic acid bacteria such as \textit{L. gasseri} directly incorporates purine mononucleotides. It is considered that lactic acid bacteria incorporate either purine mononucleotides or nucleosides more preferentially depending on the species or strain.

Reference:


KEYWORDS: \textit{Lactobacillus gasseri}, \textit{Lactococcus lactis}, purine mononucleotide, purine nucleoside
Abstract P14
Role of intracellular cytidine deaminase activity for gemcitabine metabolism in three pancreatic cancer cell lines

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Objectives: The pyrimidine nucleoside analogue gemcitabine (2’-2’-difluoro-2’-deoxycytidine, dFdC) is subject to activation and inactivation pathways, of which cytidine deaminase (CDA) is the most important for in vivo systemic inactivation and elimination kinetics (1). We assessed intra- and extracellular drug metabolite concentrations following in vitro gemcitabine treatment with and without pharmacological inhibition of CDA, and related the concentrations to the basal expression of a panel of genes and proteins in the gemcitabine pathway. The main aim was to assess the role of CDA on a cellular level, in different pancreatic ductal adenocarcinoma (PDAC) cell lines that might mimic the diversity of pancreatic tumors.

Methods: The PDAC cell lines BxPC-3, MIA PaCa-2 and PANC-1 were either incubated with 0.1, 1, 10 or 100 µM gemcitabine (spike concentration at time 0), or co-incubated with 10 or 100 µM gemcitabine and 200 µM tetrahydrodouridine (THU), a CDA enzyme inhibitor. Culture medium samples were collected after 24 hours for quantification of gemcitabine and dFdU, the main inactive gemcitabine metabolite formed via CDA. Cells were harvested and resuspended in 60% MeOH, stored at -80 °C and analyzed for the intracellular active triphosphate metabolite dFdCTP. Gemcitabine and its metabolites were quantified using liquid chromatography tandem mass spectrometric methods (LC-MS/MS) (1, 2). We also studied basal expression of a panel of transporters, activating and inactivating enzymes, including CDA, by real-time quantitative RT-PCR and Western blot, in gemcitabine-untreated cells.

Results and Discussion: After 24 hours incubation with gemcitabine, percentage turnover to dFdU was approximately 90, 25 and 10 % in BxPC-3, MIA PaCa-2 and PANC-1, respectively. After co-incubation with 10 or 100 µM gemcitabine and THU, dFdU was undetectable in either cell line, indicating effective inhibition of CDA. After 24 hours incubation with 10 µM gemcitabine, average intracellular dFdCTP concentrations were 250, 1465 and 955 pmol/10⁶ cells in BxPC-3, MIA PaCa-2 and PANC-1, respectively. After incubation with 100 µM, the dFdCTP corresponding concentrations were 850, 1240 and 660 pmol/10⁶ cells. No further increase of dFdCTP was observed between 10 and 100 µM gemcitabine in MIA PaCa-2 and PANC-1, which may reflect saturation of the activation pathways in these cell lines. Co-incubation with THU resulted in a profound increase of dFdCTP concentrations in BxPC-3, with an average of 1370 pmol/10⁶ cells, both at 10 and 100 µM gemcitabine. No increase in dFdCTP was observed in MIA PaCa-2 or PANC-1, which may reflect saturation of the activation pathways in these cell lines. Basal mRNA expression of CDA was significantly higher in BxPC-3, compared to MIA PaCa-2 and PANC-1, which fits well with the gemcitabine metabolite data. No CDA protein expression was detected in MIA PaCa-2 and PANC-1, which may be an issue of method sensitivity. The majority of the other gemcitabine pathway transporters and enzymes showed the highest mRNA and protein expressions in PANC-1.

Conclusions: Intracellular dFdCTP accumulation, a surrogate marker for cellular gemcitabine efficacy, was strongly and inversely related to intracellular CDA activity. Our findings highlight the importance of intracellular CDA activity on gemcitabine metabolism in PDAC cells. Intracellular CDA expression and activity should be taken into account in future studies of local gemcitabine delivery to pancreatic tumors. Direct quantification of gemcitabine and dFdU concentrations was superior to protein expression for the assessment of CDA activity.

References:

KEYWORDS: gemcitabine, pancreatic cancer, LC-MS/MS, mRNA, protein expression
Association between plasma xanthine oxidoreductase activity and vascular endothelial dysfunction in subjects without hypertension or diabetes – MedCity21 health examination registry

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Objectives: Administration of a xanthine oxidoreductase (XOR) inhibitor, which can reduce not only uric acid but also reactive oxygen species (ROS) production in circulating blood, has been reported to improve flow-mediated dilatation (FMD). However, the significance of a reduced level of XOR activity in the circulation in relation to improved FMD remains unclear.

Methods: This cross-sectional study included 193 subjects registered in the MedCity21 health examination registry. Plasma XOR activity was measured using our highly sensitive novel assay based on [13C2,15N2] xanthine and liquid chromatography/triple quadrupole mass spectrometry, the results of which are unaffected by the original uric acid concentration. FMD in the brachial artery was determined using ultrasound for evaluation of vascular endothelial function.

Results: The median values for serum uric acid, plasma XOR activity, and FMD were 5.6 mg/dL, 26.1 pmol/h/mL, and 6.2 %, respectively. Multivariable linear regression analysis showed no association of plasma XOR activity or serum uric acid level with FMD in the present subjects. However, among established cardiovascular disease (CVD) risk factors examined, interaction analysis found hypertension and diabetes mellitus (DM) (p for interaction=0.053 and 0.104, respectively) to be significant factors modifying the association of plasma XOR activity with FMD. In subgroup analyses, plasma XOR activity was inversely associated with FMD in subjects without hypertension (n=138) (β=-0.211, p=0.031) and in those without DM (n=168) (β=-0.151, p=0.080), whereas serum uric acid showed no such association.

Conclusions: Plasma XOR activity, but not serum uric acid, was found to be inversely associated with FMD in normotensive and non-DM subjects, in contrast to those with hypertension or DM, suggesting that XOR contributes to development of vascular endothelial dysfunction through ROS production, especially in individuals with reduced risk for CVD.

KEYWORDS: plasma XOR activity, FMD, uric acid, ROS
Abstract P16

Pannexin1 KO osteoclasts are irresponsive to Tenofovir induced differentiation

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Objectives: Tenofovir is anti-retroviral agent prescribed to human immunodeficiency virus (HIV) patients as part of the drug regimen known as highly active anti-retroviral therapy (HAART). But Tenofovir also causes osteopenia in this patients resulting in pathological fractures and hospitalisation in these patients. This is now increasingly significant since tenofovir is one of the components of HIV pre-exposure prophylaxis or PrEP, which is available for the population at risk of being exposed to HIV. Currently there are not many studies in the effect of PrEP on bone loss, but several authors have reported that this population also suffers a decrease in BMD.

A recently published study suggests that this is caused by a reduction of extracellular adenosine produced by Tenofovir mediated blockage of Pannexin-1, an ATP transporter and that this effect can be reversed by dipyridamole, that blocks adenosine re-uptake. To further confirm this study Pannexin-1 KO (PAX1KO) mice were studied and compared to C57BL/6J (WT) mice.

Methods: Primary osteoclasts were differentiated from mice bone marrow and treated with different concentrations of Tenofovir and dipyridamole. The differentiation stage and extracellular ATP levels were studied.

Results: In WT mice Dipyridamole inhibited osteoclast differentiation (p= 0.0068). The dipyridamole induced inhibition was reverted with Tenofovir in a dose dependent manner (0.0055). PAX1KO mice osteoclast differentiation was also inhibited by dipyridamole (p=0.0005), however Tenofovir was unable to revert dipyridamole induced inhibition (p= 0.9756). Additionally, opposed to the WT osteoclasts, PAX1KO mice did not decrease ATP release when treated with Tenofovir (p= 0.3292).

Conclusions: In the absence of pannexin-1 transporter tenofovir is not able to influence osteoclast differentiation and ATP release.

Relevant references:

KEYWORDS: Tenofovir, dipyridamole, bone, osteopenia
Novel mass spectrometry approaches to investigate nucleotide biosynthesis and utilization in pancreatic cancer


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Objectives: Pyrimidine nucleotide metabolism is a highly complex and regulated system with multiple pathways contributing to its plasticity and robustness. Pyrimidine nucleotides can be produced via the de novo pathway (DNP) that utilizes glucose and amino acids, and the nucleoside scavenging pathway (NSP) that utilizes extracellular uridine and cytidine. Our data suggest that nucleotide products of RNA turnover can fuel a third route for pyrimidine biosynthesis, which we term the nucleotide recycling pathway (NRP). Amongst these three major pyrimidine nucleotide biosynthetic pathways, the DNP has been studied most extensively. The biological and therapeutic significance of the NSP and NRP in cancer, as well as their regulation and coordination with DNP in the context of oncogenic signaling, are poorly understood.

Methods: Here, we describe novel mass spectrometric (MS) methods to measure the individual contributions of these convergent pyrimidine biosynthetic pathways to major biological processes including nucleic acid synthesis, phospholipid biosynthesis and protein glycosylation.

Results: Applying these methods to a panel of pancreatic ductal adenocarcinoma (PDAC) models, including patient-derived primary lines, in conjunction with clinically relevant pharmacological perturbations, revealed previously unappreciated biosynthetic heterogeneity, plasticity and robustness, suggesting the existence of distinct pyrimidine metabolic subtypes.

Conclusions: Identifying and characterizing these subtypes in the context of oncogenic KRAS signaling may guide the development of novel therapeutic approaches against PDAC and potentially other tumors.

Relevant references:


KEYWORDS: cancer nucleotide metabolism, mass spectrometry, de novo pathway, nucleoside scavenging pathway, and nucleotide recycling pathway
Response to dC+dT therapy in TK2 deficiency is influenced by nucleoside bioavailability and levels of Tk1 and Dck

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Objectives: To better understand the pharmacological mechanisms of dC+dT treatment in mtDNA Depletion Syndrome caused by Thymidine Kinase 2 (TK2) deficiency.

Methods: Using HPLC, we studied the bioavailability of dC and dT after oral gavage, intra-peritoneal (IP), and intra-venous (IV) administration to the H126N Tk2 knock-in (Tk2⁻/⁻) mouse model. We also assessed therapeutic efficacy of IP treatment and the effects of Dck and Tk1 levels (measured by immunoblotting), which are the cytosolic enzymes that may phosphorylate dC and dT.

Results: Compared to oral gavage, IP and IV injections more effectively elevated levels of dC and dT in blood, brain and liver. IP administration of Tk2⁻/⁻ mice with dC+dT (520mg/kg/day each) increased mtDNA copy number in liver and heart, compared to oral gavage; however, mtDNA depletion was present in brain as early as postnatal day 13 in brain of oral-gavage and IP treated mice. Disease onset and lifespan were also similar between the two routes of administration assessed. Immunoblotting targeting Dck and Tk1 in mice at days 4 and 29 showed up-regulation of Dck in brain and muscle and down-regulation of Tk1 in all tissues except intestine.

Conclusions: TK2 deficiency manifests predominantly as a fatal progressive mitochondrial myopathy during infancy or childhood. We previously showed that treatment with oral dC and dT, the substrates of TK2, delays disease onset and prolongs lifespan in our Tk2⁻/⁻ mouse model. Our study demonstrates that both, bioavailability of dC and dT after treatment and expression of Dck and Tk1 enzymes in target tissues contribute to the response to dC+dT therapy. Down-regulation of Tk1, and not Dck, accounts for the lack of long-term efficacy of dC+dT therapy in Tk2⁻/⁻ mice. By understanding factors contributing to efficacy of dC+dT treatment, we may be able to further optimize this therapy and translate it to patients with mtDNA Depletion Syndrome.

KEYWORDS: thymidine kinase 2, TK2, mtDNA depletion syndrome, deoxycytidine, thymidine.
Abstract P19
Tenofovir causes bone loss via decreased bone formation and increased bone resorption, which can be counteracted by adenosine A2A receptor in mice

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Objectives: Osteopenia and fragility fractures have been associated with HIV infection. Tenofovir, a common antiviral in HIV treatment, also leads to increases in bone catabolism markers and decreased bone mineral density (BMD) in children and young adults. In murine models and human cell lines, tenofovir inhibits ATP release and decreases extracellular adenosine levels. Adenosine, and adenosine A2A receptor, inhibits osteoclast formation, and increasing local adenosine concentration with dipyridamole, an agent that blocks adenosine cellular uptake, stimulates new bone formation as well as rhBMP-2. We hypothesized that tenofovir regulates bone resorption by diminishing endogenous adenosine levels and determined whether dipyridamole and adenosine A2A receptor may counteract the deleterous bone effects of tenofovir.

Methods: M-CSF/RANKL-induced-primary murine osteoclast was studied as the number of TRAP-positive-cells after challenge with tenofovir alone or in combination with dipyridamole. Differentiation markers were study by RT-PCR, and MAPK/NFkB expression by Western Blot. Male C57Bl/6 mice and A2AKO littermates were treated as follow: saline 0.9% (control), tenofovir 75mg/Kg/day, dipyridamole 25mg/Kg/day, combination tenofovir/dipyridamole (n=10, 4 weeks). Calcein/AlizarinRed-labelling of newly formed bone was used, and long bones were prepared for microCT/histology. Blood was collected at time of sacrifice and serum phosphorous was analized.

Results: Tenofovir produced a dose-dependent increase in osteoclast differentiation (EC50=44.5nM) that was reversed by dipyridamole (IC50=0.3µM). Tenofovir increased Cathepsin K and NFATc1 mRNA levels and dipyridamole reversed the effect. Dipyridamole reversed the effect of tenofovir on pERK1/2, pp38 and NFkB nuclear translocation. WT mice treated with tenofovir lost nearly 10% of body weight (p<0.001). MicroCT revealed decrease BMD and altered trabecular bone in tenofovir-treated mice, reversed by dipyridamole. TRAP-staining showed increased osteoclasts in tenofovir-treated mice (p<0.005) an effect reversed by dipyridamole. Similar results were obtained for Cathepsin K and CD68. RANKL-positive-cells were increased in tenofovir-treated mice whereas OPG-positive-cells decreased, and both effects were reversed by dipyridamole. No significant changes in phosphorus levels were found with any treatment, and neither signs of osteomalacia (measured as osteoid thickness) were found in tenofovir treated mice. Similar results were obtained for tenofovir in A2AKO mice, but dipyridamole was not able to reverse the effect.

Conclusions: These results suggest that treatment with agents that increase local adenosine concentrations, like dipyridamole, and activate adenosine A2A receptor might prevent bone loss following tenofovir treatment.

KEYWORDS: tenofovir, dipyridamole, HIV, bone turnover, osteopenia.
Abstract P20
Development of HPRT activity assay method using dried blood spot by UPLC-MS/MS

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Objectives: Lesch-Nyhan Syndrome (LNS) is an X-linked recessive disorder of purine metabolism which is caused by a deficiency of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) enzyme that transforms hypoxanthine to inosine monophosphate (IMP). LNS is characterized by the overproduction of uric acid, which is metabolized from excess hypoxanthine. HPRT activity assay using erythrocyte or fibroblast was previously reported as a reliable method\textsuperscript{1,2). Simple assay to measure HPRT activity might make it possible to diagnose LNS in its early stages and may have benefit to predict and treat symptoms. The aim of this study is to develop a convenient method to determine HPRT activity in dried blood spot (DBS) using UPLC-MS/MS.

Methods: DBS samples from 5 healthy volunteers were used to decide reaction conditions. HPRT activity of genetically confirmed male patients with LND and some suspicious pediatric patients with hyperuricemia were measured using their DBS samples. The 100 µL of water was added to 7mm diameter and dissolved on shaking at room temperature for 30 minutes. 90µL of DBS solution was mixed with 5mM phosphoribosyl diphosphate (PRPP), 2mM \textsuperscript{15}N\textsubscript{2}hypoxyaxanthine, 50mM MgCl\textsubscript{2}, BSA and 0.5M Tris-HCl buffer, then incubated at 37 °C for 20 minutes. 600mM CCL3COOH was added to stop reaction. 10µL of 200µM caffeine was added as IS. The samples were centrifuged and the supernatant was analyzed by UPLC-MS/MS. The mobile phase was 50 mM HCOONH\textsubscript{4} and acetonitrile at gradient ratio of 100:0 to 58:42. To evaluate impact of storage at various temperatures, control samples were stored at different temperatures for 30 days after three hours drying at RT. The production of \textsuperscript{15}N\textsubscript{2}IMP in each group of DBS was measured in time course.

Results: The chromatogram of enzyme reaction mixture showed a good separation between \textsuperscript{15}N\textsubscript{2}IMP, \textsuperscript{15}N\textsubscript{2}hypoxanthine, and caffeine demonstrating acceptable sensitivity. Concentration of \textsuperscript{15}N\textsubscript{2}IMP produced at each reaction time-point was directly proportional to the \textsuperscript{15}N\textsubscript{2}IMP enzyme reaction time. Average ± SD of produced \textsuperscript{15}N\textsubscript{2}IMP from 5 control DBS samples was 115.43 ± 14.95 nmol/min/7mmDBS and CV value was 12.95. No \textsuperscript{15}N\textsubscript{2}IMP peak was detected in DBS samples of LND patients. HPRT activities of pediatric patients with hyperuricemia and parents of LND patient showed within normal range. The enzyme activity of control was the highest at 1\textsuperscript{st} day, then decreased to 56% to 66% of initial \textsuperscript{15}N\textsubscript{2}IMP amount within 3 days, then kept stable for 30 days at -20 °C and 4 °C, but gradually decreased at RT.

Conclusions: The \textsuperscript{15}N\textsubscript{2}IMP converted from isotopically labeled hypoxanthine in solution from DBS was quantitatively measured by UPLC-MS/MS. HPRT enzymatic activity in DBS was unstable for first three days after collection of blood, however HPRT activities became stable after 3\textsuperscript{rd} day up to 30 days when it's was stored at -20 °C and 4 °C. In our study, we did not have sample from mild phenotype patient, but it has previously reported that HPRT activity in erythrocyte could not distinguish female heterozygote carrier from normal\textsuperscript{3}. Our method is very convenient and simple so may be useful for high risk screening to detect severe male patient in early stage, among children presenting neurological abnormalities and hyperuricemia.

Relevant references:

KEYWORDS: Lesch-Nyhan Syndrome, HPRT activity, dried blood spot, UPLC-MS/MS
Abstract P21

Venetoclax and alvocidib are cytotoxic to the nucleoside analog-resistant acute myeloid leukemia HL-60 variants with BCL-2 and MCL-1 overexpression in vitro

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Objectives: To improve therapeutic outcomes of acute myeloid leukemia (AML), new treatment regimens and agents are needed. Cytarabine (ara-C) is the key drug for more than 40 years. Ara-C is transported into the cells through a nucleoside transporter hENT1, then activated to ara-C triphosphate using deoxycytidine kinase. Ara-C triphosphate terminates DNA synthesis, thereby inhibiting the growth and inducing apoptosis\textsuperscript{1}. Clofarabine is a similar nucleoside analog, but pharmacologically more advantageous than ara-C. Clofarabine uses hENT1, 2 and hCNT3 as transporters, and deoxycytidine kinase and deoxyguanosine kinase for the intracellular activation\textsuperscript{2}. Clofarabine is widely used for AML chemotherapy in the relapsed/refractory setting. Recently, the augmented antiapoptosis is found to play a critical role in the development of the chemotherapy resistance. Clinical studies of inhibitors of antiapoptosis proteins are underway. The present study investigated antileukemic effects of antiapoptotic BCL-2 inhibitor venetoclax and MCL-1 inhibitor alvocidib on nucleoside analog-resistant HL-60 variants in vitro.

Methods: The human acute myeloid leukemia HL-60 cells, one cytarabine-resistant variant (HL-60/ara-C), and two clofarabine-resistant variants (HL-60/CAFdA4, HL-60/CAFdA30, respectively) were used. XTT assay was used for determining 50% cell-growth inhibitory effect (IC50) values, while annexin V positivity was used for calculating apoptotic cell death. Synergism (combination index) was evaluated using the computer software CalcuSyn. Intracellular cytarabine and clofarabine triphosphate production was measured by HPLC\textsuperscript{3}.

Results: Compared with HL-60, HL-60/ara-C was 10-fold more ara-C-resistant, HL-60/CAFdA4 was 4-fold more CAFdA-resistant, and HL-60/CAFdA30 was 30-fold more CAFdA-resistant. Protein expression levels of deoxycytidine kinase was reduced in all the resistant cell lines. Consequently, cytarabine triphosphate and clofarabine triphosphate productions were decreased in these cell lines compared with HL-60. Of note, antiapoptotic BCL-2 protein was overexpressed in the resistant cells, suggesting the contribution of antiapoptosis to the mechanisms of the drug resistance as well (densitometer values, 0.90 for HL-60, 1.82 for HL-60/ara-C, 1.72 for HL-60/CAFdA4, 2.12 for HL-60/CAFdA30). IC50 values of venetoclax alone were 30 nM for HL-60 and 1,200 nM for HL-60/ara-C, 330 nM for HL-60/CAFdA30, suggesting that venetoclax was cytotoxic to nucleoside analog-resistant cells with BCL-2 overexpression, but less effective for the higher BCL-2 level. Moreover, another antiapoptotic MCL-1 protein was also overexpressed in these variants (densitometer values, 0.20 for HL-60, 0.54 for HL-60/ara-C, 1.77 for HL-60/CAFdA4, 1.64 for HL-60/CAFdA30). MCL-1 inhibitor alvocidib inhibited the growth of HL-60/ara-C (IC50, 194 nM) HL-60/CAFdA4 (IC50, 196 nM) and HL-60/CAFdA30 (IC50, 163 nM) as potently as HL-60 (IC50, 87 nM). The combination of venetoclax with either nucleoside analog synergistically inhibited the cell growth of these HL-60 variants (combination index: 0.2 for HL-60/ara-C and HL-60/CAFdA4, 0.8 for HL-60/CAFdA30). This combination induced the more-than-additive apoptosis than the single agent of each.

Conclusions: Venetoclax and alvocidib were cytotoxic to the nucleoside analog-resistant HL-60 variants. Incorporation of venetoclax into conventional chemotherapeutic agents may be an effective strategy for AML in the relapsed/refractory setting.

Relevant references:

KEYWORDS: AML, Bcl-2, Mcl-1, Venetoclax, Alvocidib
Objectives: As we suggested previously\(^1\) non-purine analogue inhibitors of xanthine oxidoreductase (XOR) delayed the progress of degeneration of horns cells in ALS model mice and suggested the important role of energy supply into neuronal cells, which do not contain XOR, by salvage of hypoxanthine accumulated in blood caused by inhibition of XOR in other XOR containing organs. In this time the effects of XOR inhibitors to the Alzheimer model mice have been examined for long months to see the pathological and behavioral changes. In order to understand the mechanism, the determination of ATP and other adenine nucleotides have been performed in various model animals and cells.

Methods: We examined the effects of various XOR inhibitors to the Alzheimer model mice and observed the pathological effect and change of behaviors for more than 6 months. In order to elucidate the mechanism of these effects, we have determined the XOR activity, protein and uric acid as well as HPRT activity in various sources including marines, rabbit and human blood cells as well as neuronal cells including human iPS-induced neuronal cells. We used HPLC, NMR, LC-Mass for determination of these compounds.

Results: Marked delay of accumulation of amyloid proteins and tau-proteins in the brain cells were observed in the Alzheimer model mice by treatment of non-purine analogue inhibitors, but not significantly by allopurinol, a purine analogue. HPRT activity of red blood cells was very high in rabbit and human, but very low in murine. HPRT activity was found to be very high also in human iPS neuronal cells. We confirmed allopurinol is a substrate of HPRT and the competitive inhibitor of HPRT. In the cell level experiments, marked delay of ATP consumption of the cells was observed in the presence of hypoxanthine after the addition of mitochondrial un-coupler to the cells. It was also found significant amount of isotope labelled hypoxanthine were incorporated into ATP, but minimum amount via \textit{de novo} pathway.

Conclusions: Salvage of hypoxanthine into neuronal cells plays the key role in delay and prevention of the progression of neurodegenerative diseases by maintain ATP concentration in the cells. Administration of appropriate inhibitors of XOR could be an important strategy for neurodegenerative diseases.

Relevant references:

KEYWORDS: Xanthine oxidoreductase inhibitor, HPRTase, Salvage pathway, ATP concentration, Alzheimer disease.
Effect of female hormone and menopause on uric acid clearance

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Objectives: Serum uric acid levels in women are lower than that in men, which is supposed to be female hormone’s effect. We clarified female hormone level or menopause affects to clearance of urate by using our previous data that measured female hormone and the clearance of urate at the same time.

Methods: The original study (RICE-U study: UMIN000015142) was to decide reference range on uric acid excretion kinetics of healthy subjects using guidelines of the CLSI (Clinical and Laboratory Standards Institute). We set the target as 429 women who were registered in the RICE-U study. Menopause was determined by asking participants’ amenorrhea of 12 months or more on each questionnaire.

Results: The participants have registered in each age. There were 31 women in the postmenopausal group who were without menstruation more than 12 months on the interview and 214 women were in the premenopausal group. Regarding uric acid kinetics, the uric acid clearance value (Cua 60 min value) was 10.3 ± 3.6 mL/min in the postmenopausal and 10.4 ± 3.3 mL/min in the premenopausal group, and no significant difference was found between both groups. There was no significant difference between the premenopausal group and the postmenopausal group as to the serum uric acid value. There was no correlation between estradiol and Cua 60 min values in females. The effect of the female hormone or menopause on uric acid clearance value was almost not observed.

Conclusions: We concluded the effect of the female hormone or menopause doesn’t affect uric acid clearance value.

Relevant references:
- Studies on hyperuricemia of gout by means of the uric acid clearance. Uric Acid Research 1:45-61, 1977 (Japanese)
- Sex hormone and Uric acid Hyperuricemia and Gout. 6:47-51, 1998 (Japanese)

KEYWORDS: Uricosuria, menopause, estradiol, hyperuricemia
Abstract P24

Effect of prolonged physical exercise on nicotinamide metabolism in marathon runners

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Objectives: Running became the most popular form of aerobic exercise that is also recommended even for a patient with heart failure. However, prolonged running, such as a marathon may result in an increase of CRP and muscle damage markers as well as can affect kidney function. Nicotinamide (NA) is exogenously derived NAD precursor present in the human body. N-methylnicotinamide (MNA) and nicotinamide riboside (NR), 4-pirydone-3-carboxamide-1β-D-ribonucleoside (4PYR) and N'-methyl-2-pyridone-5-carboxamide (Met2PY), N'-methyl-4-pyridone-3-carboxamide (Met4PY) are NA metabolites and some are uremic toxins. Marathon impact on kidney function presumably could be observed as an accumulation of uremic toxins in plasma. Therefore, this study aimed to investigate the relations between prolonged physical exercise and nicotinamide metabolism.

Methods: Plasma samples of patients before, just after and 7 days post marathon were collected. Plasma nicotinamide metabolites concentrations were determined using high performance liquid chromatography - mass spectrometry (LC/MS).

Results: Our results shown that just after marathon a concentration of NR in plasma decreased (0.111±0.009 vs 0.190±0.012 µmol/l) and a concentration of Met4PY and 4PYR increased (0.308±0.037 vs 0.166±0.017 µmol/l, 0.063±0.007 vs 0.049±0.009 µmol/l) compared to the state before marathon. Concentration of compounds, measured in this study, reached similar to basic level 7 days after marathon.

Conclusions: Extreme physical exercise such as in marathon induces accumulation of NA metabolites including uremic toxins such as 4PYR. However, it returns to normal values after one week.

KEYWORDS: 4-pirydone-3-carboxamide-1β-D-ribonucleoside, marathon, uremic toxin
Impact of FLT3 modulation on cytarabine metabolism

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Objectives: Previous results of our group showed a functional link between fms-like tyrosine kinase 3 (FLT3) and the human equilibrative nucleoside transporter 1 (hENT1). Inhibition of FLT3 induced a decrease in hENT1 activity (Català \textit{et al.} 2016) modulating cytarabine cytotoxicity which is internalized by this transporter. The aim of this work is to elucidate the effect of FLT3 modulation on key enzymes of cytarabine metabolism. Deoxycytidine kinase (dCK) mediates the first and rate-limiting step of cytarabine phosphorylation, cytosolic nucleotidase II (cN-II) reverses the action of dCK, and SAM domain and HD domain-containing protein 1 (SAMHD1) is responsible for Ara-CTP deactivation.

Methods: The amounts of mRNA of the tyrosine kinase receptor FLT3 and the enzymes of nucleotide metabolism dCK, cN-II and SAMHD1 were quantified by RT-PCR in samples from pediatric acute leukemia patients. Two cell lines, SEM and MV4-11, with different expression of FLT3 were used for \textit{in vitro} experiments. RT-PCR and Western Blot were performed after FLT3 modulation (inhibition or activation) to study effects on the expression of the enzymes. Cell viability after treatment combinations was measured using the MTT assay.

Results: dCK, cN-II and SAMHD1 positively correlated with FLT3 mRNA levels in pediatric acute leukemia patients. FLT3 inhibition caused a significant increase in cN-II and SAMHD1 mRNA levels in MV4-11 cells which present a constitutively active receptor independent of their ligand (FLT3L). The activation of FLT3 in SEM cells that respond to FLT3L showed the contrary effect, confirming the FLT3 implication in that changes. The inhibition of cN-II in the combination of cytarabine and FLT3 inhibitor does not significantly decrease cell viability which can indicate that the increase of cN-II is not a major cause of resistance in this combination. Furthermore, under FLT3 inhibition SAMHD1 is activated by dephosphorylation and could be inactivating Ara-CTP, decreasing the action of this drug.

Conclusions: FLT3 can modulate cN-II and SAMHD1. This might be relevant and has to be taken in consideration in treatment schedules including FLT3 inhibitors in combination with nucleoside analogs, such as leukemia treatment.

Relevant references:

KEYWORDS: nucleotide metabolism, cytarabine, FLT3, pediatric leukemia
Objective: RX-3117 (fluorocyclopentenylcytosine) is a novel cytidine analog with unique properties compared to other cytidine analogs such as gemcitabine. RX-3117 is currently being evaluated in combination with Abraxane in metastatic pancreatic cancer and as a single agent in advanced urothelial bladder cancer. In contrast to gemcitabine, RX-3117 is not activated by deoxycytidine kinase (dCK) but selectively by uridine-cytidine kinase 2 (UCK2), which is not expressed in normal tissues. RX-3117 is not a substrate for cytidine deaminase (CDA) like most other cytidine analogs. However it is an excellent substrate for the human equilibrative nucleoside transporter (hENT) and most likely also for the human concentrative nucleoside transporter (hCNT). Notably RX-3117 inhibits DNA methyltransferase 1 (DNMT1). In order to determine which properties would be important for the sensitivity to RX-3117, we characterized the gene expression of these transporters and enzymes in human xenografts and derived cell lines, while several xenografts were also characterized for sensitivity to RX-3117.

Methods: Xenografts were grown in nude mice and originated from either established ATCC cell lines or patient derived xenografts (PDX). Tumors were removed from anesthetized mice and frozen in liquid nitrogen. The frozen tumors were pulverized and RNA was isolated and reverse transcribed to cDNA according to standard protocols. Gene expression was measured using Taqman PCR or a Roche Light Cycler FastStart DNA MasterPlus PCR kit in combination with a Lightcycler 2.0 (Roche) and related to that of β-actin or GAPDH. UCK enzyme activity was determined with radioactive uridine and RX-3117. Some tumors were characterized with a specific UCK2 antibody. RX-3117 was administered orally at 300 mg/kg three times a week.

Results: In 28 xenografts (pancreas, lung, colon, lymphoma, myeloma) and 13 cell lines, we determined gene expression. Both UCK1 and UCK2 showed a large variation; interestingly the gene expression of UCK2 was related to the UCK enzyme activity measured with RX-3117 (r= 0.63), but not with uridine, supporting the specificity of RX-3117 for UCK2. UMP-kinase showed a similar variation. hENT was expressed in all cell lines and xenografts, but the variation in hCNT was much larger and was not detectable in 2 cell lines and 11 xenografts. The dCK expression was highest in leukemic cell lines and xenografts (variation from 459-29111, with one not detectable). The gene expression of the gemcitabine targets, both ribonucleotide reductases (RRm1 and RRm2) showed a large variation in all cell lines and xenografts, while in xenograft the expression of RRm1 was higher than that of RRm2. The target of RX-3117, DNMT1, showed a large variation in both the cell line panel (from 0.58-7.3) and even larger in the xenograft panel, with 3 xenografts at the detection level. Four xenografts, selected for their UCK2 expression, were tested for their sensitivity to RX-3117, in which it was shown that sensitive (including one pancreatic cancer) tumors had a relatively high UCK2 activity and a high UCK2 protein expression.

Conclusions: the panel of cell lines and xenografts, representing most of the common human tumors, showed a large variation in the expression of pyrimidine enzymes essential for the antitumor activity of several nucleoside analogs, such as gemcitabine and the novel analog RX-3117. Interestingly in some of the selected tumors, UCK2 activity was related to RX-3117 sensitivity

KEYWORDS: RX-3117, uridine-cytidine kinase, DNA methyltransferase, ribonucleotide reductase, equilibrative nucleoside transporter
A ball milling strategy for the synthesis of dinucleotides

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Objectives: Given the importance of nucleotides and their derivatives in biological processes, numerous methods have been developed to access to these compounds and their structural analogues.\textsuperscript{[1]} Their scope ranges from mechanistic probes to versatile chemical tools for assay development and biomedical applications. Most of these syntheses present important drawbacks, such as the use of non-volatile and harmful solvents (DMF, pyridine), preparation of substrates or phosphorus reagents in their alkyl ammonium form due to solubility issues, anhydrous conditions, and fastidious purifications.

Methods: We have developed an original one-pot and protecting-group free approach, which is user-friendly and reliable, for 5',5'-dinucleotides starting from nucleoside 5'-monophosphates. The main benefits are a convenient set-up, i.e. non-dry solvent and reagents, use of substrates in their sodium or acid form, and commercially available and cheap phosphorus reagents as sodium or potassium salts.

Results: The solvent-assisted mechanochemical approach allows to prepare symmetrical and mixed dinucleoside 5,5'-polyphosphates.\textsuperscript{[2]} Under ball-milling conditions, nucleoside 5'-monophosphates are quantitatively activated using the eco-friendly N,N'-carbonyldiimidazole into their phosphorimidazolide derivatives. The addition of a nucleoside 5'-mono, di or triphosphate directly leads to the formation of the corresponding dinucleotides. Other advantages of this one-pot method include short reaction times, high conversion rates and easier purification steps.

Conclusions: Since phosphorimidazolides are essential intermediates for pyrophosphate bond formation, our studies offer new perspectives for the development of greener approaches to access a wide range of nucleotide derivatives and analogues.

Relevant references:

KEYWORDS: 1,1' carbonyldiimidazole, green chemistry, mecanochemistry, nucleotides, pyrophosphate
**Modified forearm ischemic test in hypouricemic patients**

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**Objectives:** The forearm ischemic exercise test is used for the screening of anaerobic metabolic myopathies such as glycogen storage disease V and myoadenylate deaminase deficiency. Hereditary hypouricemia sometimes leads to exercise-induced acute kidney injury (EIAKI) of unknown pathogenesis. In order to elucidate the various pathological conditions associated with hypouricemia, we analyzed the effects of low uric acid level in the body and ischemic exercise on energy metabolism.

**Methods and Results:** We have modified semi-ischemic forearm test and performed this test in four patients with hereditary renal hypouricemia and one patient with hereditary xanthinuria of Czech origin. All patients had given written informed consent to all aspects of the test. The patients were asked to rest for 30 minutes before exercise. After that blood pressure was measured and mean blood pressure was calculated. The first blood sampling was taken intravenously before exercise loading; at this time, urine sample was taken and analyzed, too. The sphyngmomanometer cuff on the upper arm was inflated to a mean blood pressure (previously described to 200 mmHg) during forearm exercise. Forearm exercise was performed by squeezing a hand dynamometer at one pace per second for 3 minutes as powerfully as possible. The sphyngmomanometer cuff pressure was kept for 10 minutes from the start of exercise. Venous blood was drawn from the antecubital vein at 3, 10, 30 and 45 minutes, respectively after the start of exercise. We measured two metabolites, lactate and hypoxanthine, which are markers of tissue hypoxia. An increase in hypoxanthine concentration indicates a lack of ATP regeneration due to tissue hypoxia. The mean plasma lactate concentration increased from a baseline of 1.3 (range 0.7-1.8 mmol/L) to 4.0 (range 2.0-5.5 mmol/L). The plasma hypoxanthine concentrations were quite low before exercise (0-2.9 μmol/L), but increased markedly to a range of 13.6-28.8 μmol/L after 10 minute forearm ischemia. Only one patient with hereditary renal hypouricemia had poor lactate and hypoxanthine response (from 1.3 to 2.0 mmol/L in lactate and from 0 to 4.3 μmol/L in hypoxanthine).

**Conclusions:** Our protocol allowed us to conclude that the load was sufficient for observing metabolic changes in temporally hypoxia and in following recovery phase. The test was well tolerated and safe, we did not observe any adverse reactions including EIAKI.

**KEYWORDS:** forearm ischemic test, hereditary xanthinuria, hereditary renal hypouricemia, hypoxanthine, lactate
Clinical manifestations and molecular aspects of phosphoribosylpyrophosphate synthetase superactivity in females

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Objectives: Phosphoribosylpyrophosphate synthetase (PRPS1) superactivity is an X-linked disorder characterized by urate overproduction Online Mendelian Inheritance in Man (OMIM) gene reference 300661. This condition is thought to rarely affect women, and when it does, the clinical presentation is mild. We describe a 16-year-old African American female who developed progressive tophi, nephrolithiasis and acute kidney failure due to urate overproduction. Family history included a mother with tophaceous gout who developed end-stage kidney disease due to nephrolithiasis and an affected sister with polyarticular gout. The main aim of this study was to describe the clinical manifestations of PRPS1 superactivity in women.

Methods: Whole exome sequencing was performed in affected females and their fathers.

Results: Mutational analysis revealed a new c.520 G > A (p.G174R) mutation in the PRPS1gene. The mutation resulted in decreased PRPS1 inhibition by ADP.

Conclusion: Clinical findings in previously reported females with PRPS1 superactivity showed a high clinical penetrance of this disorder with a mean serum urate level of 8.5 (4.1) mg/dl [506 (247) μmol/l] and a high prevalence of gout. These findings indicate that all women in families with PRPS1 superactivity should be genetically screened for a mutation (for clinical management and genetic counselling). In addition, women with tophaceous gout, gout presenting in childhood, or a strong family history of severe gout should be considered for PRPS1 mutational analysis.


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KEYWORDS: hereditary gout, PRPS1 superactivity, rare disease, chronic kidney disease, hyperuricaemia
Abstract P30

Clinical and functional characterization of a novel URAT1 dysfunctional variant in a pediatric patient with renal hypouricemia

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Objectives: Renal hypouricemia (RHUC) is caused by an inherited defect in the main renal urate transporters, URAT1 and GLUT9. RHUC is characterized by decreased concentrations of serum uric acid and an increase in its excretion fraction. Patients suffer from hypouricemia, hyperuricosuria, urolithiasis, and even acute kidney injury. We report the clinical, biochemical, and genetic findings of a pediatric patient with hypouricemia.

Methods: Sequencing analysis of the coding region of \textit{SLC22A12} and \textit{SLC2A9} and a functional study of a novel RHUC1 variant in the \textit{Xenopus} expression system were performed.

Results: The proband showed persistent hypouricemia (67–70 µmol/l; ref. range 120–360 µmol/l) and hyperuricosuria (24–34%; ref. range 7.3 ± 1.3%). The sequencing analysis identified common non-synonymous allelic variants c.73G>A, c.844G>A, c.1049C>T in the \textit{SLC2A9} gene and rare variants c.973C>T, c.1300C>T in the \textit{SLC22A12} gene. Functional characterization of the novel RHUC associated c.973C>T (p.R325W) variant showed significantly decreased urate uptake, an irregular URAT1 signal on the plasma membrane, and reduced cytoplasmic staining.

Conclusions: RHUC is an underdiagnosed disorder and unexplained hypouricemia warrants detailed metabolic and genetic investigations. The key to identifying the disorder is a greater awareness of URAT1 and GLUT9 deficiency by primary care physicians, nephrologists, and urologists.

KEYWORDS: URAT1; hypouricemia; uric acid transporters; excretion fraction of uric acid
Measurement of total purine content and free nucleosides, nucleotides and purine bases composition in Japanese anchovies (Engraulis japonicas) using high-performance liquid chromatography

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Objectives: In our laboratory, we measured the purine contents of various foods and we revealed that niboshi (which are small dried sardines) contains a large amount of purines. (1) In Japan, niboshi is used as a seasoning in various soup. Its flavor contains nucleic acid umami ingredients such as IMP and GMP. Although Japanese anchovy could be eaten as it is, it is often eaten after dried or processed by salted fermentation. We also observed the change in purine composition of a fermented fish product (i.e., fish soaked in sake lees). (2) This study aims to observe the amount and compositions of purines in raw Japanese anchovies.

Methods: Japanese anchovies are obtained from the seas near Nagasaki. After thawing, the head, internal organs, backbone, and tail fin of the anchovies are removed. We used high-performance liquid chromatography (HPLC) to measure purines. Two HPLC methods we developed previously were used. In method 1, the raw fillet was hydrolyzed with perchloric acid to obtain purine bases (adenine, guanine, hypoxanthine, and xanthine). Then it was treated with xanthine oxidase enzyme and measured using HPLC. (1) In method 2, raw filet was deproteinized by homogenization in 70% ACN without acid hydrolysis. After that, 17 types of purines (i.e., ATP, ADP, AMP, GTP, GDP, GMP, IMP, XMP, adenosine, guanosine, inosine, xanthosine, adenine, guanine, hypoxanthine, uric acid and xanthine ) were simultaneously detected using the HPLC method 2. (3)

Results: After hydrolysis through method 1, the total content of the purine bases in raw Japanese anchovies was 303.9 mg/100g. Among the raw fish, it contained a particularly large amount of purines. Hypoxanthine and guanine were included 40% and 51% of the total purine, respectively. Thus, their composition was confirmed through method 2. In the hypoxanthine group, the concentrations of IMP, with the highest concentration, inosine, and hypoxanthine were 152.3 ± 35.8, 38.5 ± 7.5, and 5.1 ± 0.7 mg per 100 g, respectively. Meanwhile, in the guanine group, guanosine had the highest concentration of 2.6 mg/ 100 g; only a small amount of guanine was found.

Conclusions: A large amount of purines belonging to the hypoxanthine group was found after hydrolysis, and it depends on IMP and inosine. Guanine is reported to exist in fish skin as guanine crystals. Therefore, it was considered that the guanine base was observed in many amounts because the guanine crystals were only dissolved in the acid solution of method 1. Future studies need to focus on measuring the fermented anchovies and studying the comparison between the raw Japanese anchovy and its fermented counterpart.

Relevant references:

KEYWORDS: Purines, Anchovy, HPLC, Umami, Fermented food
Abstract P32

Nucleotides metabolism failure as a new clue for hunting the cause of Huntington’s disease related cardiomyopathies

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Objectives: Huntington’s disease (HD) is a neurodegenerative disorder with a significant peripheral component to the disease pathology. This includes an HD-related cardiomyopathy, with an unknown pathological mechanism. In this study, we characterized changes in cardiac nucleotides metabolism in the HD mouse models. Moreover, we aimed to assess the concentrations of major adenine nucleotides and their catabolites in plasma of symptomatic HD patients.

Methods: We examined R6/2 transgenic and HdhQ150 knock-in mice and their WT littermates. To investigate changes in the nucleotides metabolism, the concentration of adenine and guanine nucleotides, creatine metabolites and nicotinamide adenine dinucleotides were measured. Activity of eNTPD, AMPD, e5'NT, ADA and PNP as well as cardiac and serum/plasma concentration of nucleotides catabolites were measured with high pressure liquid chromatography (HPLC). Protein concentration was measured with Bradford method. We evaluated cardiac substrate preferences in both of HD mouse models using 13C glucose isotopomer and LC-MS method. Analysis of genes transcripts were performed using RT-qPCR. Moreover, level of AMP regulated protein kinase phosphorylation in HD mouse models hearts was measured with ELISA KIT.

Results: We observed a notable energy metabolism deterioration in hearts of HD mice (ATP/ADP ratio = 6.39 ± 0.46 in R6/2 and 3.57 ± 0.57 in WT; 8.56 ± 1.84 in HdhQ150 and 5.98 ± 0.55 in WT). We demonstrated AMPK over-activation in hearts of HD mice that was accompanied by shift in a cardiac substrate preference from glucose to fatty acids. We found a reduced activity of AMPD (12.9 ± 1.9 in control; 7.5 ± 0.5 nmol/min/mg protein in HD) and e5'NT (11.9 ± 1.7 in control; 6.7 ± 0.7 nmol/min/mg protein in HD). Moreover, we detected an increased activity of ADA (1.3 ± 0.2 in control; 5.2 ± 0.5 nmol/min/mg protein in HD), while no changes in eNTPD and PNP activities were observed. HD mouse model hearts were characterized also by increased inosine (0.7 ± 0.01 in control; 2.7 ±0.8 nmol/mg dry tissue in HD) and reduced concentration of cardiac adenosine (0.9 ± 0.2 in control; 0.2 ± 0.08 nmol/mg dry tissue in HD). Moreover, we found a significant down-regulation of genes involved in purine de novo biosynthesis (Adenylosuccinate lyase, Phosphoribosylglycinamide formyltransferase), conversion of adenine to guanine nucleotides (Guanine monophosphate synthetase), adenine nucleotides inter-conversion (Adenylate kinase 1) and up-regulation of transcripts of genes involved in adenosine degradation (Adenosine deaminase, Dipeptidyl peptidase-4). This was accompanied by an increases in concentration of nucleotide catabolites such as inosine, hypoxanthine, xanthine, uric acid and uridine in HD mouse model serum, in comparison to their wild type littermates. Interestingly, we observed prominently increased levels of hypoxanthine and uridine also in HD patients plasma, in comparison to their healthy controls. Moreover, hypoxanthine and uridine levels strongly correlated with HD disease progression parameters.

Conclusions: This study highlights a profound deregulation in cardiac energy and nucleotides metabolism in HD mouse models. We suggest that mutant huntingtin disrupts coupling of cardiac energy metabolism with its regulatory pathways that despite its activation is unable to ensure recovery. Consequently, hearts and possibly other organs remains energy depleted that translate into elevated nucleotide catabolites concentration and suppression of nucleotide synthetic pathways. Furthermore, for the first time, our study identified biomarkers that might be linked to HD progression both in pre-clinical and clinical settings. Restoration of energy equilibrium in HD hearts may be important therapeutic target in HD.

KEYWORDS: Huntington’s disease, cardiomyopathy, energy imbalance, nucleotides catabolism
Functional interactions between de novo pyrimidine biosynthesis, innate immunity and lipogenesis in hepatocytes

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Objectives: Pyrimidine biosynthesis is regarded as a prime target in cancer therapies, autoimmune diseases and microbial infections. In the last decade, numerous drugs have been developed to target dihydroorotate dehydrogenase (DHODH), the fourth enzyme of the pathway. Interestingly, pyrimidine biosynthesis is tightly connected to the innate immune response. Indeed, the cellular stress induced by DHODH inhibition is also priming the interferon response, a pathway that is critical to antiviral and antitumoral immunity. We have contributed to the field by identifying new DHODH inhibitors and deciphering parts of the mechanisms linking pyrimidine biosynthesis to innate immunity and the antiviral response (1,2,3). More recently, we turned our interest on functional interactions between DHODH and the \textit{de novo} biosynthesis of lipids, \textit{i.e.} neolipogenesis, as the latter is also greatly intertwined to innate immunity. Furthermore, previous reports have shown that pyrimidine biosynthesis inhibition and uridine could modulate lipid biosynthesis in liver cells (4,5).

Methods: To study the impact of \textit{de novo} pyrimidine biosynthesis on neolipogenesis, we took advantage of a hepatocyte cell line recently engineered in the laboratory that, in contrast to standard hepatocellular carcinoma cells, is able accumulate and synthesize lipids alike primary hepatocytes. These cells were treated with either DHODH inhibitors or uridine, and neolipogenesis was monitored by lipid droplet staining, quantification of secreted apolipoprotein, cholesterol and phosphatidylcholine (PC).

Results: Our preliminary data show that pharmacological inhibition of DHODH is increasing triglyceride levels in treated hepatocytes as assessed by BODIPY staining. Uridine has opposite effects, decreasing intracellular levels of triglycerides.

Conclusions: Our results confirm that pyrimidine biosynthesis is tightly bound to lipogenesis, and that our cellular model is suitable to study this interaction \textit{in vitro}. Taking advantage of this model, we will now explore the mechanisms linking pyrimidine biosynthesis to neolipogenesis. We will also determine consequences of such lipid accumulation on the replication of viruses that critically depend on lipids for their replication cycle as well as antiviral and antitumoral innate immunity in the liver.

Relevant references:


KEYWORDS: dihydroorotate dehydrogenase, pyrimidine biosynthesis, lipogenesis, hepatocytes, non-alcoholic fatty liver disease.
**Abstract**

*Lactobacillus gasseri* PA-3 incorporates purine mononucleotides and utilizes them for RNA/DNA synthesis

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**Objectives:** Several studies have reported purine/pyrimidine metabolism of lactic acid bacteria are using *Lactococcus lactis* (*L. lactis*) species. J. Martinussen reported that the mononucleotides themselves cannot be incorporated but after they are dephosphorylated externally by nucleotidase to the corresponding nucleoside and then incorporated in *L. lactis*. Regardless of this report, we have previously reported that *Lactobacillus gasseri* PA-3 (PA-3), which is a kind of lactic acid bacteria, incorporates purine mononucleotides such as adenosine 5’-monophosphate (AMP), inosine 5’-monophosphate (IMP) and guanosine 5’-monophosphate (GMP). However, it remains unclear whether those bacteria directly incorporate mononucleotides or incorporate them after dephosphorylating into corresponding nucleosides. In order to clear this question, this study investigated whether PA-3 incorporated mononucleotides even when using inhibitors of nucleotide phosphatase or \(^{32}\)P-labeled AMP.

**Methods:**

- **Uptake of \(^{14}\)C-AMP with APCP by PA-3**
  PA-3 in a completely chemically defined growth medium was incubated in the presence of \(^{14}\)C-Adenosine 5’-monophosphate (\(^{14}\)C-AMP) with or without α,β-methylene ADP (APCP), known as 5’-nucleotidase inhibitor, for 0 and 15 minutes at 37 °C. The bacterial cells were collected by filtration and washed, and their radioactivity was measured by liquid scintillation counting.

- **Uptake of \(^{32}\)P-AMP by PA-3**
  \(^{32}\)P-AMP was prepared by decomposing \(^{32}\)P-Adenosine 5’-triphosphate (\(^{32}\)P-ATP) with apyrase. PA-3 in a completely chemically defined growth medium was incubated in the presence of \(^{32}\)P-AMP for 0 and 15 minutes at 37 °C. The bacterial cells were collected by filtration and washed, and their radioactivity was measured by liquid scintillation counting.

- **Utilization \(^{32}\)P-AMP for RNA/DNA by PA-3**
  PA-3 in a completely chemically defined growth medium was incubated in the presence of \(^{32}\)P-AMP for 0, 6 and 24 hrs at 37 °C. The bacterial cells were collected by centrifugation. Nucleo Spin RNA/Nucleo Spin Microbial DNA was used to extract the bacterial RNA/DNA, and their radioactivity was measured by liquid scintillation counting.

**Results:** PA-3 took in \(^{14}\)C-AMP in the presence of APCP and additionally incorporated \(^{32}\)P-AMP. Furthermore, radioactivity was detected from the RNA/DNA of the bacterial cells cultured in the presence of \(^{32}\)P-AMP.

**Conclusions:** Since PA-3 incorporated \(^{14}\)C-AMP in the presence of 5’-nucleotidase inhibitor and incorporated \(^{32}\)P-AMP, PA-3 incorporated purine mononucleotides themselves rather than incorporated them after degradation into purine nucleosides. In addition, PA-3 utilizes incorporated purine mononucleotides to synthesize RNA/DNA, probably for efficiently utilization of purines. Although further studies are required to discover purine mononucleotide transporters, they are first findings that some lactic acid bacteria directly incorporate purine mononucleotides and use them for their growth.


**KEYWORDS:** lactic acid bacteria, *Lactobacillus gasseri* PA-3, uptake of purine mononucleotides
Effect of crude drug extracts on purine metabolism in HepG2 cells

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Objective: Patients with clinical gout and hyperuricemia are generally treated with xanthine oxidase (XO) inhibitors to lower serum uric acid levels. However, these patients often also use traditional medicine and natural supplements to improve their condition. The purpose of this study was to examine whether crude drug extracts used in traditional medicine for the treatment of lifestyle-related diseases in Japan affect purine metabolism in HepG2 cells, a human liver cancer cell line.

Methods: We obtained pharmaceutical grade Ephedra Herb, Rhubarb, and other herbs approved for use in Japan. Between 2.5–5.0 g was extracted with water under reflux, and the filtrate was centrifuged to dryness. The dried extracts were dissolved in dimethyl sulfoxide, diluted to 4 different concentrations with Hank’s balanced salt solution, and added to HepG2 cells grown in a 24-well plate. Allopurinol was used as a control. The cells were collected at 24 h after the extract was added, and the intracellular amounts of 17 purines (8 nucleotides, 4 nucleosides, and 5 bases) were quantified using an HPLC method for simultaneous analysis.

Results: Our previously measured XO inhibition ratio indicated that Rhubarb and Ephedra Herb exerted a higher inhibitory effect than the other crude drug extracts. The IC\textsubscript{50} for XO inhibition was 18.0 ± 2.0 µg /mL for Rhubarb and 57.0 ±4.0 µg /mL for Ephedra Herb. We confirmed the inhibitory effect of XO with the addition of 1500µM allopurinol. The intracellular levels of purines were affected by allopurinol in a dose-dependent manner, uric acid levels decreased, and the levels of its precursor hypoxanthine increased. We confirmed that intracellular purine levels were altered with both Rhubarb and Ephedra Herb. High concentrations of Rhubarb extract induced damage in HepG2 cells, and significant nucleotide degradation was observed. Xanthosine levels increased with allopurinol but decreased with Ephedra Herb.

Conclusion: This study demonstrated the effect of crude drug extracts on purine metabolism in HepG2 cells, two of which are important for their use in traditional medicine and were found to alter intracellular purine levels. Ephedra Herb exerted an XO inhibitory effect as shown by increased intracellular hypoxanthine levels and decreased uric acid levels. Changes in the levels of other purines were not dependent on the addition of allopurinol, suggesting that other enzymes may be involved.


KEYWORDS: Crude drugs, Ephedra Herb, Purines, HepG2, HPLC
Abstract P36

Association between plasma xanthine oxidoreductase activity and blood pressure — MedCity21 health examination registry

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Objectives: Hyperuricemia has been shown to predict development of hypertension. Furthermore, administration of a xanthine oxidoreductase (XOR) inhibitor, which reduces the production of not only uric acid but also reactive oxygen species (ROS), is known to decrease blood pressure (BP), though the precise mechanism remains unclear. Here, we investigated the associations of plasma XOR activity and serum uric acid level with BP.

Methods: This cross-sectional study included 156 subjects (68 males, 88 females) registered in the MedCity21 health examination registry who were not taking anti-hypertensive or anti-hyperuricemic agents. Plasma XOR activity was measured using our highly sensitive novel assay based on [13C2,15N2] xanthine and liquid chromatography/triple quadrupole mass spectrometry, the results of which are unaffected by the original uric acid concentration. The level of uric acid in serum and BP were simultaneously determined.

Results: The median values for uric acid, plasma XOR activity, and mean BP were 5.4 mg/dL, 25.7 pmol/h/mL, and 89.0 mmHg, respectively. Plasma XOR activity (p = 0.410, p<0.001) and serum uric acid level (p = 0.263, p=0.001) were positively and significantly correlated with mean BP. Multiple regression analysis showed that plasma XOR activity (β=0.211, p=0.019), but not serum uric acid (β=0.072, p=0.502), was significantly associated with mean BP independent of other factors, including age, gender, visceral fat area, smoking habit, glycated hemoglobin, estimated glomerular filtration rate, and aldosterone-to-renin ratio. Additionally, plasma XOR activity was positively and significantly associated with systolic (β=0.200, p=0.025) and diastolic BP (β=0.192, p=0.038), whereas serum uric acid was not.

Conclusions: Our results indicate that plasma XOR activity, but not serum uric acid, has an independent positive association with BP, suggesting that XOR contributes to development of hypertension through ROS production.

KEYWORDS: plasma XOR activity, blood pressure, uric acid, ROS