Biochemical and Biophysical Research Communications 452 (2014) 157–162

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Molecular mechanism of an adverse drug–drug interaction of allopurinol and furosemide in gout treatment

Claudia Knake [a], Lisa Stamp [b], Andrew Bahn [a,*]

[a] Department of Physiology, Otago School of Medical Sciences, University of Otago, Lindo Ferguson Building, 270 Great King Street, Dunedin 9010, New Zealand
[b] Department of Medicine, University of Otago, 2 Riccarton Avenue, Christchurch, New Zealand

ARTICLE INFO

Article history:
Received 12 August 2014
Available online 21 August 2014

Keywords:
Gout
Furosemide
Oxypurinol
Xanthine oxidase
miR-448

ABSTRACT

Gout patients receiving a combination of allopurinol and furosemide require higher allopurinol doses to achieve the target serum urate (SU) of <6 mg/dl (Stamp et al., 2012) [1]. Our study aimed to identify the molecular basis for this observation. We used a fluorimetric assay to determine the impact of furosemide and oxypurinol (the active metabolite of allopurinol) on xanthine oxidase (XO) activity. Immunoblot analysis quantified expression of XO and AMP-kinase (AMPK) in drug-treated human liver (HepG2) and primary kidney (HRCE) cells. *In silico* analysis identified miR-448 as a potential XO-regulator, whose expression level in HepG2 cells was examined by qPCR.

Fluorimetric experiments revealed no direct interactions between XO and furosemide, nor did the combination of oxypurinol/furosemide alter the XO inhibition profile of oxypurinol. In HepG2 cells, we found a significant decrease in XO protein expression following oxypurinol treatment, which was abolished after co-incubation with furosemide. Probenecid alone or in combination with furosemide reduced XO protein expression significantly. qPCR analysis of miR-448 in HepG2 cells mirrored the drug-dependent changes in XO protein expression. In addition, oxypurinol and the combination of oxypurinol/furosemide significantly down-regulated AMPK protein expression in HRCE cells.

In conclusion, we show for the first time that besides the established effects of allopurinol on the purine synthetic pathway the efficiency of allopurinol treatment of gout patients is based on two further complementary mechanisms, the direct inhibition of XO activity by the allopurinol metabolite oxypurinol and a down-regulation of XO protein expression. The latter is compromised by addition of furosemide and might explain why patients receiving furosemide therapy require higher allopurinol doses. miR-448 was identified as a potential drug-dependent XO regulator. Finally, down-regulation of AMPK protein expression in HRCE cells by administration of oxypurinol/furosemide reveals a possible new mechanism of renal drug-induced hyperuricemia.

1. Introduction

The xanthine oxidase (XO) inhibitor allopurinol, or rather its pharmacologically active metabolite oxypurinol [2], is the most common clinical treatment for abnormally high serum urate (SU) levels in gout patients [3,4]. Frequently, patients with gout also suffer from co-morbidities such as cardiac or kidney problems that require additional drug treatment with diuretics, particularly furosemide. Belonging to the class of loop diuretics, furosemide primarily acts by inhibiting NKCC2, the luminal $Na^+/K^+/2Cl^-$ symporter in the thick ascending limb of the loop of Henle. Studies have shown that the combination of furosemide and oxypurinol decreases the urinary excretion of uric acid and oxypurinol [5], and initially it was speculated that this interaction might render the hypouricemic effect of allopurinol more potent. A more recent clinical report by Stamp and co-workers [1] showed that patients receiving allopurinol and furosemide indeed exhibited increased plasma oxypurinol levels as well as increased SU. Compared to patients receiving a similar allopurinol dose alone, however, they needed higher allopurinol doses to achieve the target SU of <6 mg/dl. This observation indicates that the hypouricemic effect of allopurinol is in fact attenuated by the addition of furosemide. Increased SU after co-administration of furosemide is generally thought to be due to the plasma volume reduction or secondary enhancement of sodium re-absorption that is coupled to urate. However, the molecular mechanisms underlying this drug interaction are not yet understood.

* Corresponding author. Fax: +64 3 479 7323.
E-mail address: andrew.bahn@otago.ac.nz (A. Bahn).

http://dx.doi.org/10.1016/j.bbrc.2014.08.068
0006-291X/

Oxypurinol works by competitively inhibiting the XO enzyme (Fig. 1A), but it is unknown whether furosemide exhibits a direct effect on XO as well or if it hampers the binding of oxypurinol to XO. The latter hypothesis could explain the observed adverse effect of the combination of furosemide and allopurinol. Even if direct interactions may be excluded, there are diverse regulatory pathways within the cell that could be subject to changes by the combination of furosemide and oxypurinol: (1) different expression of XO, (2) changes in levels of regulatory proteins or miRNAs, (3) changes in urate/drug transporter expression or function.

The latter has already partly been addressed. Renal secretion of diuretics such as furosemide or thiazides is crucial for their pharmacologic effect. However, this mechanisms of diuretic secretion has been recognised to cause high SU or hyperuricemia (SU > 7 mg/dl) [6,7]. We and others have shown that organic anion transporters such as OAT1 and OAT3 [8], OAT4 ([8–10]), NPT4 [11] or MRP4 [12] are involved in the renal secretion of diuretics such as furosemide (Fig. 1B). Since all of these transporters are also uric acid transporters, the concomitance of diuretics and urate alters normal excretion patterns: Diuretics can either inhibit the luminal secretion of uric acid (in case of unidirectional transporters such as NPT4 or MRP4) or the excretion of the diuretic can simultaneously facilitate the uptake of uric acid from the luminal side (OAT1–4). Both mechanisms lead to hyperuricemia.

Recently, studies have shown that AMP-kinase (AMPK) is involved in renal uric acid transport in avian renal proximal tubules [13]. Under stress conditions AMPK is activated, resulting in a decrease of uric acid secretion via MRP4, which is the only renal uric acid secreting transporter in birds. This illustrates that AMPK can be pivotal in the regulation of transporters that are involved in the clearance of urate and furosemide. Assuming a similar mechanism exists in humans, furosemide treatment could alter urate transport via activation of AMPK. Whether AMPK is affected by furosemide in human proximal renal tubule cells is currently unknown.

The aim of this study was to elucidate these drug interactions on a molecular level. Firstly, we studied direct interactions between oxypurinol and furosemide (alone and in combination) on purified XO enzyme in a cell-free assay. We then explored the effects of these drugs on expression levels of XO in cultured human liver cells (HepG2) by immunoblot. Moreover, we identified miR-448 as a potential XO-regulator and analysed its expression levels in drug-treated HepG2 cells. We also analysed AMPK expression in drug-treated primary human renal cortical epithelial (HRCE) cells

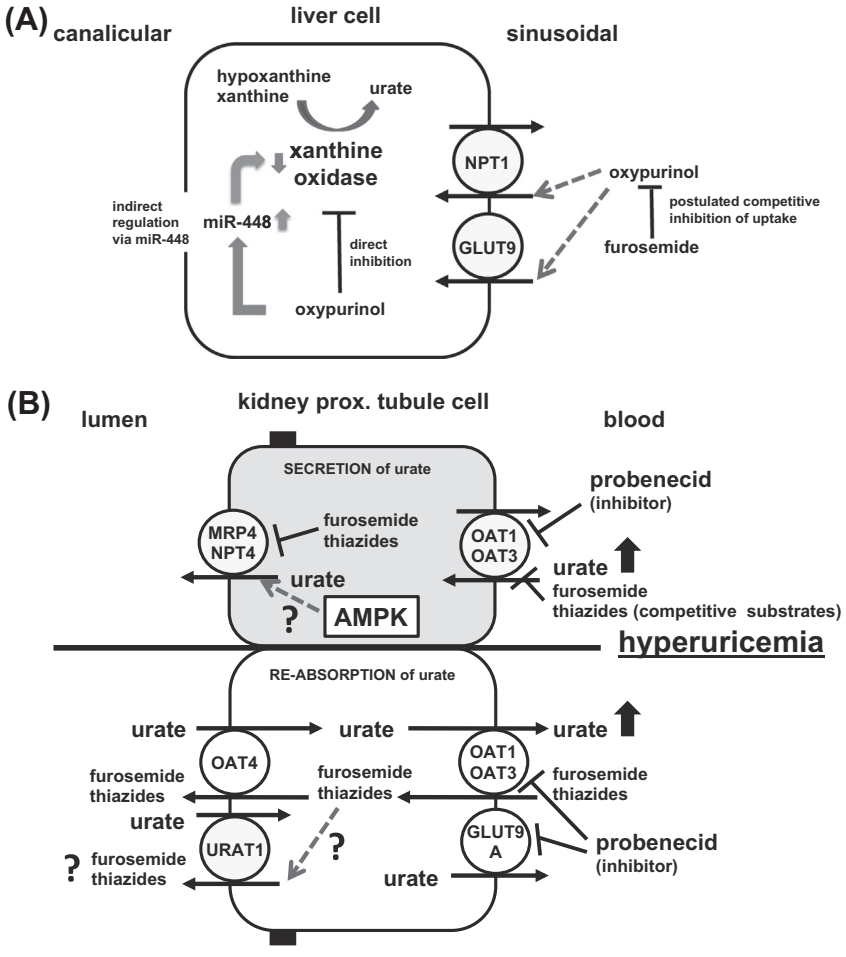

Fig. 1. Cell models for the effect of furosemide/probenecid on urate synthesis in the liver or urate transport in the kidney are shown. (A) Oxypurinol is transported into liver cells by proteins such as NPT1 or GLUT9. In the cytosol, it directly inhibits xanthine oxidase (XO) and consequently uric acid synthesis. We have identified miR-448 as potential post-transcriptional regulator of XO, which is regulated by drugs such as oxypurinol. (B) In the proximal tubule of the kidney, diuretics such as furosemide or thiazides have to be secreted from the blood into the urine to take effect. On the basolateral (blood) side, this secretion process is facilitated by organic anion transporters, namely OAT1 and OAT3. On the luminal side, furosemide and thiazides are released into the urine via MRP4 or NPT4 as well as OAT4 and URAT1. All these transporters are also urate re-absorbing (OAT4 and URAT1) or secreting (MRP4 and NPT4) transporters. Hyperuricemia (high SU) can be caused by the active secretion of diuretics (furosemide or thiazides) in exchange for urate, thus increasing the re-absorption of urate. It can also result from an inhibition of urate secretion by diuretics. Probenecid increases excretion of urate via inhibition of urate transporters on the blood and/or luminal side. In addition to these direct effects of diuretics on transporters as competitive substrates it has been reported that AMP-kinase can regulate urate transporters and consequently secretion or re-absorption of urate.

to look at kidney specific drug effects on possible regulation of uric acid transport.

2. Methods

2.1. Xanthine oxidase assay

Direct interactions between oxypurinol and/or furosemide and purified XO enzyme were tested using the fluorimetric Amplex Red Xanthine/Xanthine Oxidase Assay (Invitrogen) according to the manufacturer's protocol. For an activity standard curve, XO concentrations between 0 and 4 mU/ml were used. From this curve a concentration well within the linear part of the curve (0.75 mU/ml) was chosen for further experiments. Oxypurinol concentrations in the range from 0 to 300 μM and furosemide concentrations in the range 0–1200 μM were tested for their ability to influence XO activity. Absorbance was measured in a Synergy 2 (BioTek) plate reader at 571 nm.

2.2. Cell culture

HepG2 cells (ATCC) were grown in DMEM high glucose (Gibco) supplemented with 10% FBS and 1% antibiotic–antimycotic mix (Gibco) at 37 °C and 5% CO_2. HRCE cells (Lonza) were grown on transwells (Corning) in renal epithelial cell medium (REBM™ plus supplements) at 37 °C and 5% CO_2. At around 80% confluence, the cells were incubated with the respective drug(s) for 24 h at 37 °C. For these experiments we used concentrations of 250 μM for oxypurinol/probenecid and 1 mM for furosemide.

2.3. Protein extraction and immunoblot

After 24 h of drug-treatment, HepG2 and HRCE cells were harvested using Trypsin–EDTA (TrypLE™, Gibco) and cell pellets were stored in RNA later (Ambion) at −20 °C for further analysis.

Proteins were extracted in CelLytic M buffer (Sigma) according to the manufacturer's protocol. Protein concentration of the samples was determined by DC-assay (BioRad). Protein samples were mixed with SDS–PAGE sample buffer and incubated at 70 °C for 10 min prior to loading onto a 10% SDS polyacrylamide gel. After electrophoresis, the gels were blotted onto PVDF membranes (Amersham). Membranes were then blocked in 5% low-fat milk powder for at least 1 h at 37 °C. Dilutions of primary antibodies were as follows: rabbit anti-xanthine oxidase (ab109235, Abcam) 1:200, rabbit anti-AMPK (ab32047, Abcam) 1:500, mouse anti-β-Actin-HRP (ab49900, Abcam) 1:25,000. Blots were incubated with primary antibodies at 4 °C over night. After several washing steps secondary anti-rabbit-HRP (ab6721, Abcam) was applied at a 1:10,000 dilution for 30 min at 37 °C. Luminescence was detected using the ECL immunoblotting detection system (Amersham).

2.4. miRNA extraction, RT and qPCR

Total RNA was extracted from HepG2 cells using the NucleoSpin miRNA Kit (Macherey & Nagel) according to the manufacturer's protocol. For each sample, 10 ng of total RNA was used for the reverse transcription (RT). miRNA RT and qPCR primers were obtained from Life Technologies (miR-448: # 001029; U6 snoRNA: # 001973). RT was performed using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. qPCR reactions were carried out according to TaqMan Small RNA Assay protocol. U6 snoRNA was used as internal control.

2.5. In silico sequence analysis

We used the TargetScan 6.2 database to find miRNA candidates that bind in the 3′UTR region of the XO mRNA.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, USA). We used two-tailed unpaired *t*-tests (Welch-corrected) or one-way ANOVAs for comparisons between treatments. *p*-Values below 0.05 were considered significant.

3. Results

3.1. No direct interactions between furosemide and XO

We first examined the activity of a series of XO concentrations in the range of 0–4 mU/ml (Fig. 2A). From this curve we chose the concentration of 0.75 mU/ml for further experiments and tested the effect of various concentrations of oxypurinol or furosemide (Fig. 2B) on XO activity. As expected for a direct inhibitor, oxypurinol revealed a dose-dependent inhibition of XO activity. Contrary, furosemide alone (up to 1200 μM) had no effect on XO activity, nor did it alter oxypurinol-mediated XO inhibition (Fig. 2C).

3.2. XO expression in human liver cells (HepG2) after drug treatment

To investigate possible regulatory mechanisms within a cellular environment, we examined XO protein expression in HepG2 cells, which had been treated with 250 μM oxypurinol and/or 1 mM furosemide for 24 h. Interestingly, we found a significant down-regulation of XO protein expression upon oxypurinol treatment, which was not seen with furosemide alone or a combination of both drugs (Fig. 3A).

Looking for an alternative for allopurinol in the clinic that eliminates the adverse drug–drug interaction of the oxypurinol/

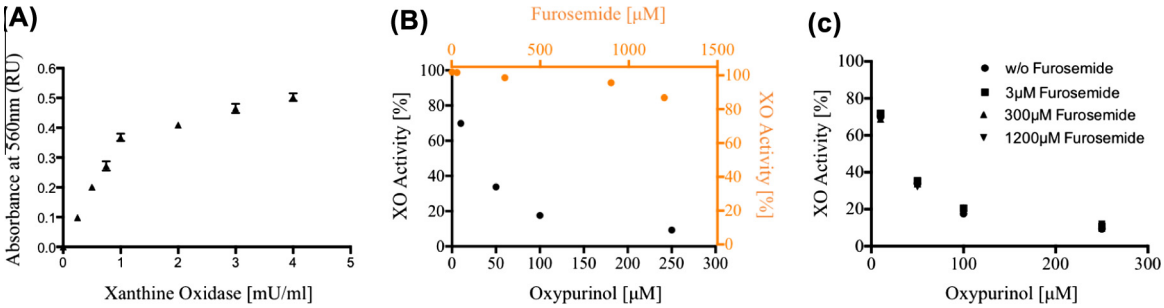

Fig. 2. The direct interactions between oxypurinol, furosemide and purified XO enzyme were examined. (A) XO activity standard curve; (B) inhibition of XO activity (0.75 mU/ml) with increasing concentrations of oxypurinol (black dots) or furosemide (orange dots); (C) inhibition of XO activity (0.75 mU/ml) with increasing concentrations of oxypurinol and furosemide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

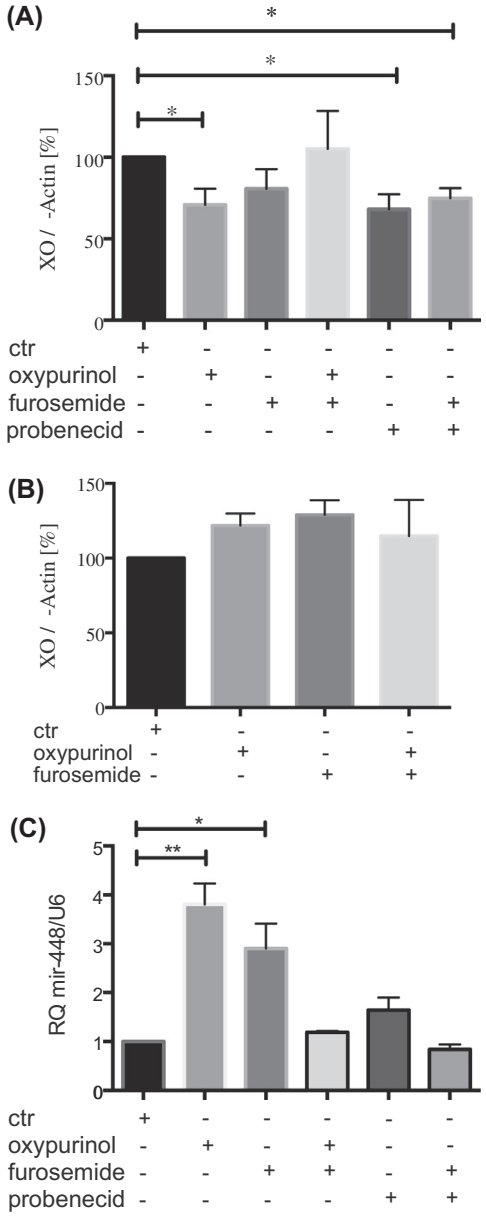

Fig. 3. (A) XO protein expression was determined in HepG2 cells following 24 h drug treatment by immunoblot. HepG2 cells were treated with oxypurinol (250 μM), probenecid (250 μM), furosemide (1 mM) or a combination of these drugs for 24 h. Band intensities were determined using densitometry and were normalised to β-actin band intensities. Statistical analysis was assessed by two-tailed unpaired *t*-test; $n = 5$; $^{*}p < 0.05$. (B) XO protein expression in human renal cortical epithelial (HRCE) cells was tested following 24 h drug treatment. HRCE cells were treated with oxypurinol (250 μM), probenecid (250 μM), furosemide (1 mM) or a combination of these drugs for 24 h. XO protein expression was determined by immunoblot. Band intensities were determined using densitometry and were normalised to β-actin band intensities. Statistical analysis was assessed by two-tailed unpaired *t*-test; $n = 2$. (C) qPCR analysis of miR-448 expression in HepG2 cells was performed following 24 h drug treatment. HepG2 cells were treated with oxypurinol (250 μM), probenecid (250 μM), furosemide (1 mM) or a combination of these drugs for 24 h. Total RNA was isolated and qPCR for miR-448 was carried out. U6 snoRNA was used for internal normalisation and relative quantities (RQ) are displayed. Statistical significance was assessed by one-way ANOVA; $n = 2$; $^{*}p < 0.05$; $^{**}p < 0.01$.

furosemide combination, we tested the effect of probenecid on XO expression. Probenecid is a drug prescribed to gout patients that show adverse effects to XO inhibitors or where treatment with XO inhibitors is not well tolerated. Hence, we tested extracts of HepG2 cells that had been treated with probenecid (250 μM) or a combination of probenecid (250 μM) and furosemide (1 mM) for

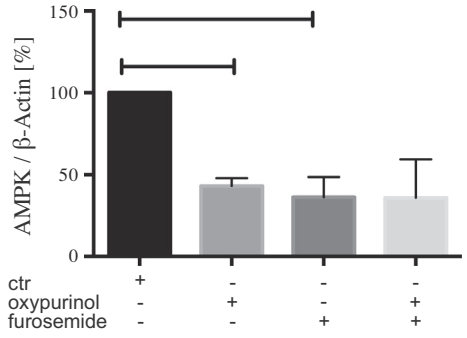

Fig. 4. AMPK protein expression in HRCE cells was examined following 24 h drug treatment. HRCE cells were treated with oxypurinol (250 μM), probenecid (250 μM), furosemide (1 mM) or a combination of these drugs for 24 h. XO protein expression was determined by western blot. Band intensities were determined densitometrically and were normalised to β-actin band intensities. Statistical analysis was assessed by two-tailed unpaired *t*-test; $n = 2$; $^{*}p < 0.05$; $^{**}p < 0.01$.

24 h. Both treatments resulted in significantly decreased XO protein expression (Fig. 3A). Here we demonstrate for the first time a negative XO expression regulation by oxypurinol and probenecid in human liver cells.

3.3. XO and AMPK expression in human kidney cells (HRCE) after drug treatment

We tested the effect of oxypurinol and furosemide on XO expression in human primary renal cortical epithelial (HRCE) cells by immunoblot analysis. Interestingly, we did not detect any changes in XO protein expression due to oxypurinol or furosemide (Fig. 3B).

Even though oxypurinol and furosemide treatment did not alter XO expression in this cell line, we were able to track down other drug-dependent changes in these cells. We examined the expression of AMPK expression, a key enzyme in cellular energy homeostasis, and found a dramatic decrease of AMPK protein expression after treatment with oxypurinol or furosemide (Fig. 4). This decrease was highly significant after treatment with oxypurinol alone ($p = 0.0068$), significant after treatment with furosemide alone ($p = 0.0357$), but not significant after treatment with the combination of both drugs. These experiments reveal different targets for oxypurinol and furosemide in kidney and liver cells, with AMPK being a potential key regulator of diverse down-stream effects in the kidney.

3.4. XO regulation by miRNA-448

To further explore the regulation of XO protein expression via oxypurinol and furosemide, we used the TargetScan 6.2 [14] database to identify regulatory miRNAs. We identified miR-448 that has a binding site in the 3′UTR of the XO gene as a possible candidate and performed qPCR analysis for miR-448 expression in HepG2 cells that had been treated with oxypurinol, furosemide, probenecid and a combination of these drugs (Fig. 3C). Treatment with oxypurinol or furosemide resulted in the up-regulation of miR-448 expression, whereas the combination of both drugs or the administration of probenecid did not have a significant impact on miR-448 expression. These results indicate that miR-448 might work as a regulator of XO expression.

4. Discussion

From our experiments on the interactions of oxypurinol and furosemide on purified XO protein, we conclude that furosemide does not directly interact with XO and does not influence binding

of oxypurinol to XO. These findings imply a more complex drug effect within the cellular environment.

We also demonstrate for the first time that the efficiency of oxypurinol in lowering SU could also be due to a suppression of XO protein expression in addition to its known direct inhibitory effect on XO activity [2]. Both effects will synergistically lead to a decrease of urate synthesis. The combination of oxypurinol and furosemide abolishes the effect of oxypurinol on XO expression. A possible explanation for this observation could be that oxypurinol and furosemide might use the same transporter to enter the liver cell. If true, the addition of furosemide would lead to a competitive inhibition of oxypurinol uptake into the cell, which in turn would diminish the effect of oxypurinol on XO expression. It is not clear which proteins are involved in the transport of oxypurinol. Stocker and co-workers [15] studied the pharmacokinetics of oxypurinol in people with gout and suggested GLUT9 and inorganic phosphate transporter 1 (NPT1) as candidates that may transport oxypurinol. It has already been shown that oxypurinol reduces GLUT9-mediated [^{14}C]urate uptake by about 60% [16], indicating affinity of GLUT9 for oxypurinol. Furosemide on the other hand, did not reduce GLUT9-mediated [^{14}C]urate uptake, but it was shown to inhibit [^{14}C]faropenem uptake in mouse NPT1-expressing oocytes [17]. Thus, NPT1 remains a candidate for the uptake of both, oxypurinol and furosemide that might explain the observed effects.

Furosemide alone did not alter XO protein expression significantly. However, the combination of oxypurinol and furosemide was found to revert XO protein expression back to control conditions, thereby counteracting the inhibitory effect of oxypurinol on XO protein expression and leading to increased XO protein levels. Consequently, this would explain why higher amounts of oxypurinol are needed to lower urate levels in gout patients, who also receive furosemide treatment.

Probenecid is well known as a classical OAT inhibitor and is used as uricosuric drug facilitating renal excretion of uric acid by inhibiting apical uric acid reabsorbing transport proteins such as OAT4 [15], URAT1 [16] or glucose transporter 9 (GLUT9) [16]. Consequently probenecid should lead to decreased SU. Our data demonstrate the down-regulation of XO expression by probenecid, which might also contribute to decreasing SU. This down-regulation is not altered when probenecid is combined with furosemide. The fact that probenecid has a significant impact on XO protein expression has never been reported and warrants further investigation to explore the underlying mechanism. However, the use of probenecid as alternative to allopurinol in the clinic may be limited, as shown by a recent study that reported that only ~1/3 of patients achieve the target SU of <6 mg/dl with probenecid therapy alone or in combination with allopurinol [18].

XO is mainly expressed in the liver and to a lower extent in a variety of other tissues such as kidney [19]. We did not detect any changes in XO protein expression in primary kidney epithelial cells (HRCE cells) upon oxypurinol or furosemide treatment, indicating a liver specific effect of these drugs and hinting at a different regulation in the kidney. It was recently demonstrated that uric acid transport can be altered by an activation of AMPK under stress conditions [20] and subsequently changes urate transport by MRP4. MRP4 in turn has been shown to possess affinity for both oxypurinol and furosemide [12] and might therefore be a key regulator in the interplay between oxypurinol, furosemide and urate in the kidney. The observed effect of oxypurinol and furosemide on the expression of AMPK adds another layer of complexity to renal uric acid transport and needs further investigation. These findings again illustrate the potential tissue specificity of drug-induced changes in metabolic pathways and stress the importance of considering pre-existing medical conditions such as liver or kidney insufficiencies when choosing urate-lowering therapy.

miRNAs are small non-coding RNAs, which regulate protein expression of a gene post-transcriptionally [21]. As expected for a miRNA that acts as a negative regulator of gene expression, the miR-448 expression profile resulted in an almost perfect mirror of the XO protein expression profile, suggesting that miR-448 is regulated in a drug-dependent manner and might regulate XO expression.

Over the last couple of years, the role of miRNAs as regulators for pharmacology-related genes and as mediators of drug response has become more and more appreciated, creating the field of miRNA pharmacogenomics [22,23]. Pharmacogenomics aim to combine individual genomic and transcriptomic data (e.g. SNPs, copy number variations, alternative splicing or gene expression levels, miRNA status) to pave the way for personalised drug treatment. It also sheds new light on so-called 'silent mutations' or SNPs in the coding region or 3′UTR of a gene – if these sequences happen to be within the binding site of a miRNA, then these mutations could have an effect on protein expression levels and thus at least partly explain different patient reactions to equal drug concentrations [24]. If miR-448 can be verified as a direct regulator of XO, it might prove helpful as a marker to predict the efficacy of drug treatment, as it can easily be amplified from blood plasma samples (unpublished results). Since XO is not only involved in the metabolism of allopurinol, but also in the metabolism of common drugs like the immunosuppressive 6-mercaptopurine and the antituberculous pyrazinamide [25], miR-448 levels might also help to predict outcome for these treatments.

5. Conclusion

Our findings widen the understanding of the molecular mechanisms of these commonly used drugs and pinpoint adverse drug interactions. Thus, this study could pave the way to designing further studies on drug-induced molecular changes and interactions, eventually leading to the finding of more efficient treatment options for gout or other rheumatic diseases. Furthermore, this study highlights the potential usefulness of incorporating the patient's miRNA status into the treatment strategy, getting us one step closer to the realisation of personalised medicine. Given the central role of xanthine oxidase as mediator of inflammation in various pathophysiologies ranging from cancer to neurodegeneration, these findings might have even broader implications for the clinic.

Conflict of interest

The authors report no competing interests.

Funding

This work was supported by an Otago Medical School Laurenson Research Grant (Otago Medical Research Foundation).

Acknowledgments

We would like to acknowledge Dr. Martin Fronius and Dr. Jeff Erickson for help with editing and proofreading of the manuscript.

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