Enterohepatic Recirculation: A Powerful Incentive for Drug Discovery in the Inosine Monophosphate Dehydrogenase Field

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Abstract: With the exception of organ transplant immunosuppression, the treatment of various IMPDH-dependent hyperproliferative diseases by MPA has failed due to the drug's EHC-induced GIT adverse effects. To influence its therapeutic index, novel formulations such as gastro-resistant MPA-Na (ERL080) or MPA/cholestyramine combinations have been developed. Structurally novel IMPDH inhibitors have been discovered based on high throughput screening (pyridazoles) and rational design (methoxyphenyloxazoles). The clinical data on methoxyphenyloxazole derivatives such as VX-497 that is not expected to undergo EHC, will bring improved understanding of the relationship between IMPDH blockade and GIT toxicity.

## ISSUES RELATED TO ENTEROHEPATIC RECIRCULATION (EHC).

Enterohepatic recirculation is the process by which a drug is reabsorbed in the gastrointestinal track (GIT) after biliary excretion. A major prerequisite for such a process is the conjugation of the drug by a phase II biotransformation in the liver and the excretion into the bile of the resulting conjugate (glucuronide, glycoside, sulfate). This conjugate may then be enzymatically hydrolyzed by intestinal flora in the GIT releasing the parent drug for reabsorbtion into the portal circulation. Extensive EHC may result in secondary absorption peaks, erratic plasma concentration-time profiles, and long terminal half-life in spite of high metabolic clearance [1]. Consequently, if EHC is not appropriately accounted for in the total disposition of the parent drug, erroneous calculations of pharmacokinetic (PK) parameters may lead to inappropriate drug dosing. Moreover, the PK parameters of the drug can be dramatically altered by disturbances in the intestinal flora due to concomitant medication or alteration of the biliary function. Although the large majority of drugs undergoing EHC show only one cycle of reabsorption after single dose administration, there have been reported rare cases of drugs characterized by two cycles [2, 3].

EHC not only greatly influences the PK parameters of a drug but may also contribute to its side-effect profile, predominantly by causing GIT damage. This relationship concept is supported by the outcome of extensive investigations with nonsteroidal anti-inflammatory agents (NSAIDs). "Fig. (**1**)". Although there is general acceptance that NSAID-induced inhibition of cyclooxygenase (COX) 1 is responsible for the GI toxicity of these drugs that can
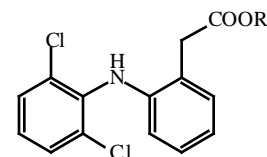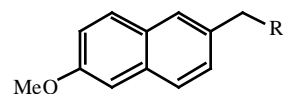

dichlofenac   R = H

nitrofenac R = (CH$_2$)$_4$ONO$_2$
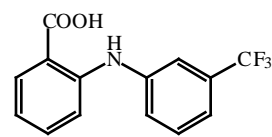
flufenamic acid

nabumetone   R = CH$_2$C(O)CH$_3$
" " metabolite R=COOH

Fig. (1). Structures of COX inhibitors.

severely damage the gastric mucosa and exacerbate preexisting ulcers [4], EHC can also influence the mechanism of NSAID-mediated small intestinal injury as first reported in data on flufenamic acid [5]. This effect was comprehensively addressed in rat models by studying the consequences of repeated administration of diclofenac and nitrofenac, two pharmacologically equipotent compounds that are subjected to significantly different extent of EHC. In contrast to diclofenac that undergoes extensive EHC and damages the intestinal epithelium as judged by the elevation of the enteric bacterial numbers, nitrofenac undergoes less pronounced EHC and has an intestinal-sparing effect. The above data is also supported by the observation that two

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NSAID drugs that do not undergo EHC, aspirin and nabumetone's bioactive metabolite -a potent, moderately selective COX-2 inhibitor-, fail to cause detectable intestinal toxicity in this rat model. Both of these observations support the role of EHC in the etiology of GIT side-effects observed in this species [6, 7, 8]. In conclusion, NSAID-induced small intestinal injury is not only attributable to the COX-1 inhibition but also to EHC. However, since the extend a drug is subjected to EHC is species dependent, the relative therapeutic impact of HEC versus COX selectivity on the GIT adverse effects can only be assessed in clinical trials.

ISSUES RELATED TO DRUGS TARGETING INOSINE MONOPHOSPHATE DEHYDROGENASE (IMPDH).

The nicotinamide adenine dinucleotide (NAD)-dependent catalysis of the conversion of inosine-5'-monophosphate (IMP) to xanthosine-5'- monophosphate (XMP) by the enzyme IMPDH is the rate limiting step in the *de novo* pathway of guanosine biosynthesis. Rapidly proliferating cells are heavily dependent on the availability of large nucleotide pools to meet their metabolic requirements. Compounds blocking this *de novo* biosynthesis pathway will act selectively on these cell types and leave the other ones unaffected. For example, nucleotide pool production via the salvage pathway alone is sufficient for neural cell and kidney-tissue cell proliferation but not for lymphocytes or cancer cells [9]. As a consequence of these cell requirements, IMPDH inhibition is a recognized target for immunosuppression, anti-cancer treatment and viral chemotherapy. The biochemical mechanism and structural aspects of enzymatic catalysis and inhibition have been recently reviewed [10, 11].

There are currently three IMPDH inhibitors on the market. The nucleosides Ribavirin and Mizoribine (Bredinin) that are used clinically as antiviral and immunosuppressive drugs, respectively "Fig. (**2**)", and the non-nucleoside agent mycophenolate mofetil (MMF), an immunosuppressant used in combination with calcineurin inhibitors such as cyclosporin A or FK-506 in many treatment regimens for the prophylaxis of transplant rejection [12]. None of the above IMPDH inhibitors are typically used as monotherapy because their efficacious dosing is limited by adverse events, in particular GI or bone marrow toxicity. These toxicities result either from lack of enzyme specificity or unfavorable pharmacokinetics. For efficacy, the nucleoside drugs require metabolic activation to the corresponding 5'-monophosphates that competitively bind to the nucleotide site of IMPDH (IMP). Nucleotide-binding domains being conserved among many enzymes, the action of Ribavirin and Mizoribin is not IMPDH specific. Ribavirin's interaction with guanine monophosphate reductase, guanine phosphoribosyl transferase, deoxycytidine kinase and thymidine kinase has been reported [13]. Moreover, these two compounds can be further phosphorylated which allows their interference with additional enzymes or their incorporation into DNA. The reported reversible myelotoxicity of Ribavirin in man is typical for cytotoxic

drugs [14]. No toxicology data based on clinical practice with Mizoribin is publicly available [15]. In recognition of the above specificity issues, extensive work in the nucleoside / nucleotide field has been and continues to be carried out [16].
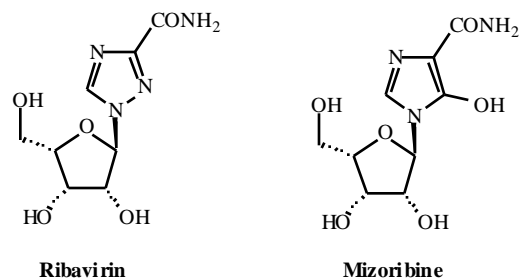

Ribavirin   **Mizoribine**

Fig. (2). Structures of marketed nucleoside inhibitors of IMPDH.

The unique non-nucleoside drug MMF is a prodrug of the fungal agent mycophenolic acid (MPA); a highly potent, selective, reversible, uncompetitive IMPDH inhibitor binding at the enzyme's NAD binding site. "Fig. (**3**)". However, the favorable activity profile of MMF does not translate into a compound with high clinical efficacy or large therapeutic index. For example, the suppressive effect of MMF on cancer cell lines could not be confirmed in vivo [17]. The relative efficacy of MPA administered on a compassionate-use basis (mean oral dose: 3.7 g/d for the first year followed by 3.0 g/d for >10years) to psoriasis patients refractory to conventional therapy was limited by a high incidence of GI toxicity (in 72% of patients during first year) [18]. Significant clinical improvement has been seen in many MMF-treated rheumatoid arthritis (RA) patients refractory to other disease-modifying anti-rheumatic drugs. Administration of 2 g/d orally b.i.d reduced rheumatoid factor titters in patient peripheral blood (IgG, IgM, IgA titters T-cell number). However, in spite of the low dose used in comparison to the one employed in the psoriasis trial, GI adverse-effects were again of concern [19, 20]. The only indication for which MMF has been approved (2- and 3 g/d given orally b.i.d) is for the prevention of solid transplant organ rejection [12, 21]. In addition to the presence of GI adverse effects, a new finding of leukopenia was also observed [22]. In spite of its narrow therapeutic index, MMF is widely being used in immunosuppressive regimens for organ transplantation because of the documented substantial reduction of the incidence and intensity of acute organ loss in this life-saving indication.

Numerous PK investigations have established that MPA, the bioactive component resulting after the rapid and complete enzymatic hydrolysis of orally administered MMF, undergoes extensive EHC [23]. Approximately 80-90% of MPA is efficiently conjugated into the biologically inactive glucuronide (MPAG) mainly in the liver. MPAG is then excreted into the bile, de-glucuronidated by colonic bacteria and absorbed in the GIT, entering the systemic circulation via the portal flow as MPA. "Fig.(**3**)". As a result of this pronounced EHC, huge concentrations of MPA are present in the GIT resulting in local damage to the intestinal epithelium. This process is manifested as symptoms including nausea, vomiting, loose bowel movements,
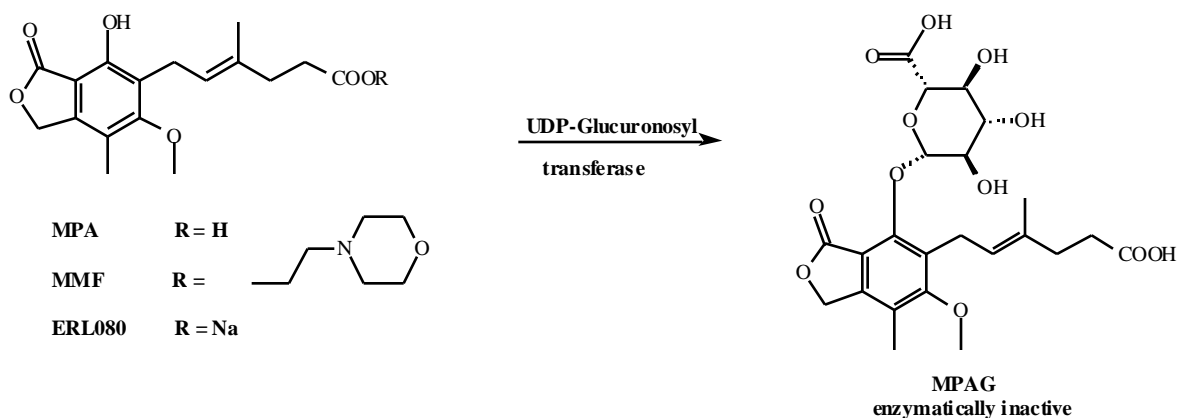
Fig. (3). Structures of MPA derivatives.

abdominal pain, and diarrhea. As demonstrated in renal transplanted patients, the PK parameter most closely linked to the pharmacodynamic effect and therapeutic efficacy of MMF is the systemic MPA exposure (area under the time-concentration curve, AUC). Consequently, high doses of MMF are required to maintain systemic therapeutic levels [24, 25]. Any process that would tend to decrease the systemic concentration of MPA will also decrease the therapeutic efficacy of MPA. Therefore, it is reasonable to assume that the reported lack of anti-cancer activity of MPA *in vivo* can be attributed to its rapid removal from the circulation via drastic glucuronidation by cancer cells [26].

These data taken collectively clearly suggest that novel MPA-analogues or mimetics that are resistant to glucuronidation and maintain the activity of MPA will have a huge therapeutic potential for the treatment of immunological disorders as well as cancer.

APPROACHES AIMING AT THE DISCOVERY OF NON-NUCLEOSIDE IMPDH INHIBITORS WITH IMPROVED THERAPEUTIC INDEX.

a) MPA-Based Approaches

The alteration of MPA's metabolism and potency via modification of the parent compound has been extensively tried. "Fig. (**4**)". The replacement or protection of the phenolic group to avoid glucuronidation, the modification of the phthalide ring or the replacement of the lactone oxygen was found to be detrimental for potent IMPDH inhibition. Among the numerous derivatives synthesized, the substitution of the methoxy group by an ethyl chain resulted in compound (**I**) that manifesting a two-fold higher *in vitro* and 3.5-fold higher in vivo potency in the murine plaque-forming assay than MPA [27]. The enzymatic activity was also very sensitive to alterations of MPA's hexenoic chain. While the large majority of these alterations led to compounds of limited biological utility, some interesting MPA derivatives were produced [28]. For example, the conformationally restricted cyclopentenyl analogue (**II**) inhibited IMPDH with an IC_{50} of 8nM versus 20nM for MPA due to the entropic energy gained by locking MPA into its bioactive conformation [29]. However, the presence

of the phenolic function responsible for EHC in both analogues (**I**) and (**II**), suggests that, in spite of their minor superior potency, no considerable improvement in the therapeutic indexes of these two inhibitors can be expected. Additionally, MPA derivatives carrying at the hexenoic side chain either -substituents (benzyl, thiomethyl, methoxymethyl, p-hydroxyphenyl, trifluoroacetamidophenyl) or a methyl at the -position were shown to be less susceptible to glucuronidation as assessed using the HT29 cell line which rapidly transforms MPA to MPAG. However, their *in vitro* efficacy was greatly reduced with the exception of the racemic methoxymethyl derivative (**III**) which manifest 29% higher *in vitro* activity than MPA [30]. This racemic compound is twice as resistant to glucuronidation as MPA and it would be interesting to thoroughly evaluate the PK and efficacy profiles of the corresponding optically pure isomers.
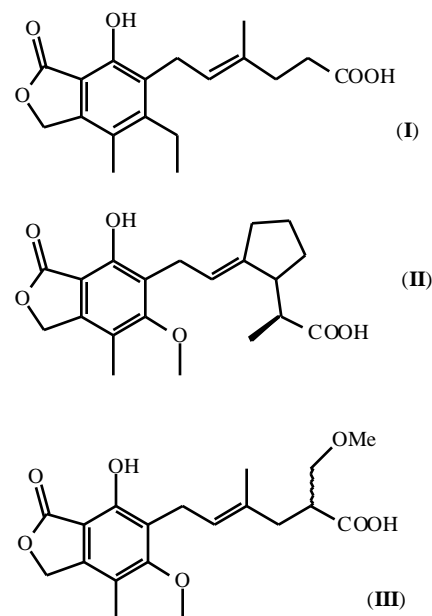

Fig. (4). Structures of MPA-derivatives with improved activity.

In contrast to the structure-activity relationships that can be rationally interpreted based on the architecture of the complex between MPA and IMPDH, the reasons for the

resistance of some phenolic compounds to glucuronyltransferases are unclear [31, 32]. It has been suggested that the additional substituents lead to unfavourable steric interactions with the enzyme's active site [30].

In addition to the extensive medicinal chemistry work aimed at improving the profile of MPA, a recent approach to increase its therapeutic index by influencing the severity of the adverse effects through an alternative galenic formulation has been reported.

The sodium salt of MPA (MPS), "Fig. (**3**)", in an enteric coated delivery form, coded as ERL080, is currently in phase III clinical studies for the prevention of acute renal allograft rejection [33]. This novel formulation is expected to release the drug in or near the small intestine and thus alleviate the adverse effects of MPA related to high local concentrations in the upper GIT such as anorexia, abdominal pain, nausea or vomiting. The functioning enteric coating of ERL080 was apparent based on the delayed MPA Tmax measured in PK studies carried out in renal transplanted patients (2.0 versus 0.8 hours for MMF) [34]. Indeed, the ERL gastro-resistant tablets were rapidly absorbed upon oral administration, leading to systemic MPA exposure bioequivalent to that of MMF capsules. This study also clearly showed that a MPA prodrug form such as MMF is not necessary for the efficient systemic delivery of MPA via the oral route. The outcome of the clinical evaluation will be highly informative regarding the influence of this formulation on the GIT adverse effects of MPA.

Another formulation claimed to improve the therapeutic range of anti-proliferative drugs undergoing EHC, consists of the combination of MMF or MPA with cholestyramine [35]. Cholestyramine is a non-absorbable, cationic resin that unspecifically binds bile-acids and any large-sized acidic drug such as MPA and thus blocks the recycling of the parent compound via the EHC route. When cholestyramine was administered to healthy subjects receiving single doses of MMF, exposure to MPA was significantly decreased (mean reduction 37%), this result being consistent with a strong EHC process [23]. Taking into account the established correlation between MPA's pharmacological effects and systemic AUC, [24, 25] a formulation allowing the co-delivery of cholestyramine and MMF would be expected to drastically lower MPA AUC and, consequently, the efficacy of MPA. No pharmacological data resulting from the above combination treatment has been reported.

b) MPA-Mimetic Search and Identification

Undoubtedly, the most challenging approach for the finding of effective IMPDH inhibitors is either via rational design or high throughput screening of available compound collections. The high clinical value of a structurally novel, non-nucleoside, inhibitor with greater metabolic stability than MPA definitely warrants research investment.

A rational discovery effort based on the incorporation of structural information into an iterative drug-design procedure afforded the methoxyphenyloxazole derivative VX-497 (**IV**),

a selective, reversible and uncompetitive IMPDH inhibitor of similar potency to that of MPA (Ki 10 and 11 nM, respectively). "Fig. (**5**)". In analogy to the MPA / IMPDH interaction, VX-497 is also characterized by the packing of the phenyloxazole moiety underneath XMP but makes several new interactions that were not observed in the binding of MPA [36, 37]. The compound is a potent immunosuppressive agent *in vitro* and *in vivo*. Due to its greater antiviral effect as compared to that of Ribavirin, VX-497 will enter clinical phase III trials in combination with interferon-alpha (IFN-) as an alternative to the use of Ribavirin / IFN- treatment against hepatitis C (HCV) infection [38, 39]. Another derivative, VX-944, is currently in phase II trials in psoriasis while a third one, VX-147, is in early development for RA. The efficacy data disclosed on VX-497 and its apparent lack of structural elements responsible for EHC, indicate that this compound has a promising therapeutic benefit. For its optimal oral uptake, carbamate prodrugs of VX-497 have been obtained [40]. The methoxyphenyloxazole moiety appears to be a key element for the interaction of a compound with IMPDH. Indeed, in addition to the carbamate (**V**) and acetamido (**VI**) derivatives of VX-497 [41], the corresponding benzimidazole (**VII**), benthiazole (**VIII**) and benzoxazole (**IX**) derivatives are also potent enzyme inhibitors [42]. Other variants of the above

X = NH (IV; X = O (V); X = CH₂ (VI)

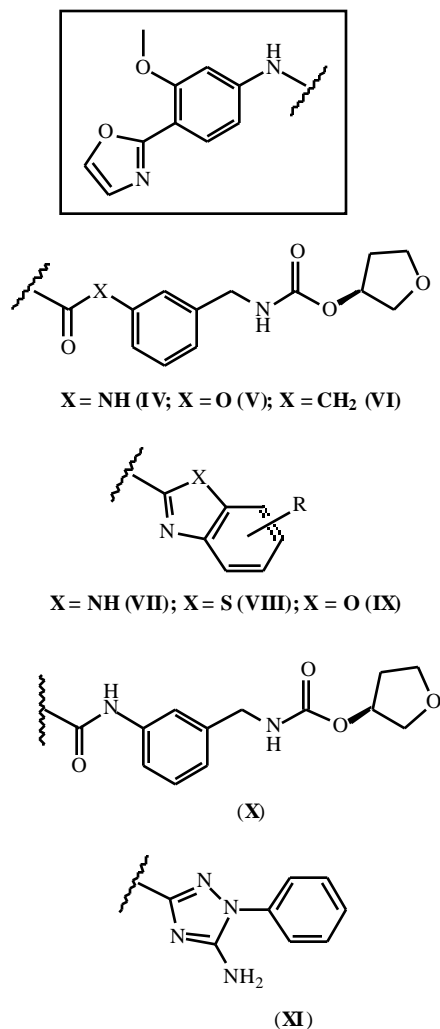**X = NH (VII); X = S (VIII); X = O (IX)**

(**X**)

(**XI**)

Fig. (5). Structures of methoxyphenyloxazol inhibitors.

compounds, including structures (**X**) and (**XI**), are also claimed to be novel IMPDH inhibitors, however no biological data has been disclosed so far [43, 44].

In a different approach, a high throughput screen for inhibitors of IMPDH of a corporate collection of approximately 80,000 compounds led to the identification of (**XII**), a novel non-nucleoside inhibitor belonging to the pyridazine compound class (IC_{50} = 1.9 μM). "Fig. (**6**)". Like MPA and VX-497, (**XII**) is an uncompetitive inhibitor of the enzyme mediating its action through the trapping of a covalent intermediate formed during the conversion of IMP to XMP. The analogues (**XIII**) and (**XIV**), obtained by chemical derivation of the initial hit, have enhanced potency against IMPDH (IC_{50} = 0.7 μM) and block DNA synthesis from human peripheral blood mononuclear cells stimulated by anti-CD3 antibody with IC_{50} = 0.37 and 1.63 μM, respectively. Compound (**XIV**) demonstrated dose-dependent immunosuppressive activity in a mouse delayed type hypersensitivity (DTH) model when administered i.p twice daily [45]. However, its higher efficacy than MPA in this model is at least in part due to the species employed, MPA's EHC being low in the mouse [46]. In spite of the oral effect-bioavailability of (**XIV**) at high dose, the efficacy of pyrazines after oral administration could be severely limited by the very low aqueous solubility of this substance class.
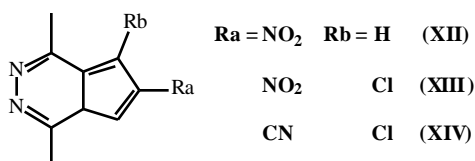

Ra = NO₂   Rb = H   (**XII**)

NO₂   Cl   (**XIII**)

CN   Cl   (**XIV**)

Fig. (6) Structures of pyridazine inhibitors.

Diazabicyclohexanones represent an additional substance class of IMPDH inhibitors. They are derivatives of pyrazolinediones that were originally identified as antineoplastic agents [47]. Interestingly and in contrast to the other non-nucleoside compounds, these by-cyclic compounds are competitive enzyme inhibitors with respect to IMP and highly selective for the type II isoform of IMPDH. The most potent compound (**XV**) (Ki = 5.1 μM, IC_{50} = 22 μM for type II, IC_{50} > 500μM for type I) offers a starting point for the development of novel and isoenzyme specific therapeutic agents. "Fig. (**7**)".
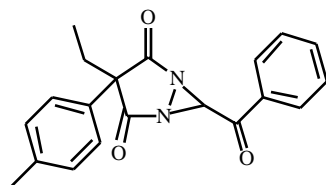

Fig. (7) Structure of inhibitor (**XV**).

CONCLUSION

Due to the continuous drug release in the GIT, EHC can be a major limiting factor for the long-term administration of drugs. The IMPDH inhibition field is a typical example of a therapeutically relevant area that suffers from the lack of potent and metabolically inert agents that could be used for the treatment of hyperproliferative diseases. Indeed, the efficacy and therapeutic index of the highly selective enzyme inhibitor MPA are severely hampered by its extensive glucuronidation and subsequent EHC. Not surprisingly, the administration of MMF to transplanted patients, the marketed prodrug of MPA, is often linked to GIT tolerability issues. In order to broaden the clinical applications of MMF or MPA to psoriasis, cancer and RA patients, the extensive derivation of MPA was undertaken aiming at the blockade of the MPAG formation. The very limited success of this medicinal chemistry approach stimulated alternative strategies based on novel formulations of MPA. The influence of ERL080, MPA's sodium salt (MPS) administered in gastro-resistant tablets, on the GIT adverse effects is currently in investigation in phase III clinical trials. Capitalising on the wealth of information concerning the MPA / IMPDH interaction, a structurally novel class of inhibitors has been discovered via rational design. The methoxyphenyloxazole template has afforded very potent, orally active compounds devoid of structural elements that lead to EHC. The most advanced representative, VX-497, is being currently evaluated clinically for the treatment of HCV and psoriasis. The data on the GIT adverse effects of this compound and, more generally, of the entire compound class will bring improved understanding of the role of MPAG in the narrow therapeutic index of MMF. The biological data disclosed on some pyridazine derivatives, a novel IMPDH class of inhibitors identified from high throughput screening, indicated only moderate efficacy which together with unfavorable physicochemical properties make this substance class a less attractive alternative to the current methoxyphenyloxazole or MPA derivatives. Diazabicyclohexanediones may serve as lead compounds for the development of novel, isoform-selective IMPDH inhibitors; however, it is difficult to assess their potential at the current stage of investigations.

The heavy intellectual and technological investment over many years in the IMPDH field has opened the post-MMF era. The therapeutic benefit of the two novel, conceptually diverging principles is currently being assessed in late clinical trials. Independently of the outcome of these studies, the development of alternative treatments for hyperproliferative diseases will remain a major focus of pharmaceutical research.

ABBREVIATION LIST

AUC	=	Area under the time-concentration curve
COX	=	Cyclooxygenase
DTH	=	Delayed type hypersensitivity

GIT   =   Gastrointestinal track

EHC   =   Enterohepatic recirculation

HCV   =   Hepatitis C virus

IFN-   =   Interferon-alpha

IMP   =   Inosine-5'-monophosphate

IMPDH   =   Inosine-5'-monophosphate dehydrogenase

MMF   =   Mycophenolate mofetil

MPA   =   Mycophenolic acid

MPS   =   Mycophenolate sodium

NAD   =   Nicotinamide adenine dinucleotide

NSAIDs   =   Nonsteroidal anti-inflammatory drugs

PK   =   Pharmacokinetic

RA   =   Rheumatoid arthritis

XMP   =   Xanthosine-5'- monophosphate

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