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Mechanisms of NSAID-Induced Hepatotoxicity

Focus on Nimesulide

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) have been associated with idiosyncratic hepatotoxicity in susceptible patients. The molecular mechanisms underlying this toxicity have not yet been fully elucidated. However, experimental evidence suggests that they include increased concentration of the drugs in the hepatobiliary compartment, formation of reactive metabolites that covalently modify proteins and produce oxidative stress, and mitochondrial injury. Genetic and/or acquired patient factors can either augment the pathways leading to hepatic toxicity or impede the protective and detoxifying pathways. An example is nimesulide, a selective cyclo-oxygenase-2 inhibitor widely used for the treatment of inflammatory and pain conditions, which has been recently associated with

rare but serious and unpredictable adverse reactions in the liver (increases in serum aminotransferase activities, hepatocellular necrosis, and/or intrahepatic cholestasis). Similar to other drugs causing idiosyncratic hepatotoxicity, both the molecule and the patient contribute to the hazard. Here, the weakly acidic sulfonanilide drug undergoes bioreductive metabolism of the nitroarene group to reactive intermediates that have been implicated in oxidative stress, covalent binding, and mitochondrial injury. It is only in a small number of susceptible patients, however, that genetic or nongenetic factors will cause this potential toxicity to become clinically manifest. In view of the very large recipient population, the incidence of nimesulide-induced liver injury has been low (approximately 0.1 per 100 000 patients treated). Although this estimation is based on spontaneous reporting data versus sales units and needs correction due to the classical bias of this system, the type and incidence of these rare but severe hepatic adverse reactions are comparable to that of other NSAIDs.

Nimesulide (4-nitro-2-phenoxymethane-sulfoanilide) is a non-carboxylic acid nonsteroidal anti-inflammatory drug (NSAID) that has been widely and effectively used for the treatment of a variety of inflammatory and pain conditions including osteoarthritis (figure 1). As a selective cyclooxygenase (COX)-2 inhibitor,[1] nimesulide features a low profile of gastrointestinal adverse effects and generally exhibits good tolerability.[2,3] Despite the fact that nimesulide has been on the market for over 16 years, isolated cases indicating that this compound may be associated with rare but serious cases of liver dysfunction and hepatic injury have been reported only recently. These findings raise the question of whether the incidence and pathogenesis of nimesulide-induced hepatotoxicity are compound-specific or whether these characteristics are shared with other NSAIDs.

The aim of this review is to revisit the pathogenesis of idiosyncratic reactions in the liver caused by NSAIDs and in this light critically analyse the hepatic safety profile of nimesulide. In addition, based on metabolic data and mechanistic considerations, a unifying pathway is proposed that may help to better understand the liver liability associated with nimesulide and other NSAIDs in susceptible patients.

1. Hepatotoxicity of Nonsteroidal Anti-Inflammatory Drugs (NSAIDs)

1.1 Idiosyncratic Drug Reactions in the Liver

NSAIDs have generally been considered well tolerated drugs. Nevertheless, in view of the enormous consumption of NSAIDs worldwide, it is not surprising that cases of adverse drug reactions, including those in the gastrointestinal tract, kidney, and skin, but also in liver, have been reported.[4-14]

In the liver, the severity of NSAID-associated liver reactions can widely differ. On the one hand, a mild form of liver dysfunction is frequently observed, manifested as small increases in serum aminotransferase activities and other markers of hepatic integrity, in the absence of overt parenchymal tissue injury.[15] These changes are often tran-

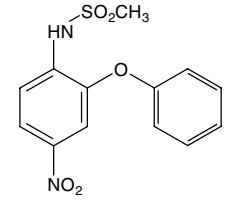

Fig. 1. Chemical structure of nimesulide, featuring an aromatic nitro group, which undergoes reductive metabolism, and a sulfoanilide group, which is responsible for the weakly acidic properties.

sient in nature and possibly reflect an adaptive response to the drug. The second form of hepatic adverse effects consists of more serious reactions and can result in cholestasis, hepatic necrosis, and even fulminant liver failure.[5,11] Although the incidence for developing such serious hepatic adverse reactions is low (typically 1 to 5 among 100 000 recipients),[16,17] the absolute number of affected individuals becomes impressive, given the extremely large population of recipients. To date, for a new NSAID on the market, it is not possible to estimate the risk for developing hepatic adverse reactions in a patient population, even though the drug might feature a favourable preclinical safety profile. Furthermore, it is not yet possible to establish a typical patient susceptibility profile and to predict which individuals will be prone to developing serious hepatic adverse reactions, while the vast majority of recipients will tolerate the drug.

This unpredictability is a reflection of our incomplete understanding of the mechanisms that underlie drug-induced liver injury. The current belief is that the hepatic adverse effects of most NSAIDs are caused by abnormally high or prolonged exposure of the liver to the drug, due to, e.g. abnormal renal or hepatobiliary excretory mechanisms. In addition, and importantly, this concept implies that biotransformation pathways of the drug are gated to the production of reactive metabolites that interact with molecular targets in the liver cells, resulting in covalent binding, oxidative stress, mitochondrial injury, or apoptosis (figure 2).

Poor predictability also exists from the perspective of the patient. For example, a reliable pharmacokinetic-based calculation of the risk of developing hepatic adverse reactions is confounded by multiple individual host factors which ultimately determine whether an individual will tolerate the drug or whether failure of the normally powerful protective and compensatory mechanisms or repair systems will make the individual succumb to the potential intrinsic toxicity of an NSAID.

These host factors that are relevant to the liver have not yet been identified. However, it is generally assumed that they include inherited abnormalities in genes encoding for, e.g. bioactivating enzymes, or of acquired alterations in differential gene expression.[18] Host factors also include epigenetic factors relevant to the underlying disease, infections, or multi-medication, which can greatly influence the disposition and metabolism of the drug and its interactions with cellular targets. Finally, host factors may also inhibit the defence systems, interfere with repair mechanisms, or allow for breaking of immune tolerance (figure 2).[19]

Such host factor-driven, rare adverse reactions are termed idiosyncratic, implying that they depend on individual patient-specific characteristics. The term 'idiosyncrasy' is, unfortunately, poorly defined and differentially used by immunologists, clinicians, or toxicologists. For the sake of clarity, idiosyncratic adverse reactions will be defined here as abnormal reactions to drugs determined by genetic or acquired (environmental) factors. These reactions are rare and become only manifest in a very small subset of patients in whom these genetically-determined or environmentally-induced abnormalities 'unmask' the inherent bioreactivity of the compound. The reactions are unexpected because, within the range of therapeutic exposure, the adverse effects do not become manifest in the vast majority of exposed patients.

Efforts to unravel the enigma of idiosyncratic toxicity of NSAIDs in the liver should therefore concentrate both on the identification of the critical host factors and the intrinsic toxic potential of the drug itself. One important clue to achieve this is a more detailed understanding of the underlying molecular mechanisms.[20]

1.2 Mechanisms Underlying the Hepatotoxicity of NSAIDs

The networks of interdependent molecular and cellular mechanisms underlying NSAID-associated liver injury are only incompletely known. Although the entire therapeutic group has been labelled with the potential to cause hepatotoxicity through a common class effect,[21] it is more likely

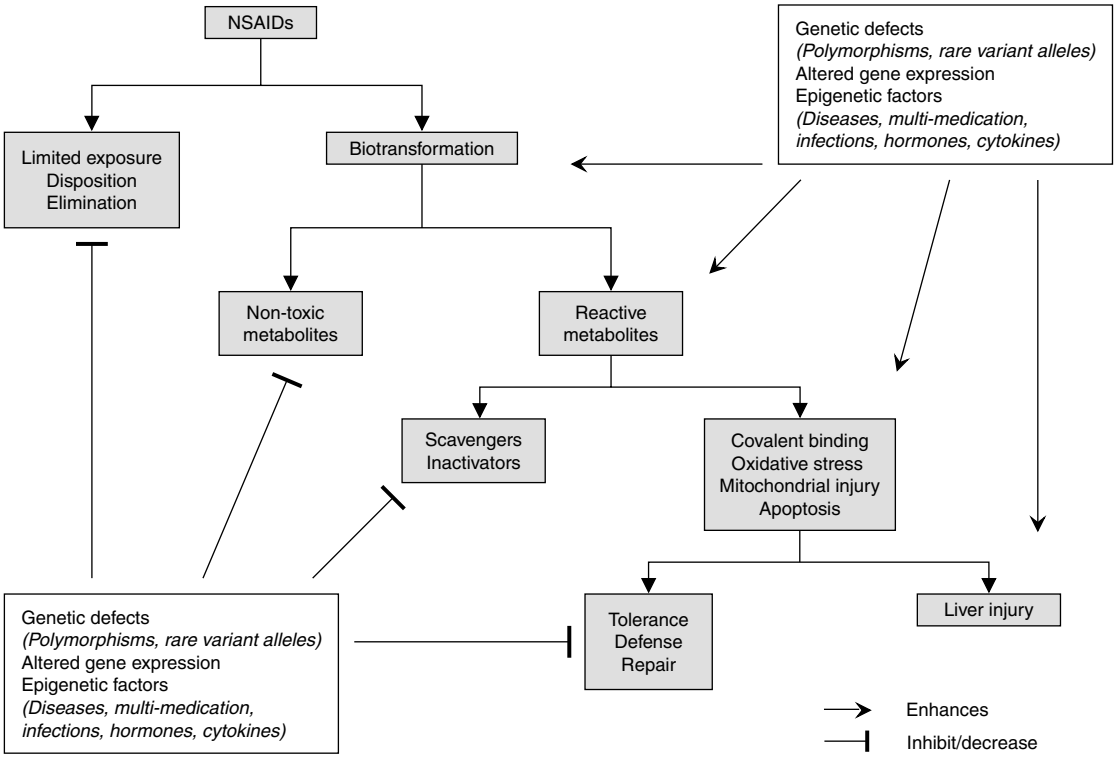

Fig. 2. Key steps that lead to a nonsteroidal anti-inflammatory drug (NSAID) to reach high exposure levels and/or produce large amounts of reactive metabolites that cause primary tissue injury. According to this model, both genetic and epigenetic factors can either enhance these steps, or inhibit/decrease protective and detoxifying pathways, resulting in the development of rare idiosyncratic liver toxicity.

that a number of distinct and compound-selective mechanisms act together or are even superimposed on each other. Among these, three key mechanisms underlying NSAID-induced liver injury have been emerging: mitochondrial injury, induction of cholestasis, and protein adduct formation by reactive drug metabolites. Recently, possible direct consequences of COX inhibition have also been revisited as an additional factor.

1.2.1 Mitochondrial Injury

Mitochondrial injury has been incriminated as a general mechanism in the toxicity of many NSAIDs.[22-27] The lipophilic and weakly acidic drugs can easily penetrate the outer mitochondrial membrane and act as protonophores, shuttling protons from the intermembraneous space across the inner mitochondrial membrane back into the matrix. By doing so, this futile proton cycling dissipates the proton gradient, which had been built up and which is maintained by the electron transport chain. This leads to a decrease in ATP synthetase activity, which is normally driven by this proton gradient. The result of mitochondrial uncoupling of oxidative phosphorylation is a cellular energy crisis and, ultimately, cell demise.

Although there is experimental evidence in support of this concept, most studies have used isolated mitochondria or cultured cells exposed to high concentrations of the drugs, and the relevance *in vivo* is not clear. For example, the critical concentrations needed to cause uncoupling effects are

rarely attained *in vivo* because all NSAIDs exhibit a high degree of plasma protein binding, which greatly reduces the concentrations of 'free' (unbound) drug. Furthermore, there is no clear correlation between the potential, in relative terms, of a given NSAID to cause liver injury in humans and the degree by which it is able to cause mitochondrial uncoupling and ATP depletion *in vitro*.

Mitochondrial injury can also be triggered by other mechanisms, including stress-induced opening of the mitochondrial permeability transition pore (MPTP).[28-31] The fact that some NSAIDs pose an intracellular oxidative stress which can cause NAD(P)H oxidation and/or protein thiol oxidation is an attractive hypothesis to alternatively explain mitochondrial injury.

1.2.2 Cholestasis

The clinical picture of NSAID-associated hepatotoxicity often includes intrahepatic cholestasis. In many cases, however, it is difficult to assess whether cholestasis is the cause or the consequence of liver injury. In either case, cholestasis can further exacerbate the disease. For example, some NSAIDs or their metabolites have been demonstrated to compete with bile salts or bile acid conjugates for hepatobiliary transmembrane transport.[32-34] Enterohepatic circulation, which is common for many NSAIDs, might further increase the hepatocellular exposure and thus prolong the interference with transporters. One consequence would be the retention and/or accumulation of toxic bile salts in the liver cells, which in turn will cause oxidative stress and apoptosis if the impairment of bile salt export will remain uncompensated. Although some alternative carriers can be up-regulated under conditions of cholestasis, to compensate for the loss of other transporters,[35] the exposure of hepatocytes towards both toxic bile salts and the NSAID or its metabolites will continue to be abnormally high.

Alternatively, if there is pre-existing cholestasis and the liver is subsequently loaded with an NSAID, then the compromised ability of the liver to excrete the NSAID or their metabolites by the biliary route will similarly lead to increased intracellular concentrations of the drug and cause toxicity.

1.2.3 Protein-Reactive Metabolites

A number of acidic NSAIDs have been implicated in forming covalent adducts to hepatic proteins. This protein-reactivity is the result of enzymatic bioactivation of the drug to a reactive metabolite. One of the best studied examples are the carboxylic acid-containing NSAIDs which are normally conjugated to glucuronic acid to form acyl glucuronides. Acyl glucuronides are electrophilic intermediates which are unstable and which can interact with nucleophilic amino acid residues to form covalent protein adducts to hepatocytes, the biliary tree (into which they are secreted), and more distally the small intestinal epithelia.[32,34,36-45] Thus, target proteins can be covalently modified and, if the protein is critical or if the adduct density is high enough, result in functional impairment[46-48] or even in haptenation, causing an immune response against the drug-altered peptide.[49-51] Although never proven *in vivo*, this covalent binding mechanism has been implicated in the hepatic toxicity of many NSAIDs. It has also been suggested to play a role in the formation of neoantigens and the precipitation of immune-mediated hypersensitivity reactions in the liver.

1.2.4 Inhibition of Cyclo-Oxygenase-2

Recent insights into the molecular regulatory processes indicate that prostaglandin (PG) E2 might play a crucial role in maintaining a line of defence against drug toxicity, and that inhibition of COX by NSAIDs may therefore interfere with these defence mechanisms. For example, COX-2, which is normally not expressed in the liver but which can be induced in Kupffer cells under pathophysiological conditions such as cholestasis,[52] catalyses the production of prostanoids in the liver. These prostanoids in turn up-regulate the anti-apoptotic mitochondrial protein Bcl-2, which can protect against bile acid-induced apoptosis. Inhibition of PGE2 production by COX-2 inhibitors

may therefore block this anti-apoptotic signal. These findings might provide an intriguing explanation for the increased susceptibility of mitochondria towards oxidative stress in the presence of COX inhibitors.[52] Indeed, a study with COX-2 knockout mice demonstrated that the absence of PGE2 rendered these mice more susceptible to the hepatotoxic effects of paracetamol (acetaminophen),[53] suggesting that PGE2 might be a important regulatory signal involved in protection from drug-induced injury. Whether this mechanism might contribute to the hepatic toxicity of NSAIDs *in vivo* is, however, still unclear.

1.3 Genetic and Epigenetic Factors Sensitising the Liver to Adverse Effects from NSAIDs

A number of possible host factors are likely to account for a greater hepatic sensitivity of individuals to some NSAIDs, promoting the development of the liver disease via the mechanisms already described. For example, rare genetic abnormalities that: (i) render mitochondria more susceptible to drug-induced injury; (ii) aggravate cholestasis and enhance the accumulation of toxic bile salts; and (iii) gate the bioactivating pathways towards higher rates of protein-reactive metabolites, can be postulated to play a role. However, such genetic abnormalities have not yet been demonstrated in patients. In addition, epigenetic causes, such as environmental stimuli that would up- or down-regulate proteins involved in drug uptake and/or export from the liver cells, could also increase the risk. Finally, factors including the underlying disease itself, such as rheumatoid arthritis, in particular in the chronic stage, is associated with unchecked up-regulated pro-inflammatory cytokines.[54] Similarly, the non-selective COX inhibitors may injure the intestinal mucosa, increase the permeability, and release bacterial lipopolysaccharide (LPS) into the bloodstream, which in turn may exacerbate liver injury by the hepatic production of tumour necrosis factor (TNF)-α and other proinflammatory cytokines.

It has been noted that a similar hepatotoxic phenotype can be produced by NSAIDs with different chemical structures,[16] and nimesulide is no exception. What is not known, however, is whether the majority of NSAIDs also share similar mechanisms underlying the idiosyncratic toxicity in the liver. Nimesulide is unique in that it bears a number of compound-specific structural and pharmacological features, as well as follows differential pathways of metabolism and disposition in the liver. To better understand the hepatic liability of nimesulide, and to be able to weigh the risk of hepatotoxicity with this agent against that associated with other NSAIDs, the following part of this review will focus on these nimesulide-specific characteristics.

2. Nimesulide

2.1 Molecular Characteristics

From the molecular viewpoint and with respect to the relevance for hepatic safety assessment, nimesulide is an atypical NSAID for a number of reasons. First, it does not possess a carboxylic acid moiety, nor is it metabolised to a carboxylic acid. Hence, nimesulide does not undergo conjugation to a reactive acyl glucuronide. Second, nimesulide is a sulfonanilide derivative (a structural feature facilitating access to the COX-2 binding site) and is only weakly acidic, with a pKa of 6.5.[55,56] This is a favourable feature, rendering uptake of nimesulide and intracellular trapping in the gastrointestinal mucosa more difficult than for other, more acidic NSAIDs. Third, nimesulide is a selective COX-2 inhibitor, with only residual activity against COX-1 after therapeutic doses.[1,57-61] This characteristic most likely accounts for the good gastric tolerability of nimesulide, which is shared with other COX-2 inhibitors.[62] Fourth, besides COX-2, nimesulide selectively interacts with other molecular targets, too.[63] For example, and again in contrast to classical NSAIDs, nimesulide inhibits phosphodiesterase (PDE) IV, the major enzyme involved in cAMP degradation. This effect, which has been demonstrated *in vitro*, can bear important

toxicological implications, as nimesulide is able, through PDE IV inhibition, to inhibit macrophage and neutrophil function and attenuate the release of proinflammatory cytokines and reactive oxygen (ROS) and nitrogen species released by these phagocytes.[64] *In vivo* evidence, however, has yet to be provided.

Although these and other characteristics clearly distinguish nimesulide from classical acidic NSAIDs, nimesulide has been associated with idiosyncratic adverse reactions in the liver, similar to other NSAIDs.

2.2 Incidence and Phenotype of Nimesulide-Induced Hepatotoxicity

During clinical trials (15 to 30 days of treatment), nimesulide was associated with a low incidence (0.4%) of the mild form of hepatic reactions, reflected as small increases [>2 × upper limit of normal (ULN)] in aminotransferase activities in the plasma.[65] This number was slightly higher (1.5%) when the duration of treatment was prolonged to 6 to 12 months. This is clearly a low value which can be expected for an NSAID.[7]

As to the rare but more serious forms of liver injury, no long-term pharmacoepidemiological studies are available which would allow for a direct comparison among several NSAIDs in the same population. Therefore, both historical data and postmarketing surveillance data have to be evoked in order make estimations about incidence rates. A number of case reports describing an association between nimesulide treatment and the development of liver injury have appeared in the recent past, despite the fact that the product has been on the market since 1985.[65-76] Although multi-medication or pre-existing liver disease can always contribute to the severity of liver injury, and although causality assessment in drug-induced hepatotoxicity can be ambiguous,[74] it is generally accepted that nimesulide, similar to many other NSAIDs, bears an intrinsic hazard and has the potential to be causally involved in precipitating liver dysfunction.

Global reports of adverse events, however, are an unreliable measure of risk because they may be biased by underreporting and differences in the reporting rate from one country to another.[77,78] The number of reported cases should be clearly seen against the background of the large number of patients exposed to the drug and should be corrected for the appearance of the same adverse effect but unrelated to the drug.[14,79] In a non-exposed population, and provided that exposure to other known hepatotoxicants or viral hepatitis can be excluded, serious liver disease is a very rare event. In fact, the background incidence for acute liver failure has been estimated to be 1 to 2 cases per million.[80] Therefore, even a slight increase in the incidence of liver disease caused by NSAIDs would become evident.[17]

Accordingly, the incidence of nimesulide-related hepatic injury can be calculated from the number of globally reported cases divided by the total number of patients treated, based on estimates from the total unit sales.[65] According to Helsinn Healthcare, the major manufacturer of nimesulide, between 1985 and 2001, a total number of 195 hepatic adverse effects were reported to the company, of which 123 were considered to be serious and 72 classified as non-serious.[81] A rough estimation of the total number of patients treated in the 15-year period revealed that approximately 304 million patients have been receiving the drug. This was calculated on the basis of a mean treatment time of 2 weeks for acute treatment and of 6 months for long-term treatment.[81] From these numbers, an incidence of approximately 0.1 case of hepatic liability per 100 000 patients can be calculated. This number may be too low due to underreporting, and long-term treatment may be more common than assumed, making the recipient population smaller. Nevertheless, even after correction the incidence is still low. Furthermore, by comparing the incidence of nimesulide-associated liver injury with that caused by other NSAIDs, it becomes evident that the risk for nimesulide is comparable to that of most other currently used

NSAIDs.[17,82] There are exceptions, such as sulindac, where the incidence is approximately 10-fold higher,[17] or the coxibs, which have been introduced only recently and for which no reports are available that would point to liver toxicity.

Analysis of the published cases of nimesulide-associated serious liver adverse reactions[66-76] revealed three typical features. First, in some patients, centrilobular or massive panlobular hepatocellular necrosis, sometimes with mild or moderate inflammatory infiltration, had occurred. This was accompanied by highly increased serum aminotransferase activities (ALT mean 18 × ULN, range 3 to 89 × ULN; AST mean 21 × ULN, range 2 to 86 × ULN) with or without increases in bilirubin levels (mean 14 × ULN, range 2 to 52 × ULN). In others, the injury was defined histopathologically as intrahepatic cholestasis. In some individuals, the type of injury was mixed hepatocellular and cholestatic. Second, the presence of these two types of liver injury was gender-selective. For example, in one study hepatocellular necrosis occurred in female patients (4 of 6), while in males bland intrahepatic cholestasis with biliary pigment both in canaliculi and in hepatocytes was present (2 of 6).[68] Third, typical signs of hypersensitivity reactions were found in the minority of cases only. Thus, immune mechanisms do not seem to be the rule for nimesulide-induced hepatic reactions. This would also be compatible with the relatively long time period between the start of treatment and the onset of symptoms.

Taken together, in view of the very large population of patients treated worldwide with nimesulide, the absolute risk of developing hepatic adverse effects including fulminant hepatitis is very low. This is a feature that nimesulide shares with some other widely used NSAIDs, e.g. diclofenac.

2.3 Molecular Mechanisms of Liver Injury: Contribution of the Drug

In common with other NSAIDs, the molecular mechanisms underlying the rare hepatic toxicity of nimesulide have remained enigmatic, and there are no reliable animal models available which would allow one to reproduce the effects and to explore the mechanisms. However, it has become clear that the hepatic metabolism and disposition of nimesulide and its metabolites play a pivotal role.

2.3.1 Hepatic Metabolism and Disposition of Nimesulide

Nimesulide is almost exclusively metabolised and cleared by the liver.[83] Therefore, it is inviting to speculate that patients with hepatic insufficiency and individuals with genetic or acquired abnormalities in drug metabolising enzymes or transmembrane carriers, may be at an increased risk for higher nimesulide concentrations in the liver.

Metabolic biotransformation of nimesulide in the liver can occur at both the phenoxy ring moiety and the aromatic nitro group (figure 1). The major oxidative metabolite found in plasma is 4′-hydroxy nimesulide.[83] This metabolite is normally conjugated with sulphate or glucuronic acid and excreted renally or via biliary elimination (figure 3). Nitroreduction is an equally important pathway of biotransformation, and toxicologically of great importance, because it results in the formation of an aromatic amine. The amine metabolite is further metabolised by *N*-acetylation, catalysed by *N*-acetyltransferases (NAT) [figure 3]. These intermediates can also be further sulpho- or glucuronoconjugated and excreted in urine.

In common with other drugs causing idiosyncratic liver injury,[84] it is the presence of reactive metabolites generated in the liver that seems to be crucial and which may play a central role in nimesulide-associated adverse hepatic reactions. In particular, the nitroreductive pathway produces potentially reactive species (figure 4). Although not yet proven *in vivo*, evidence stemming from *in vitro* systems suggests that these reactive intermediates have the potential to pose an oxidoreductive stress and/or undergo covalent binding to selective target proteins (see sections 2.3.2 and 2.3.3). A particularly sensitive organelle are the mitochondria.

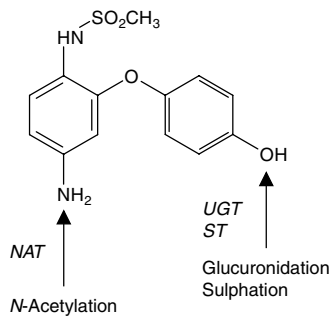

Fig. 3. Major pathways of nimesulide metabolism in the liver. The nitroarene is reduced to the amine, followed by *N*-acetylation. The phenoxy ring undergoes 4′-hydroxylation and subsequent conjugation to glucuronic acid or sulphate. Cross-combinations of these metabolic steps are common and all metabolites have been identified in humans. **NAT** = *N*-acetyltransferase; **ST** = sulfotransferase; **UGT** = uridine diphosphate glucuronosyltransferase.

2.3.2 Oxidative Stress

There is compelling evidence that nitroaromatic compounds, including nimesulide, can induce intracellular oxidative stress in *in vitro*.[85-89] The underlying cause is the relative ease by which nitroaromatic compounds can be enzymatically reduced by nitroreductase-catalysed one- or two-electron transfer pathways (figure 4). These nitroreductases, which are flavoproteins, are located in the cytosol and the endoplasmic reticulum but, importantly, are also active in mitochondria.[90] The one-electron transfer pathway produces the nitro anion radical, a highly reactive species which, under aerobic conditions, is able to transfer the electron to molecular oxygen, thereby producing superoxide anion radicals. The production of this intermediate has indeed been demonstrated *in vitro* by electrochemical generation from nimesulide and its reactivity has been studied.[91] *In vivo*, however, this intermediate has not been demonstrated, probably because it rapidly autooxidises back to the parent compound. Redox cycling is potentially hazardous because one molecule of nitro compound can theoretically give rise to a large number of superoxide anion molecules (figure 4). The resulting downstream production of superoxide anion-derived ROS can, if not compensated by antioxidant defence mechanisms, alter cellular signalling, induce mitochondrial alterations, or even trigger apoptosis.

2.3.3 Covalent Protein Binding

The two-electron transfer pathway of nitroarenes leads to the production of the amine, with the nitroso and the hydroxylamine as intermediate metabolites (figure 4). This multi-step reduction of aromatic nitro compounds is a common pathway for, e.g. sulphonamide drugs and has been well characterised.[92,93] Importantly, both the hydroxylamine and the nitroso intermediate are electrophilic, protein-reactive species that can deplete glutathione and form covalent adducts with proteins.[92]

The aromatic amine (and its *N*-acetylated conjugate) are indeed major metabolites of nimesulide.[94] In contrast, the reactive nitroso and hydroxylamine intermediates have not been demonstrated *in vivo*, probably because they are readily reduced and because they are short-lived. Nevertheless, covalent binding of [^{14}C]nimesulide to protein has been demonstrated in rat liver microsomal or S9 fractions,[95] lending further evidence for the generation of protein-reactive intermediates Covalent

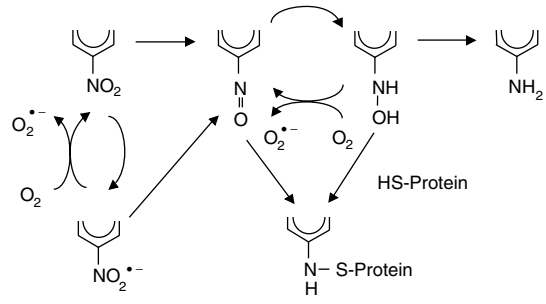

Fig. 4. Nitroreductive metabolism of nimesulide leading to the putative formation of reactive intermediates. Redox cycling between the nitro anion radical and the parent compound can produce superoxide anion and pose an oxidative stress, while production of the nitroso and *N*-hydroxy compound can cause both oxidative stress and covalent protein adduct formation. The latter process will be stimulated if, for example, the reductive step from the *N*-hydroxy to the amine is inhibited, or if the rates of *N*-acetylation and subsequent elimination of the amine are decreased.

binding of critical target proteins is thus a cellular reaction that nimesulide shares with other acidic NSAIDs, although through a completely different mechanism. If remaining uncompensated or not repaired, persistent adducts could alter a protein's function, or, if occurring in mitochondria, alter mitochondrial function.

2.3.4 Mitochondrial Injury

Recent studies have indicated that nimesulide is able to uncouple oxidative phosphorylation in isolated mitochondria.[27,96,97] Specifically, the drug increased basal respiration, decreased the transmembrane potential and inhibited ATP synthesis with an EC50 (concentration required to inhibit synthesis by 50%) ranging between 5 and 27 μmol/L. It should be noted, however, that studies with isolated mitochondria are usually done in the absence of serum proteins added to the incubation media. Therefore, the EC50 values obtained (in the low micromolar range) may be misleading. To assess the *in vivo* relevance of the mitochondrial effects, the nimesulide concentrations entailing uncoupling activity *in vitro* have to be compared with the free (non-plasma protein-bound) therapeutic plasma concentrations in humans. Assuming peak plasma concentrations in the range of 20 μmol/L and the unbound fraction of nimesulide being 1%,[94] then the concentrations used in the mitochondrial studies were approximately 100-fold higher than therapeutically relevant concentrations. Thus, nimesulide concentrations in patients are unlikely to reach levels that are high enough to induce substantial uncoupling *in vivo*. This assumption is corroborated by an *in vivo* rat study where mitochondrial uncoupling of respiration (determined indirectly as increases in intestinal permeability) could be induced at the high (supratherapeutic) dose of 60 mg/kg only.[98]

Indeed, experimental evidence suggests that the protonophoretic activity of nimesulide is unlikely to be the only mechanism by which mitochondria are injured. Incubation of mitochondria with nimesulide caused osmotic swelling of mitochondria and NAD(P)H oxidation.[96] This indicates that activation and opening of the MPTP had occurred. The MPTP is a redox-sensitive megachannel across the inner and outer mitochondrial membrane which is normally closed but opens upon increased oxidative stress. The consequences of MPTP opening are several-fold and include dissipation of the intermembraneous proton gradient, leading to ATP depletion, mitochondrial swelling and rupture of the outer mitochondrial membrane, and release of apoptosis-inducing mediators into the cytosol. To date, however, no *in vivo* data are published that would demonstrate whether nimesulide might be able to induce apoptosis in the liver.

Because the uncoupling effects were lost when the reduced arylamine metabolite of nimesulide, as opposed to the parent compound, was incubated with mitochondria, the conclusion that the nitro group was responsible for the mitochondrial liability is further supported.[96] In fact, the combined data allow one to provide a working hypothesis of the molecular mechanisms underlying nimesulide-associated mitochondrial injury (figure 5). This hypothesis implies that high concentrations of nimesulide penetrate into mitochondria, where bioreductive metabolism produces redox cycling and oxidative stress, leading to opening of the MPTP. In addition, bioreductive metabolism of nimesulide in the cytosol and/or endoplasmic reticulum causes both oxidative stress and covalent modification of target proteins which also can lead to direct or indirect (via Ca^{2+} mobilisation) activation of the MPTP. It is also likely that covalent modification of intracellular proteins, together with oxidative stress-induced depletion of glutathione, might activate signalling pathways that lead to hepatocellular necrosis.

2.3.5 Role of Tumour Necrosis Factor-α

Proinflammatory cytokines including TNFα have been implicated in exacerbating drug-induced liver injury.[99] TNFα expression is normally regulated by prostaglandins. For example, inhibition of COX-2 causes up-regulation of TNFα via a multi-step feedback loop.[100] While

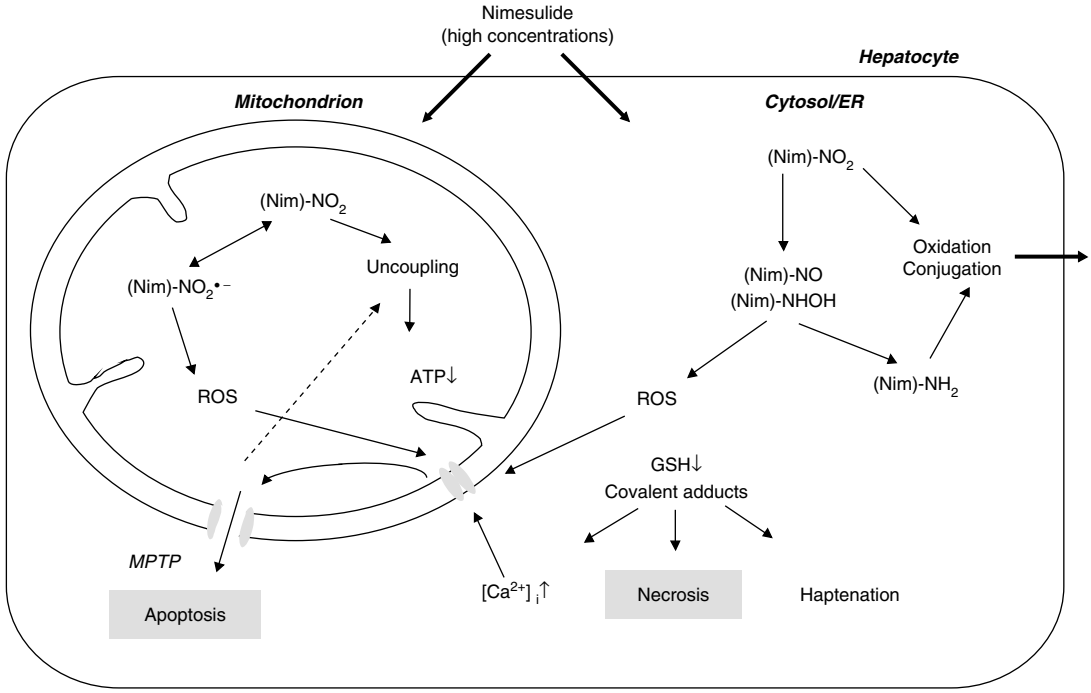

Fig. 5. Working hypothesis of the molecular events leading to nimesulide-induced mitochondrial injury and induction of cell death. If high concentrations of nimesulide are taken up by hepatocytes, the drug penetrates into mitochondria, where bioreductive metabolism produces redox cycling and oxidative stress. In addition, bioreductive metabolism of nimesulide in the cytosol and/or endoplasmic reticulum causes both oxidative stress and covalent modification of target proteins. This can lead to direct (or possibly indirect, via Ca^{2+} mobilisation) activation of the mitochondrial membrane permeability transition pore (MPTP). It is also reasonable to assume that covalent modification of intracellular proteins, together with oxidative stress-induced depletion of glutathione, might activate signalling pathways that lead to hepatocellular necrosis. **$[Ca^{2+}]_i$** = intracellular concentrations of free calcium; **ER =** endoplasmic reticulum; **GSH** = glutathione; **Nim** = nimesulide; **ROS** = reactive oxygen species; ↑ = increase; ↓ = decrease.

most NSAIDs indeed produce local and systemic increases in TNFα, which could lead to pro-inflammatory changes or even tissue injury,[101] nimesulide is an exception in that it causes inhibition rather than stimulation of TNFα production (via PDE IV inhibition, see section 3.1). In fact, in contrast to other NSAIDs (for example, indomethacin, ibuprofen) nimesulide *in vivo* totally prevented TNFα production in rat liver even upon challenge with the powerful TNFα-stimulating agent lipopolysaccharide.[102] Because TNFα is also essential in liver injury because it stimulates the production of cytoprotective factors,[103] it is not clear to date whether this suppression of TNF-α might play a role in nimesulide-associated hepatocellular injury.

2.4 Molecular Mechanisms of Liver Injury: Contribution of the Susceptible Patient

Although host factors play a pivotal role in idiosyncratic drug reactions, little is known about these individual susceptibility factors. Increasing awareness of relevant genetic abnormalities will undoubtedly shed more light on this aspect in the future. For example, a genetic candidate factor that would determine a key step in the bioactivation cascade (figure 2) is the genetic polymorphism in NAT which is critically involved in detoxication

and elimination of nimesulide. Indeed, for sulphonamides, evidence indicates that 90% of patients with hypersensitivity reactions belonged to the slow acetylator phenotype.[104] Meanwhile, a large number of additional allelic variants of NAT1 and NAT2, with their multiple single-nucleotide polymorphisms (SNPs) have been well characterised.[105,106] In general, it can be assumed that a fast acetylator phenotype may protect an individual from the toxic consequences of reactive metabolites of sulphonamides.[107] However, this has not been demonstrated for nimesulide.

Another putative susceptibility factor is an aberration in the gene coding for hydroxylamine reductase, resulting in incomplete reduction of the aromatic hydroxylamine to the amine.[108] This could reduce drug elimination and favour redox cycling and covalent binding of the reactive drug intermediates.

Finally, the underlying disease itself, or inflammatory reactions associated with the disease, can pose another susceptibility factor. For example, in a rat arthritis model, the extent of mitochondrial liability after incubation of isolated mitochondria with nimesulide was substantially higher than that in mitochondria from normal rats.[97] Although nimesulide today is mainly used against osteoarthritis and as short-term anti-inflammatory treatment, rather than to treat rheumatoid arthritis, one should be aware of these patient factors.

3. Conclusions

3.1 Assessment of Hepatic Safety of Nimesulide: Hazard

A number of general as well as nimesulide-specific features that are relevant for the hepatic safety have been identified. They can be summarised as follows.

First, nimesulide bioactivation in the liver produces potentially protein-reactive intermediates and poses an oxidoreductive stress. This is a hazard that nimesulide shares with other NSAIDs. Although nimesulide does not form acyl glucuronides, it bears an aromatic nitro group which undergoes nitroreductive activation and which has been implicated in the toxicity of other nitroaromatic compounds,[86,109-111] or aromatic amines [92,112,113]

Second, one of the features that differentiates nimesulide from other NSAIDs is the fact that nimesulide is a powerful inhibitor of PDE IV. This effect has been linked with the ability of nimesulide to inhibit TNFα production,[100] as PDE IV inhibitors increase intracellular levels of cAMP, which in turn will down-regulate TNFα expression.[114-116] PDE IV inhibitors also exhibit a range of other effects besides inhibition of TNFα synthesis. For example, they can protect from NSAID-induced enteropathy by non-TNFα-mediated mechanisms.[117] As a consequence, intestinal injury, which can have serious repercussions on the liver, is attenuated, and possibly deleterious effects of LPS on the liver parenchyma are prevented.

3.2 Assessment of Hepatic Safety of Nimesulide: Risk

When human exposure data and mechanistic considerations are used to estimate the actual nimesulide-associated risk for patients to develop liver dysfunction or overt injury, the following key points have emerged.

First, nimesulide is widely used and in fact in 2000 ranked fifth among NSAIDs in the worldwide market.[61] Therefore, the appearance of adverse effects including those in the liver has to be evaluated with respect to the large population exposed to the drug. However, by weighing the relative risk of nimesulide-associated serious liver reactions, rather than by focusing on individual case reports, it becomes clear that this risk is low and comparable with a number of other commonly used NSAIDs.

Second, the individual risk factors that can predispose a patient to an increased risk for developing nimesulide-associated hepatic adverse reactions, such as specific gene abnormalities, alterations in specific gene expression, or epigenetic factors, are currently not known. Therefore, the occurrence of these rare reactions will remain unpredictable. A

more detailed understanding of the complex molecular mechanisms underlying the hepatic liability of nimesulide and other NSAIDs and a better knowledge of the genetic and epigenetic predisposing factors will facilitate the identification of the small populations of susceptible patients.

Finally, a small number of clinical trials using endoscopic endpoints have revealed that nimesulide has a smaller potential to induce gastrointestinal injury than other, non-selective COX inhibitors.[2,62,118-121] This was recently supported by a postmarketing surveillance study in Northern Italy.[3] In view of the fact that gastrointestinal adverse effects are a major problem in NSAID therapy and that nimesulide in humans exhibits a high selectivity for COX-2,[1] a global safety assessment of nimesulide must also take this GI-sparing effect of nimesulide into account.[61,122]

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