Toxicology in Vitro 26 (2012) 197–205

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

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

Differential involvement of mitochondrial dysfunction, cytochrome P450 activity, and active transport in the toxicity of structurally related NSAIDs

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ARTICLE INFO

Article history:
Received 18 April 2011
Accepted 17 November 2011
Available online 26 November 2011

Keywords:
NSAID
Yeast
Cytochrome P450
Reactive oxygen species
Multidrug resistance

ABSTRACT

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of pain and inflammation. However, this group of drugs is associated with serious adverse drug reactions. Previously, we studied the mechanisms underlying toxicity of the NSAID diclofenac using *Saccharomyces cerevisiae* as model system. We identified the involvement of several mitochondrial proteins, a transporter and cytochrome P450 activity in diclofenac toxicity. In this study, we investigated if these processes are also involved in the toxicity of other NSAIDs. We divided the NSAIDs into three classes based on their toxicity mechanisms. Class I consists of diclofenac, indomethacin and ketoprofen. Mitochondrial respiration and reactive oxygen species (ROS) play a major role in the toxicity of this class. Metabolism by cytochrome P450s further increases their toxicity, while ABC-transporters decrease the toxicity. Mitochondria and oxidative metabolism also contribute to toxicity of class II drugs ibuprofen and naproxen, but another cellular target dominates their toxicity. Interestingly, ibuprofen was the only NSAID that was unable to induce upregulation of the multidrug resistance response. The class III NSAIDs sulindac, ketorolac and zomepirac were relatively non-toxic in yeast. In conclusion, we demonstrate the use of yeast to investigate the mechanisms underlying the toxicity of structurally related drugs.

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used class of medication worldwide. They inhibit cyclooxygenases involved in the synthesis of prostaglandins and are widely used in the treatment of pain and inflammation. Unfortunately, NSAIDs can cause serious adverse drug reactions that mainly target the upper gastrointestines (Lewis et al., 2002). Also heart (Fosbol et al., 2009), kidney (Lafrance and Miller, 2009) and liver (Laine et al., 2009) toxicity have been reported after human NSAID use. Additionally, glucuronidation of NSAIDs can lead to instable glucuronide-conjugates that, via protein adduct formation, can activate the immune system leading to allergic reactions such as anaphylaxis and Stevens–Johnson syndrome (Sawamura et al., 2010). Gastrointestinal and kidney toxicity have been related to non-selective inhibition of cyclooxygenases, and have decreased after development of selective COX-2 inhibitors (Lafrance and Miller, 2009; Mitchell and Warner, 1999). However, the occurrence of heart and liver failure remains (Fosbol et al., 2009; Laine et al., 2009), indicating that additional mechanisms of toxicity exist.

In earlier studies, we have used *Saccharomyces cerevisiae* as model organism to study the toxicity of diclofenac (van Leeuwen et al., 2011a,b,c). Advantages of yeast as model in toxicity studies are its well-annotated genome, straightforward genetics, cost-effectiveness and rapid growth (Hoon et al., 2008; Yasokawa and Iwahashi, 2010). Genetic screens using the genome spanning collections of deletion strains or overexpression constructs have led to the identification of on- and off-targets of various drugs (Ho et al., 2011). Many P450s have been heterologously expressed in yeast to study metabolism related (geno)toxicity. Additionally, yeast lacks cyclooxygenases, which simplifies the test system, whereas many of the mechanisms underlying toxicity, such as mitochondrial dysfunction and DNA damage, are conserved (Steinmetz et al., 2002; Yu et al., 2008).

In mammalian cell lines, the toxicity of NSAIDs has been associated with mitochondrial dysfunction (Moreno-Sanchez et al., 1999), metabolism by cytochrome P450s (Jurima-Romet et al., 1994) and efflux by transporters (Mima et al., 2007). However, the underlying mechanisms and relative contribution of these processes to NSAID toxicity remain largely unknown. Previously, we showed that yeast cells lacking mitochondrial DNA (so called rho^0 cells) and thereby missing the mitochondrial electron transport chain (ETC) are resistant to diclofenac. Furthermore,

Abbreviations: DIC, diclofenac; IBU, ibuprofen; IND, indomethacin; ETC, electron transport chain; KEP, ketoprofen; KER, ketorolac; MDR, multidrug resistance; NAP, naproxen; NSAID, non-steroidal anti-inflammatory drug; P450, cytochrome P450; ROS, reactive oxygen species; SUL, sulindac; ZOM, zomepirac.

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doi:10.1016/j.tiv.2011.11.013

ROS formation induced by diclofenac in yeast is dependent on the presence of the ETC. Within the ETC, we identified subunits Rip1p and Cox9p of the respiratory chain complex III and IV, respectively, as major mitochondrial targets of diclofenac (van Leeuwen et al., 2011a). Additionally, expression of bacterial cytochrome P450 (P450) BM3 mutant M11, which metabolizes diclofenac in a similar way as human P450s, increased the toxicity of diclofenac in yeast (van Leeuwen et al., 2011b). Cytochrome P450 BM3 is extensively genetically modified to mimic drug metabolism by human P450s and has the advantage that it is a soluble protein composed of both an oxidase and reductase domain (Kumar, 2010). Furthermore, we used microarray analysis to study the genetic responses of yeast towards diclofenac exposure and showed that the multidrug resistance (MDR) response and especially ABC-transporter Pdr5p were involved in resistance against diclofenac (van Leeuwen et al., 2011c).

In the current study, we will explore if these proteins and pathways are also involved in toxicity and tolerance of other NSAIDs and compare their relative contribution to toxicity as well as investigate whether yeast cells provide an additional model system to detect toxicity pathways *in vitro*. For this purpose, we selected a set of structurally related NSAIDs, all either acetic acid or propionic acid derivatives (Fig. S1) and studied their toxicity in various yeast strains to identify underlying toxicity mechanisms. In this way, we can subdivide the NSAIDs in three classes based on their mechanisms of toxicity in yeast.

2. Materials and methods

2.1. Chemicals and stock solutions

All NSAIDs were purchased from Sigma Aldrich at highest purity. Naproxen was dissolved in ethanol (100 mM) and all other NSAIDs in DMSO (100 mM). 2′,7′-Dichlorodihydrofluorescein diacetate was obtained from Alexis Biochemicals and dissolved in ethanol (4 mM). All stock solutions were stored at −20 °C and protected from light. All other chemicals were purchased from Sigma Aldrich at the highest purity.

2.2. Yeast strains and plasmids

The haploid BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) wild type and deletion strains were obtained from EUROSCARF. Strains without mitochondrial DNA (rho^0) were generated by growth with ethidium bromide (10 μg/ml) as described previously (Goldring et al., 1970). Loss of mitochondrial DNA was confirmed by staining the DNA with DAPI (4′-6-diamidino-2-phenylindole). BM3 M11 was expressed in yeast using a previously described, galactose-inducible construct (van Leeuwen et al., 2011b).

2.3. Growth conditions and NSAID incubation

Strains were grown overnight on minimal media (YNB: 0.67% yeast nitrogen base without amino acids, 2% glucose, supplemented amino acids and bases) at 30 °C. Overnight cultures were diluted in minimal media and grown at 30 °C to an optical density at 600 nm (OD_{600}) of ~0.2. At this point, 25–500 μM of the NSAID was added. Controls were treated with equal amounts of DMSO or ethanol (max 1% v/v). All cultures were growing at similar, exponential, growth rates before drugs or DMSO/EtOH was added. The OD_{600} of control and NSAID-treated cultures was followed for 24–48 h.

2.4. Measurement of reactive oxygen species (ROS) production

Exponentially growing yeast cultures (2 ml) of OD_{600} ~0.2 were treated with the NSAID in the presence of 10 μM of the fluorescent, ROS-sensitive 2′,7′-dichlorodihydrofluorescein diacetate. After 3 h at 30 °C the cultures were centrifuged (3 min 3000 rpm) and cell pellets were washed and resuspended in 1 ml water. Fluorescence (λ_{ex} = 485 nm, λ_{em} = 535 nm) was measured as relative fluorescence units (RFU) and corrected for the cell density in the samples.

2.5. ß-Galactosidase reporter assay

The *TRP5*- and *PDR5*-lacZ reporter constructs in the low copy number vector pSEYC102 were a kind gift from Prof. Scott Moye-Rowley (Katzmann et al., 1994). The plasmids were transformed into yeast using the freeze–thaw method (Klebe et al., 1983). Yeast cultures (150 μl in 96-well plates) of OD_{600} ~0.2 were treated with the NSAID. After 3 h, the OD_{600} was measured and cells were lysed by addition of 50 μl 2% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) in lacZ buffer (40 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-mercaptoethanol). After incubating 30 min, 20 μl of chlorophenolred-ß-D-galactopyranoside (1.2 mg/ml in lacZ buffer) was added and the samples were incubated 1–2 h at 30 °C. Absorption at 575 nm was measured and corrected for the cell density in the samples.

2.6. Statistical analysis

To compare yeast cell growth, ROS formation or *lacZ* expression between samples, the two-sided Student's *t*-test for unpaired samples was used. *P* values less than 0.01 were considered statistically significant. When applicable, *P* values were corrected for multiple comparisons.

3. Results

3.1. Measurement of growth inhibition and general NSAID toxicity in yeast

To assess the general toxicity of the NSAIDs in yeast, wild type yeast cells were incubated with 50–500 μM of the drugs and growth of the cultures was followed in time (Fig. 1). The highest concentration used of sulindac (SUL) and zomepirac (ZOM) was 250 μM since both drugs precipitated at higher concentrations. A characteristic growth experiment is shown in Fig. 1A. Most NSAIDs caused a growth delay, but when growth resumed had little effect on the growth rate (Fig. 1A). Likely, adaptation is required for surviving cells to resume growth in the presence of the drug (van Leeuwen et al., 2011c). We used the growth delay (Δt) as measure for toxicity (Fig. 1A and B).

Diclofenac (DIC), indomethacin (IND) and ibuprofen (IBU) were most toxic and no growth was observed at concentrations above 100 μM. Ketoprofen (KEP) and naproxen (NAP) showed significant toxicity at 500 μM, while sulindac was only slightly toxic in the range between 50–250 μM (Fig. 1B). Ketorolac (KER) and zomepirac did not cause a significant delay in growth at the concentrations used.

Previously, we described an increase in the formation of reactive oxygen species (ROS) during diclofenac incubation using both 2′,7′-dichlorodihydrofluorescein diacetate and dihydroethidium as ROS-sensitive probes (van Leeuwen et al., 2011a). Here, we found that also other NSAIDs induce ROS formation in a concentration-dependent manner (Fig. 2). For most NSAIDs, concentrations that caused a growth delay also induced significant ROS formation. Ketorolac and zomepirac, that did not affect growth, also hardly induced ROS formation. However, there is no obvious correlation between the length of the growth delay and the amount of ROS. Cultures incubated with indomethacin and sulindac produced relatively high amounts of ROS compared to the growth delay, while

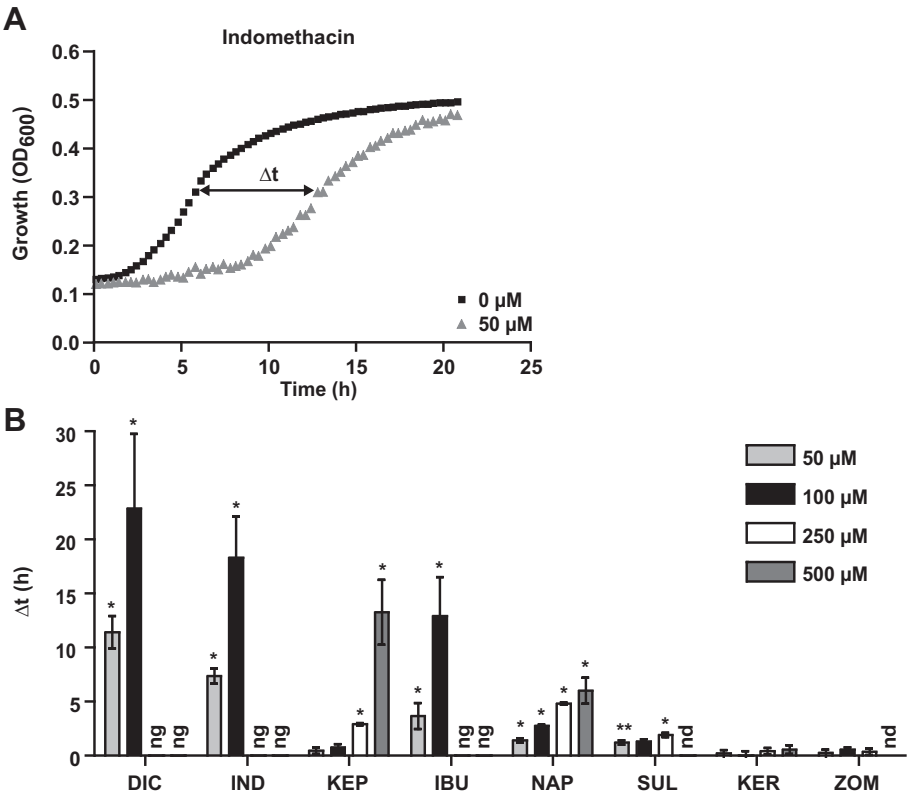

Fig. 1. NSAID toxicity in yeast. (A) Growth of wild type BY4741 yeast cells incubated with 0 μM (black squares) or 50 μM (grey triangles) indomethacin in YNB medium supplemented with glucose. Measurement of the growth delay as Δt is indicated. (B) Delay in growth of BY4741 wild type cells incubated with 50, 100, 250 or 500 μM diclofenac (DIC), indomethacin (IND), ketoprofen (KEP), ibuprofen (IBU), naproxen (NAP), sulindac (SUL), ketorolac (KER) or zomepirac (ZOM). The delay in growth (Δt) is expressed in hours ± SD. $^*P < 0.01$ compared to cells incubated without drug. N.d. = not determined. N.g. = no growth during 24 h.

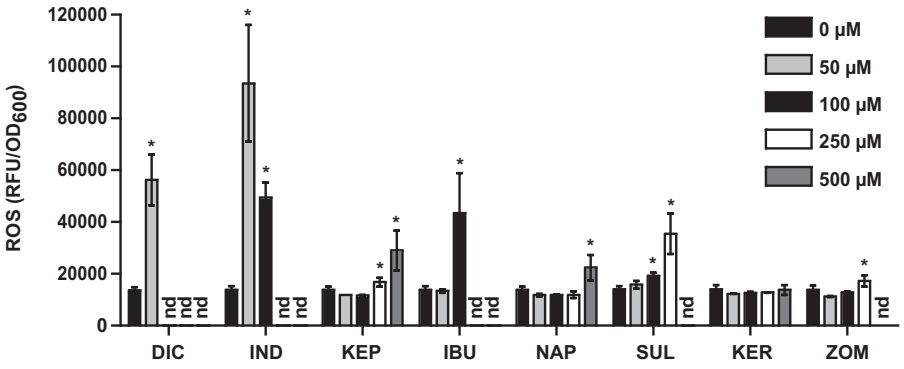

Fig. 2. NSAID-induced ROS formation. ROS levels in BY4741 wild type cells incubated for 3 h with 0, 50, 100, 250 or 500 μM diclofenac (DIC), indomethacin (IND), ketoprofen (KEP), ibuprofen (IBU), naproxen (NAP), sulindac (SUL), ketorolac (KER) or zomepirac (ZOM) in the presence of the ROS-sensitive, fluorescent 2′,7′-dichlorodihydrofluorescein diacetate in YNB medium supplemented with glucose. The ROS levels are expressed as fluorescence units (RFU), corrected for the cell density in the samples, ±SD. $^*P < 0.01$ compared to cells incubated without drug. N.d. = not determined.

ketoprofen induced relatively low ROS formation. For instance, at 50 μM indomethacin, we see ~7× increase in ROS formation, which only corresponds to a Δt of 7.5 h. In contrast, Δt's longer than 10 h are seen for 50 μM diclofenac, 500 μM ketoprofen and 100 μM ibuprofen although ROS levels are increased no more than ~2–4×. Also, 250 μM sulindac has a comparable ROS level as 500 μM ketoprofen, but a much shorter Δt (2 and 13 h, respectively). Interestingly, 50 μM ibuprofen and 250 μM naproxen already caused a significant growth delay (Fig. 1B) without an increase in ROS formation (Fig. 2). The reduced ROS formation at 100 μM versus 50 μM for IND probably reflects the reduced number of viable cells, as also growth was severely inhibited at this concentration (Fig. 1B).

3.2. Mitochondrial toxicity of NSAIDs

The mitochondrial respiratory chain is the main source of ROS in the cell (Kowaltowski et al., 2009). Recently, we found that diclofenac inhibits oxygen consumption in yeast and that toxicity is enhanced when yeast cells rely more on respiration for their energy production (van Leeuwen et al., 2011a). We have shown that rho^0 cells, that lack mitochondrial DNA and are therefore unable to

respire, show increased resistance to diclofenac (van Leeuwen et al., 2011a). Mitochondria are possibly also involved in the toxicity of other NSAIDs (Lal et al., 2009; Moreno-Sanchez et al., 1999). We investigated the role of mitochondria in NSAID toxicity by comparing the resistance of a rho^0 strain to wild type cells. Since sulindac, ketorolac and zomepirac were not or only slightly toxic to the wild type strain (Fig. 1B) we did not test the resistance of rho^0 cells to these drugs. We found that, similar to diclofenac, rho^0 cells were also much more resistant to indomethacin and ketoprofen, but lack of mitochondrial DNA did not affect the toxicity of ibuprofen and naproxen to the same extent (Fig. 3) indicative for a different major cellular drug target.

We used yeast deletion strains lacking genes encoding subunits of the mitochondrial electron transfer chain to identify the position of interference of the NSAID with respiration. Especially strains lacking subunit Rip1p or Cox9p of respiratory chain complex III and IV, respectively, were shown to be significantly more resistant to diclofenac (van Leeuwen et al., 2011a). Here, we tested the NSAID-resistance of strains in which the genes were deleted encoding the yeast equivalent of complex I (Ndi1p) or subunits of complex II (Sdh1p), III (Qcr2p or Rip1p) or IV (Cox6p or Cox9p). Deletion of other complex II, III or IV subunits (Sdh2p, Cor1p or Cox5ap, respectively) revealed similar phenotypes as Δsdh1, Δqcr2 and Δcox6 (data not shown). Resistance to indomethacin and ketoprofen was greatly increased in cells lacking *RIP1* or *COX9* as shown by a reduction in Δ*t* by more than 50% (Fig. 3), indicating an underlying mechanism of toxicity identical to diclofenac. Sensitivity to ibuprofen and naproxen showed only a minor change in Δ*t* of 10–30% in these strains. Apparently, mitochondrial respiration only plays a minor role in the toxicity of these compounds. Notably, although with 100 μM diclofenac the sensitivity of Δndi1, Δsdh1, Δqcr2 and Δcox6 is comparable to that of wild type cells (van Leeuwen et al., 2011a), with 50 μM diclofenac these strains had an increase in resistance compared to wild type (Fig. 3). Also with indomethacin, Δcox6 and Δsdh1 showed increased resistance. However, Rip1p and Cox9p are the main respiratory contributors to diclofenac, indomethacin and ketoprofen toxicity.

Previously, we have described a clear correlation between diclofenac toxicity and diclofenac-induced ROS formation (van Leeuwen et al., 2011a). Here, we investigated the ROS levels induced by the other NSAIDs in the various respiratory deficient strains at drug-concentrations where ROS levels were prominent. Indeed, rho^0 cells were not only more resistant to diclofenac, indomethacin and ketoprofen, the ROS levels were also significantly lower in this strain compared to wild type cells (Fig. 4). Interestingly, although rho^0 cells did not show increased resistance to ibuprofen, the ROS levels induced by this drug were significantly lower in the rho^0 strain compared to wild type. Also Δrip1 and Δcox9 cells hardly showed any ROS formation at all for diclofenac,

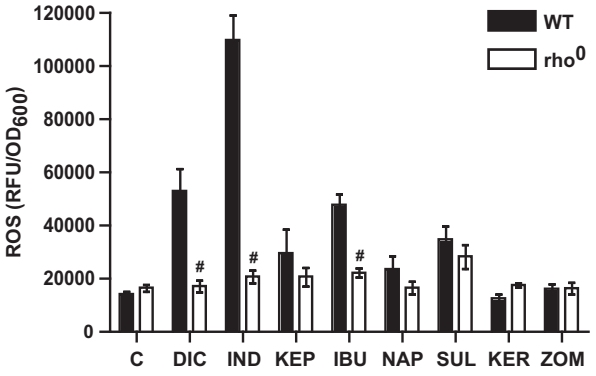

Fig. 4. Decreased ROS formation in rho^0 cells. Wild type cells (WT) and cells lacking mitochondrial DNA (rho^0) were incubated for 3 h with 50 μM diclofenac (DIC), 50 μM indomethacin (IND), 500 μM ketoprofen (KEP), 100 μM ibuprofen (IBU), 500 μM naproxen (NAP), 250 μM sulindac (SUL), 500 μM ketorolac (KER), 250 μM zomepirac (ZOM) or without drug (C) in the presence of the ROS-sensitive, fluorescent 2′,7′-dichlorodihydrofluorescein diacetate in YNB medium supplemented with glucose. The ROS levels are expressed as RFU, corrected for the cell density in the samples, ±SD. $^{\#}P < 0.01$ compared to WT cells treated with the same drug.

indomethacin and ibuprofen while the ROS levels in Δndi1, Δsdh1, Δqcr2 and Δcox6 were comparable to wild type levels (data not shown). Apparently, although Rip1p and Cox9p contribute to ibuprofen-induced ROS formation, this ROS formation is not the main cause of toxicity of ibuprofen. The ROS levels of sulindac-treated rho^0 cells were ~170% of those of untreated rho^0 cells, showing that sulindac-induced production of ROS is apparently not related to respiration. Ketorolac and zomepirac hardly induced ROS formation in wild type cells, so no decrease in ROS levels could be observed for these drugs.

3.3. Oxidative metabolism-dependent NSAID toxicity

Another suggested cause of NSAID toxicity is metabolism by cytochrome P450s (Agundez et al., 2009; Jurima-Romet et al., 1994). A previously established model system expressing cytochrome P450 BM3 M11 in yeast (van Leeuwen et al., 2011b) was used to examine the toxicity of NSAID oxidative metabolism. BM3 M11 is a model P450 that metabolizes various drugs, including the NSAIDs paracetamol and diclofenac, in a similar way as human P450s (Damsten et al., 2008; van Leeuwen et al., 2011b). Strains expressing BM3 M11 or control strains transformed with an empty vector were incubated with the NSAID and growth and ROS formation were examined (Fig. 5). In contrast to what we saw in the other experiments with glucose as carbon-source

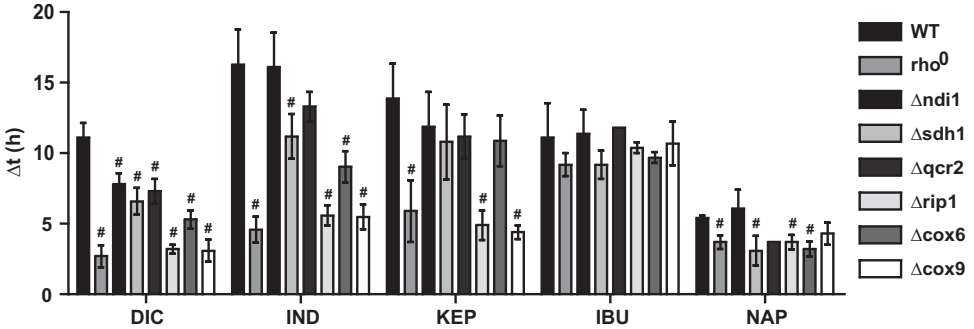

Fig. 3. Involvement of mitochondria in NSAID toxicity. Growth of BY4741 wild type (WT) cells, cells lacking mitochondrial DNA (rho^0) or cells in which a gene encoding a subunit of a respiratory chain complex has been deleted in YNB medium supplemented with glucose. The strains were grown in the presence of 50 μM diclofenac (DIC), 100 μM indomethacin (IND), 500 μM ketoprofen (KEP), 100 μM ibuprofen (IBU) or 500 μM naproxen (NAP). The delay in growth (Δ*t*) is expressed in hours compared to unexposed cells ± SD. $^{\#}P < 0.01$ compared to WT cells treated with the same drug.

(Fig. 1A), on the galactose medium necessary to induce BM3 M11 expression, the NSAIDs did not just lead to a delay in growth but also decreased the growth rate (Fig. 5A). This might be due to the increased respiration on galactose-medium, which was shown to enhance diclofenac toxicity (van Leeuwen et al., 2011a). In the absence of NSAIDs, strains expressing BM3 M11 or controls had comparable growth and ROS levels. BM3 M11 activity increased the toxicity of diclofenac, indomethacin, ketoprofen and naproxen (Fig. 5A), which corresponded with increased ROS levels (Fig. 5B). For diclofenac, indomethacin and ketoprofen, the increase in ROS due to BM3 M11 was most prominent (1.5–2 times). With ibuprofen, a slight increase in ROS formation was seen in the BM3 M11 expressing strain, however this difference was not significant ($p > 0.01$), while the modest increase in ROS for naproxen was significant ($p < 0.01$). BM3 M11 expression did not increase toxicity (data not shown) and ROS levels of cells incubated with sulindac, ketorolac or zomepirac.

3.4. Active transport and the multidrug resistance response

Previously, we showed that yeast ABC-transporter Pdr5p contributes to diclofenac-resistance (van Leeuwen et al., 2011c). In this research, we explored the role of the two main ABC-transporters Pdr5p and Snq2p in NSAID resistance in yeast. WT, Δpdr5 and Δsnq2 cells were incubated with the NSAIDs and growth was monitored (Fig. 6A). Indeed, lack of Pdr5p increased diclofenac toxicity. The Δpdr5 strain was also more sensitive to ketoprofen compared to wild type cells. Deletion of *SNQ2* increased the sensitivity to indomethacin, ketoprofen and sulindac. The toxicity of ibuprofen, naproxen, ketorolac and zomepirac was not enhanced by deletion of *PDR5* or *SNQ2*. These results show that the two promiscuous transporters show substrate-specificity towards these structurally related NSAIDs.

NSAIDs are known to induce the expression of various drug transporters in human cells (Mima et al., 2007; Takara et al., 2009; Tatebe et al., 2002). Previously, we showed that diclofenac also increased expression of multidrug resistance genes in yeast (van Leeuwen et al., 2011c). In yeast, transcription factors Pdr1p and Pdr3p regulate expression of multidrug resistance genes like *PDR5*, *SNQ2* and *RSB1*. Using a lacZ reporter construct we tested the effect of the NSAIDs on expression of *PDR5* (Fig. 6B). Expression of *TRP5*, encoding a protein involved in tryptophan biosynthesis, was assayed as a control (Fig. 6C). Diclofenac, indomethacin, ketoprofen, naproxen, sulindac, ketorolac and zomepirac all induced

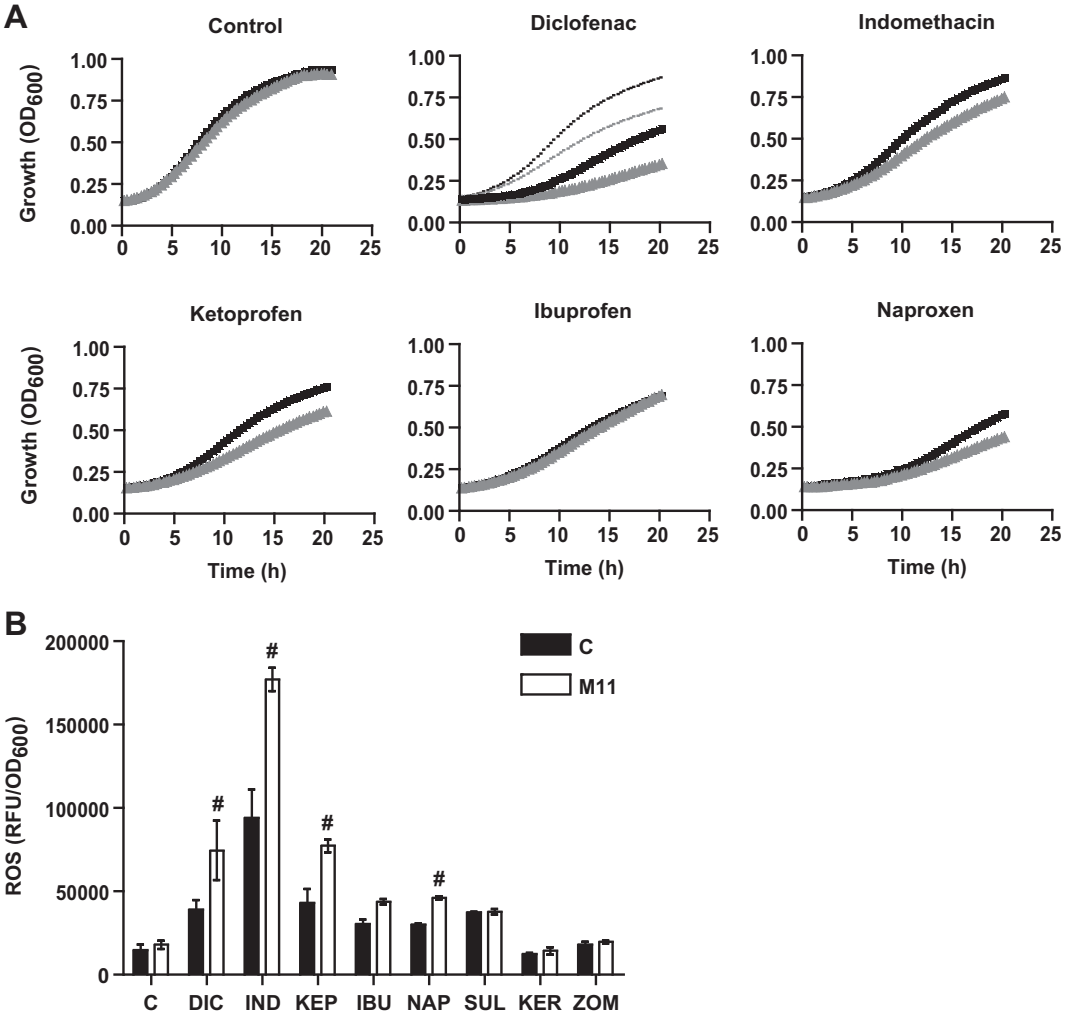

Fig. 5. Metabolism-dependent toxicity. (A) Growth curves of BY4741 strains expressing cytochrome P450 BM3 M11 (grey lines) or transformed with an empty vector (black lines). Growth is expressed as OD_{600}. Representative growth curves are shown. (B) ROS levels in BY4741 cells expressing BM3 M11 (M11) or transformed with an empty vector (C). The ROS levels are expressed as RFU, corrected for the cell density in the samples, ±SD. $^{\#}P < 0.01$ compared to cells transformed with an empty vector treated with the same drug. The strains were incubated without drug or with 50 μM diclofenac (DIC), 50 μM indomethacin (IND), 500 μM ketoprofen (KEP), 50 μM ibuprofen (IBU), 500 μM naproxen (NAP), 250 μM sulindac (SUL), 500 μM ketorolac (KER) or 250 μM zomepirac (ZOM) in YNB medium supplemented with galactose.

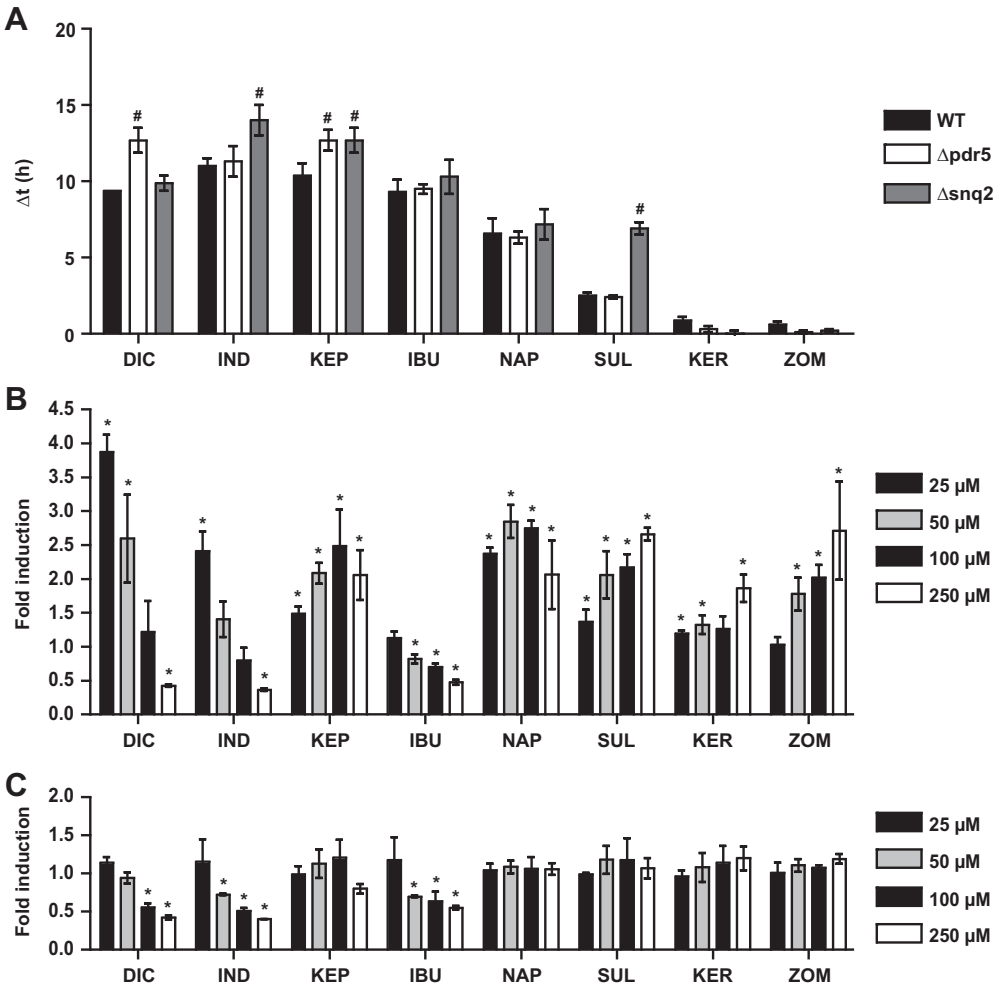

Fig. 6. Involvement of transporters and MDR in NSAID toxicity. (A) Growth of BY4741 wild type cells (WT) or cells lacking the genes encoding ABC-transporter Pdr5p (Δpdr5) or Snq2p (Δsnq2) incubated with 50 μM diclofenac (DIC), 100 μM indomethacin (IND), 500 μM ketoprofen (KEP), 100 μM ibuprofen (IBU), 500 μM naproxen (NAP), 250 μM sulindac (SUL), 500 μM ketorolac (KER) or 250 μM zomepirac (ZOM) in YNB medium supplemented with glucose. The delay in growth (Δt) is expressed in hours ± SD. $^{\#}P < 0.01$ compared to WT cells treated with the same drug. (B,C) *PDR5*- (B) and *TRP5*-lacZ (C) expression in wild type strains incubated with 25, 50, 100 or 250 μM of the indicated NSAID. Data are expressed as fold induction of ß-galactosidase activity after correction for cell density in the samples ±SD. $^{*}P < 0.01$ compared to cells incubated without drug.

expression of *PDR5*, while *TRP5* expression levels were not increased. Higher concentrations of diclofenac, indomethacin and ibuprofen decreased *PDR5* and *TRP5* expression, probably due to decreased viability of the cells. *RSB1* and *SNQ2* showed a similar induction-pattern as *PDR5*, although *SNQ2* induction was generally 2-fold lower (data not shown). Surprisingly, ibuprofen did not increase expression of multidrug resistance genes *PDR5*, *RSB1* or *SNQ2*.

4. Discussion

In this study we used yeast as eukaryotic model to investigate the toxicity of a set of structurally related NSAIDs containing a carboxylic acid group (Fig. S1). We have focused on the role of mitochondrial respiration, metabolism by cytochrome P450 BM3 M11 and active transport in NSAID toxicity, since these mechanisms have not only been implicated in NSAID toxicity in humans (Jurima-Romet et al., 1994; Lal et al., 2009; Mima et al., 2007) but we have also identified these as important in diclofenac toxicity in yeast (van Leeuwen et al., 2011a,b,c). We have used a set of yeast strains lacking mitochondrial respiration or transporter encoding genes or expressing bacterial cytochrome P450 BM3 mutant M11. By investigating these three toxicity mechanisms in a single study and model organism, we could compare the relative contribution of the processes to NSAID toxicity. The results obtained in yeast can provide a short cut into the investigation of the toxicity of these drugs on human cells. An example of such a spin-off is provided by Yu et al. (2008), who showed for structurally related imidalo-pyridines and -pyrimidines a differential involvement of mitochondrial dysfunction in their toxicity in yeast and confirmed these results in cultured human cells. Therefore, using a selected set of yeast mitochondrial deletion strains is not only a fast and cost-efficient way to determine the involvement of mitochondrial respiration in the toxicity of drugs, but potentially also relevant to humans.

Further support for the suitability of yeast is the observation that the general 'order' of NSAID-toxicity in yeast (Fig. 1) is comparable to what was found in rat hepatocytes and human epithelial cell cultures although the concentrations we used were generally tenfold lower than what was used in the mammalian cells (Allen et al., 1991; Jurima-Romet et al., 1994; Masubuchi et al., 1998). In all model systems, the highest toxicity was observed with indomethacin and diclofenac. Interestingly, naproxen seems to be relatively more toxic to yeast than to the mammalian cells.

Historically, NSAIDs are classified based on their structure (i.e. acetic acid derivatives, salicylates) or on their ability to inhibit

cyclooxygenase 2 (selective COX-2 inhibitors or 'coxibs'). Based on the experimental results obtained in this study, we have grouped the NSAIDs in classes according to the mechanisms involved in their toxicity (Table 1). Class I is characterized by toxicity related to the generation of ROS at the mitochondrial respiratory chain and ROS formation is enhanced by cytochrome P450 BM3 M11 metabolism. Class II is represented by compounds, which mainly cause toxicity independent of ROS production and respiration. Class III is defined by a relative lack of toxicity at the tested concentrations in yeast, although a PDR response is elicited. Both class II and III NSAIDs show little or no P450 BM3 M11-related toxicity.

4.1. Mitochondrial toxicity of NSAIDs

The first class of NSAIDs consists of diclofenac, indomethacin and ketoprofen, whose toxicity is clearly linked to the mitochondrial respiratory chain with its subunits Rip1p and Cox9p as potential drug off-targets (Fig. 3). Deletion of the gene for either of these subunits or removal of the complete electron transfer chain by using rho^0 cells greatly diminished the toxicity of class I compounds. Although the mitochondrial effects of diclofenac and indomethacin have been widely described in literature using isolated rat liver mitochondria (Lal et al., 2009; Moreno-Sanchez et al., 1999), this is to our knowledge the first clear mechanistic understanding of the toxicity of ketoprofen in disturbing mitochondrial respiration. Translational research is needed to confirm the mitochondrial toxicity of ketoprofen in mammalian cells. Interference of the respiratory chain results in ROS production that has a negative impact on growth and viability of the yeast cells as shown for diclofenac in a survival assay (van Leeuwen et al., 2011a).

Ibuprofen and naproxen are grouped as a separate class of NSAIDs that only slightly affect mitochondrial function. Although ibuprofen showed significantly lower ROS production in rho^0 cells, this did not lead to a major decrease in toxicity as for class I NSAIDs (Figs. 3 and 4). Additionally, ibuprofen (at 50 μM) and naproxen (at 250 μM) cause a growth delay in the absence of ROS induction, indicating that ROS formation is not a primary cause of toxicity of these drugs. Although for ibuprofen uncoupling has been described in isolated mitochondria (Lal et al., 2009; Moreno-Sanchez et al., 1999), mitochondrial dysfunction does not seem to be involved in toxicity in yeast. Previously, Campos et al. (2004) showed that mitochondrial dysfunction was also not involved in ibuprofen-induced cell death in mammalian tumor cells. Together these results show that another, as yet unknown target dominates the toxicity of ibuprofen and naproxen.

Table 1
Summary of toxicity parameters obtained in yeast.

		Toxicity[a]			ROS[b]		P450[c]	Transporter[d]	MDR[e]
		WT	rho^0	Δrip1	WT	rho^0			
I	DIC	+++	+	+	++	–	++	PDR5	++
	IND	+++	+	+	++	–	++	SNQ2	++
	KEP	++	+	+	+	–	++	PDR5 + SNQ2	++
II	IBU	+++	+++	+++	++	–	–	–	–
	NAP	++	+	+	+	–	+	–	++
III	SUL	+	nd	nd	+	+	–	SNQ2	++
	KER	–	nd	nd	–	–	–	–	+
	ZOM	–	nd	nd	–	–	–	–	++

"+" indicates the relative increase compared to controls, "–" means no change compared to controls. "nd" = not determined.

[a] Ability of the NSAID to delay growth of the WT, rho^0 or Δrip1 strain.

[b] Ability of the NSAID to induce ROS formation in the WT or rho^0 strain.

[c] Increase in toxicity in the cytochrome P450 BM3 M11 expressing strain compared to a control strain.

[d] ABC-transporter whose deletion increased toxicity of the NSAID.

[e] Ability of the NSAID to induce expression of multidrug resistance genes.

The class III NSAIDs, consisting of sulindac, ketorolac and zomepirac, are relatively non-toxic to yeast. However, SUL did increase ROS formation in both WT and rho^0 cells. Apparently, an as yet unknown source and cellular localization (but not the ETC) of ROS formation does not result in a major growth defect. A possible other source of ROS could be the interference of NSAIDs with fatty acid oxidation (Yang et al., 1998). As the class III NSAIDs did induce expression of *PDR5*, the low toxicity is probably not a result of decreased cellular uptake of the drugs.

4.2. Metabolism-related toxicity of NSAIDs

All NSAIDs used in this study contain a carboxylic acid moiety that is readily glucuronidated. The resulting glucuronides are reactive and can form protein-adducts associated with adverse immune reactions (Sawamura et al., 2010). In yeast, glucuronidation does not occur due to the lack of glucuronosyl-transferases. However, glucuronidation and protein-adduct formation do not correlate with hepatotoxicity (Obach et al., 2008) suggesting that another mechanism such as metabolism by cytochrome P450s may contribute to the toxicity.

Jurima-Romet et al. (1994) compared the effect of P450 activity on the toxicity of a range of NSAIDs in rat hepatocytes using a P450-inducer or inhibitor. They found that especially with diclofenac and ketoprofen differences in P450 expression had a large effect on toxicity. We expressed the versatile P450 BM3 M11 in yeast, which can metabolize a wide variety of drugs in a similar way as human P450s (Damsten et al., 2008). Using this model, we also found increased toxicity related to diclofenac and ketoprofen metabolism, but found additionally P450-related toxicity for indomethacin and naproxen (Fig. 5). This increase in toxicity could be indicative of the generation of a more toxic compound and can both be the result or the cause of the observed increase in ROS formation. No clear role for P450-metabolism in ibuprofen toxicity could be determined. Sulindac, zomepirac and ketorolac toxicity was clearly not related to metabolism by cytochrome P450 BM3 M11 in yeast. In mammalian cells, sulindac is reduced by methionine sulfoxide reductases to the pharmacologically active sulindac sulfide that can cause mitochondrial uncoupling (Brunell et al., 2011; Leite et al., 2006). Although yeast contains two methionine sulfoxide reductases, Mxr1p and Mxr2p, it is unknown if these can metabolize sulindac. This may be useful to further investigate in future toxicity studies using yeast and sulindac.

The oxidative metabolism of diclofenac is extensively studied in various models, including the BM3 M11 enzyme used here, and multiple major and minor metabolites and their conjugates have been identified (Damsten et al., 2008; van Leeuwen et al., 2011b). However, less is known about the P450-related metabolism of the other NSAIDs, for which mainly the primary metabolic route has been described (Agundez et al., 2009). Both indomethacin and naproxen are subject to O-demethylation by human P450s (Nakajima et al., 1998; Rodrigues et al., 1996). For diclofenac, indomethacin and ketoprofen a decarboxylated metabolite has been described, whose formation might be involved in toxicity (Grillo et al., 2008; Komuro et al., 1995; van Leeuwen et al., 2011b). The various NSAIDs can also be hydroxylated on the aromatic rings, which may proceed via potentially toxic epoxides as has been described for diclofenac and zomepirac (Chen et al., 2006; Yan et al., 2005). The resulting hydroxyl-metabolite may lead to quinone-like reactive species (Shen et al., 1999), however, diclofenac quinone-imines are not toxic in yeast (van Leeuwen et al., 2011b). Further research into the oxidative metabolite profiles of the NSAIDs will be needed to identify the metabolic route(s) responsible for the increase in toxicity.

4.3. MDR and transport

Decreased NSAID-efflux might also cause toxicity by increasing the cellular concentration of the drug. Indeed, polymorphisms in *ABCC2* (encoding MRP2) may contribute to toxicity in NSAID-users (Daly et al., 2007). Diclofenac, indomethacin, ketoprofen, ibuprofen, naproxen and sulindac were found to interact with the ABC-transporters MRP4, MRP2, MRP1 and/or BCRP (El-Sheikh et al., 2007; Nozaki et al., 2007; Reid et al., 2003). In yeast, overexpression of major facilitator family-member Tpo1p increases resistance to diclofenac, indomethacin and ibuprofen (Mima et al., 2007). Also overexpression of the mammalian Tpo1p homolog, TETRAN, conveys resistance to diclofenac and indomethacin in human adenocarcinoma gastric cells, while silencing increases sensitivity (Mima et al., 2007). We found that ABC-transporter Pdr5p contributes to the resistance to diclofenac and ketoprofen while Snq2p augments resistance to indomethacin, ketoprofen and sulindac.

Although sequence identity is relatively low, there is a strong functional analogy between MDR-regulators Pdr1p/Pdr3p in yeast and PXR in mammals (Thakur et al., 2008). Both Pdr1p/Pdr3p and PXR can directly bind a large variety of xenobiotics and thereby induce expression of multidrug resistance transporters (Kliewer et al., 2002; Thakur et al., 2008). Both up- and downregulation of ABC-transporters by NSAIDs has been described in various mammalian cells, since expression levels of transporters are strongly affected by variations in cell type, COX-2 levels and incubation time (Patel et al., 2002; Roller et al., 1999; Zrieki et al., 2008). Diclofenac, indomethacin and sulindac can increase expression of MDR1, MRP1, MRP3 and/or TETRAN in human cancer cell lines (Mima et al., 2007; Takara et al., 2009; Tatebe et al., 2002). Additionally, we found that also ketoprofen, naproxen, ketorolac and zomepirac induce the MDR response (Fig. 6B). For ibuprofen, no increase in MDR1 expression was observed in human cells (Takara et al., 2009) and, interestingly, we neither observed an increase in PDR5 transporter-expression in yeast (Fig. 6B). Although both Pdr1p/Pdr3p and PXR are very promiscuous, ibuprofen is apparently unable to interact in such a way to promote MDR induction.

5. Conclusion

Using yeast as model system we investigated the involvement of mitochondrial dysfunction, cytochrome P450-related metabolism and active transport in NSAID toxicity and used these processes to classify the NSAIDs tested. We found that generation of ROS by the mitochondrial respiratory chain is the main toxicological event in diclofenac, indomethacin and ketoprofen toxicity, while this is not the primary cause of toxicity for ibuprofen or naproxen. Intracellular metabolism by cytochrome P450 BM3 M11 increased the toxicity of diclofenac, indomethacin, ketoprofen and naproxen but had no significant effect on the toxicity of the other NSAIDs tested. Finally, ABC-transporters Pdr5p and/or Snq2p were involved in the efflux of diclofenac, indomethacin, ketoprofen and sulindac. Ibuprofen is the only NSAID we tested that was unable to induce a pleiotropic drug response in yeast. Together, our results endorse that yeast provides a relatively easy, fast and cost-efficient way to determine various major mechanisms of drug toxicity.

Conflict of interest statement

None declared.

Acknowledgment

We thank Scott Moye-Rowley for the *lacZ* reporter constructs and Pedro Ramos for experimental assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tiv.2011.11.013.

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