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Influence of mustard group structure on pathways of *in vitro* metabolism of anticancer N-(2-hydroxyethyl)-3,5-dinitrobenzamide 2-mustard prodrugs.

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Hepatic metabolism of dinitrobenzamide mustard prodrugs

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DNBM, dinitrobenzamide mustard, Rt, retention time

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Abstract

The dinitrobenzamide mustards (DNBM) are a class of bioreductive nitroaromatic anticancer prodrugs, of which a phosphorylated analogue (PR-104) is currently in clinical development. They are bioactivated by tumour reductases to form DNA cross-linking cytotoxins. However their biotransformation in normal tissues has not been examined. Here we report the aerobic *in vitro* metabolism of three N-(2 hydroxyethyl)-3,5-dinitrobenzamide 2-mustards and the corresponding non-mustard analogue in human, mouse, rat and dog hepatic S9 preparations. These compounds have a range of mustard structures ($-N(CH_2CH_2X)_2$ where X=H, Cl, Br, OSO_2Me). Four metabolic routes were identified: reduction of either nitro group, *N*-dealkylation of the mustard, plus *O*-acetylation and *O*-glucuronidation of the hydroxyethyl side chain. Reduction of the nitro group *ortho* to the mustard resulted in intramolecular alkylation and is considered to be an inactivation pathway, while reduction of the nitro group *para* to the mustard generated potential DNA cross linking cytotoxins. *N*-dealkylation inactivated the mustard moiety but may result in the formation of toxic acetaldehyde derivatives. Increasing the size of the nitrogen mustard leaving group abrogated the *ortho*-nitroreduction and *N*-dealkylation routes and thereby improved overall metabolic stability, but had little effect on aerobic *para*-nitroreduction. All four compounds underwent *O*-glucuronidation of the hydroxyethyl side chain and further studies to elucidate the relative importance of this pathway *in vivo* are in progress.

The dinitrobenzamide mustards (DNBM) are bioreductive anticancer prodrugs designed to be activated by nitroreduction, either by endogenous one-electron reductases in hypoxic regions of tumours or by exogenous oxygen-independent two-electron reductases expressed in tumours using gene therapy. Enzymatic nitroreduction converts an electron-withdrawing nitro group (σ_p +0.78) to a hydroxylamine (σ_p –0.32) or amine (σ_p -0.66) metabolite; this "electronic switch" can be used to activate the latent nitrogen mustard moiety (Denny and Wilson, 1986; Palmer *et al.*, 1990; Siim *et al.*, 1997) The 2,4-dinitrobenzamide-5-mustard class (2,4-DNBM) was investigated first, and shown to provide cytotoxicity and DNA cross-linking selectively under hypoxic conditions (Palmer *et al.*, 1992). The 2,4-DNBM were subsequently shown to be substrates for the oxygen-insensitive nitroreductase *nfsB* from *E. coli* (Anlezark *et al.*, 1995; Wilson *et al.*, 2002; Atwell *et al.*, 2007), which is of interest for prodrug activation in the context of gene- or virus-directed enzyme prodrug therapy (GDEPT or VDEPT). We have shown that SN 23862 and other 2,4-DNBM provide efficient bystander effects (killing of adjoining cells) when activated by either endogenous one-electron reductases or the *nfsB* nitroreductase through the diffusion of an activated metabolite in which the nitro group *para* to the mustard has been reduced to the corresponding amine (Wilson *et al.*, 2002; Helsby *et al.*, 2004; Wilson *et al.*, 2007). In addition to its metabolic activation in tumour cells, the 2,4-DNBM SN 23862 is extensively metabolised in tumour-bearing mice, with nitroreduction of either the 4- or 2- nitro group as well as sequential oxidative *N*-dealkylation of the mustard moiety

(Kestell *et al.*, 2000). The latter route yields the non-toxic 2-4-dinitro-5 amine, but the dechloroethylation reactions also generate chloroacetaldehyde which is a reactive cytotoxin (Sood and O'Brien, 1993). Formation of chloroacetaldehyde by oxidative dealkylation has been suggested to contribute to the toxicity of oxazaphosphorine mustards (Springate, 1997) and hence may lead to normal tissue toxicity of DNBM.

Evaluation of structure-activity relationships for DNBM as hypoxic cytotoxins has identified the 3,5-dinitrobenzamide-2-mustards (3,5-DNBM) as the preferred regioisomers; this class of compounds are also good substrates for the *nfsB* nitroreductase (Singleton *et al.*, 2007). Low aqueous solubility of these prodrugs led to development of phosphate esters of 3,5-DNBM with hydroxylalkylcarboxamide side chains, with PR-104 (2-((2-Bromoethyl)-2-{[(2-hydroxyethyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate phosphate ester) possessing activity against hypoxic cells in human tumour xenografts (Patterson *et al.*, 2007). PR-104 is hydrolysed rapidly to the corresponding alcohol PR-104A (SN 27858; **1** in the present study) *in vivo,* which is reduced in hypoxic tumour cells to the DNA cross-linking *para*- hydroxylamine, PR-104H (**1a** in the present study) (Guise *et al.*, 2007; Patterson *et al.*, 2007). PR-104 is currently in clinical trial as an antitumour agent (http://www.proacta.com).

3,5-DNBM, such as PR-104, with hydroxyethylcarboxamide side chains represent a new pharmacophore for which routes of biotransformation in normal tissues are not yet well understood. A recent report has demonstrated

metabolites resulting from oxidative dealkylation and thiol conjugation of the mustard, and glucuronidation of the hydroxylethyl side chain, in mouse plasma (Patel *et al.*, 2007). The aim of the present study was to establish the chemical identity of metabolites of hydroxyethyl 3,5-DNBM in liver S9 preparations from liver of mice, rats, dogs and humans under aerobic conditions. We have previously demonstrated the *in vitro* aerobic NAD(P)H-dependent nitroreduction of the 2, 4-dinitrobenzamide-5-aziridine CB 1954 by human liver S9 (Tang *et al.*, 2005), suggesting that this will be a useful model for evaluating the sensitivity of DNBM oxygen-insensitive nitroreduction. Liver S9 preparations were used for these studies rather than microsomes as we have shown previously that nitro reduction of the related dinitrobenzamide-aziridine compound, CB 1954, involved a complex interplay of microsomal and cytosolic enzymes (Tang *et al.*, 2005). The four compounds studied here, SN 27858 (PR-104A; **1**), SN 29546 (**2**), SN 27686 (**3**), and SN 29893 (**4**), were chosen to clarify the effect of structural changes at the nitrogen mustard position on the potentially cytotoxic routes of metabolism, namely nitroreduction and *N*-dealkylation. We also compared the sensitivity of each compound to glucuronidation by liver microsomes from each species as this is pathway has potential implications for metabolic clearance and host toxicity via regeneration of the parent drug in the gastrointestinal tract.

Materials and methods

2-((2-Bromoethyl)-2-{[(2-hydroxyethyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (SN 27858, PR-104A **1**), 2-((2-chloroethyl)-2-{[(2-hydroxyethyl)amino]carbonyl}-4,6-dinitroanilino)ethyl methanesulfonate (SN 29546, **2**), 2-[*bis*(2-bromoethyl)amino]-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (SN 27686, **3**), 2-(diethylamino)-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide (SN 29893, **4**), SN 27858 glucuronide (PR-104 glucuronide;**1m**), metabolite **1a** (PR-104H), **1b** (PR-104M), metabolite **3f** (SN 29839) metabolite **4f** and metabolite **4j** were synthesised and kindly supplied by the Auckland Cancer Society Research Centre. NADPH was obtained from Applichem GmbH and NADH was purchased from Roche Applied Science, New Zealand. Ammonium formate was purchased from Acros Organics, USA. Dimethyl sulfoxide (DMSO), ethyl acetate and acetonitrile were purchased from Scharlau Chemie S.A., Spain. All other reagents and solutions used were of analytical grade.

Aerobic S9 metabolism of dinitrobenzamide analogues (**1-4**)

In vitro hepatic metabolism of compounds **1-4** was studied using the 9000*g* post-mitochondrial supernatant (S9) of human (HL5 and HL18), dog (beagle), rat (male and female, Sprague-Dawley) and mouse (pooled male and female CD-1 athymic *nu/nu*) livers, prepared as described previously (Tang *et al.*, 2005). The human livers selected were chosen because we have previously shown that they are representative of drug metabolism across a diverse range of cancer chemotherapeutics (Zhou *et al* ., 2000 ; Lu *et al.*, 2004; Tang *et al.*, 2004).

Hepatic S9 preparations (8 mg protein/mL) and dinitrobenzamide prodrug substrate (250 µM) were incubated for 30 min at 37^{o}C in a final volume of 0.5 mL sodium-potassium phosphate buffer (67 mM, pH 7.4) under air in the presence of cofactor (NADPH or NADH, 1 mM). The reaction was terminated and metabolites extracted by two sequential additions of 0.5 mL ice cold ethyl acetate, with centrifugation at 10,600*g* for 5 min following each extraction. The supernatant from the two sequential ethyl acetate extractions were combined and evaporated to dryness under vacuum. The samples were resuspended in 100 µL of mobile phase consisting of 80% ammonium formate (45 mM, pH 4.5) and 20% acetonitrile and an aliquot (80 µL) injected onto the HPLC column. All experiments were performed in quadruplicate and incubations with boiled S9 preparations were used as controls. Mass spectrometry was used for identification of the metabolites and metabolite formation was determined using peak area absorbance at 254 nm.

In vitro glucuronidation of dinitrobenzamide compounds

Microsomes (2 mg/mL) prepared from human (HL18), dog (beagle), rat (pooled male and female Sprague-Dawley) and mouse (pooled male and female CD-1 athymic *nu/nu*) liver were used to detect formation of glucuronide metabolites. Microsomes were incubated with UDPGA (5 mM) and drug substrate (250 µM) for 60 min at 37^{o}C in the presence of Brij 58 (0.15 µg/incubation) in a final volume of 0.5 mL in Tris-HCL (50 mM) buffer, pH 7.4. Following extraction,

formation of glucuronide metabolites was determined by LC/MS analysis as above.

LC/MS analysis

The HPLC system consisted of an Agilent 1100 HPLC interfaced with a diode array detector. Separation was carried out on a 2.1 x 150 mm Alltima 5µ C8 column (Alltech, USA). The mobile phase consisted of 80% $^{v}/_{v}$ acetonitrile in water (A) and 45 mM ammonium formate buffer, pH 4.5 (B) at a flow rate of 0.3 mL/min. The gradient conditions were: 0-4 min 20% of A, 4-21 min 90% A, 21-22 min 90% A, 22-26 min 20% A, with a total run time of 40 minutes. Absorbance detection was at 254 nm and 370 nm (bandwidth 4 nm, reference 550 nm). Online mass spectra were obtained with a single quadruple mass spectrometer (Agilent LC/MSD model A). Products from metabolism of the analogues were identified using positive and negative mode atmospheric pressure electrospray ionisation with nitrogen as the vaporizing and drying gas. The following parameters were used: fragmentor voltage 100 V, capillary spray voltage 4 kV, nebulizer pressure 45 psi, gas temperature 350°C, auxiliary drying gas flow 12 L/min and the mass/charge (*m/z*) ratio was scanned from 100-1000. An Agilent LC/MSD trap-SL equipped with Agilent capillary HPLC was also used for the further identification of some metabolic products. Chromatographic separation was as above with the exception that a 150 x 0.5mm, 5 µ column at a flow rate of 15 µl was used. The electrospray ionisation source was set at positive ionisation mode with auto MS(n).

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Results

In vitro hepatic S9 metabolism of SN 27858 (**1**)

Human liver S9 metabolism of the asymmetric bromomesylate compound, **1**, resulted in the formation of three metabolites (figure 1a), the inferred structures are shown in figure 2, where X= CH_2Br and Y= CH_2OSO_2Me. The reduction products the of nitro group *para* to the mustard were identified as the *para*-hydroxylamine (**1a**; $[M+H]^+$ 485 *m/z;* Rt 13.9 min) and the *para*-amine metabolite (**1b**; $[M+H]^+$ 469 *m/z*: Rt 14.5 min), as observed previously in tumour cell lines (Guise *et al.*, 2007; Patterson *et al.*, 2007). *Ortho*-nitro reduction of compound **1** in tumour cell lines under hypoxia results in intramolecular alkylation to form tetrahydroquinoxaline products (Patterson *et al.*, 2007) which have a distinctive UV spectrum (λ_{max} 400 nm). However, there was no evidence of formation of the tetrahydroquinoxaline products of *ortho*-nitro reduction of compound **1** in human liver S9. In addition there was no detectable N-dealkylation of compound **1.** However, a more lipophilic metabolite with a molecular mass of 540 (X+42 amu) was observed (**1j**) which was tentatively identified as a product of *O*-acetylation of the alcohol side chain. Nucleophilic displacement of the bromine by chloride ion from the incubation buffer resulted in the formation of the product **1k** (X= CH_2Cl and Y= CH_2OSO_2Me) with a molecular mass of 454. Other unidentified peaks were also observed in boiled S9 and are likely to be hydrolysis products of the parent compound. No new metabolites were observed following incubation with NADH as cofactor and metabolite levels were lower with this co-factor than with NADPH (data not shown).

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In vitro hepatic S9 metabolism of SN 29546 (**2**)

Human liver S9 metabolism of the asymmetric chloromesylate compound (**2**) also resulted in the formation of three metabolites (figure 1b). The inferred structures are shown in figure 2, where X= CH_2Cl and Y= CH_2OSO_2Me. The metabolites were identified as the hydroxylamine (**2a**; $[M+H]^+$ 440.9 *m/z*) and the amine metabolite (**2b**; $[M+H]^+$ 424.9 *m/z*). These products have a similar retention time and UV spectrum as **1a** and **1b** and hence are expected to be the products of reduction of the nitro group *para* to the mustard. A lipophilic metabolite with an increase in molecular mass of X+ 42 amu was observed and tentatively identified as an *O*-acetylated metabolite **2j.** Minor products presumably due to nucleophilic displacement of the mesylate moiety by hydroxyl ion (Rt 14.7; $[M+H]^+$ 377.0 m/z) or chloride ion (**2k;** Rt 16.7 min) were observed. In addition the hydrolysis product of the *para*-nitro reduced metabolite (**2b**) was also tentatively identified (Rt 12.5 min). As with the asymmetric bromomesylate compound (**1**), the chloromesylate (**2**) did not undergo any detectable *N*-dealkylation or *ortho*-nitro reduction. Similar to compound **1** no new metabolites were observed following incubation with NADH as cofactor.

In vitro hepatic S9 metabolism of SN 27686 (**3**)

Incubation of the dibromo mustard (**3**), with human liver S9 plus NADPH resulted in the formation of a complex set of products (figure 1c), of which seven were identified (the inferred structures are shown in figure 2, where X=Y=CH_2Br). The major metabolites were products of nitro reduction, **3b**, **3d** and **3e** and the *N*-dealkylated product (**3f**). Metabolite **3b** was identified as an amine metabolite

due to the decrease in mass (X-30 amu). This product had a molecular ion of $[M+H]^+$ 453 *m/z* with an adduct (+44) in the negative mode. The presence of two bromine molecules was apparent from the clear signature pattern of the satellite peaks. Although the intermediate *ortho*-hydroxylamine and *ortho*-amine metabolites were not observed, the products of intramolecular alkylation, **3d** and **3e**, were detected and characterized by the distinctive UV spectra (λ_{max} 400 nm), the presence of a single bromine satellite peak signature pattern and molecular mass (388 and 372 respectively). The metabolite, **3f** (Rt 15.9 min) had a distinctive UV spectrum (λ_{max} 365 nm) and a molecular mass of 375.5 with a satellite peak +2 amu with an intensity 98% of the base peak, indicative of a single bromine in the molecule. This metabolite was identified as the product of *N*-dealkylation and confirmed by independent synthesis. Peak **3h**, (Rt 15.2 min, molecular mass 418) also had this distinctive UV spectrum as well as the presence of a single bromine satellite peak in the mass spectrum and an increase in molecular mass of X+42 amu indicating that it may be an *O*-acetylated derivative of **3f**.

The late eluting peak (Rt 21.7 min) with a molecular mass of 524 was identified as an *O*-acetylated metabolite (**3j**) of the hydroxyethyl side chain, as described for compound **1** and **2**. Nucleophilic displacement of the bromo group by chloride ion to form a less lipophilic product (**3k**) was also observed.

Similar to compound **1** and **2** no new metabolites were observed following incubation with NADH as cofactor and metabolite levels were lower with this co-factor than with NADPH (data not shown).

In vitro hepatic S9 metabolism of SN 29893 (**4**)

Incubation of the diethylamine compound (**4**) with human liver S9 plus NADPH resulted in the formation of six metabolites (figure 1d); the inferred structures are shown in figure 2, where X =Y= CH_3. The products of nitroreduction were identified by mass spectrometry as an amine (**4b**) ($[M+H]^+$ 297 *m/z* and $[M-H]^-$ 295 *m/z*) and hydroxylamine (**4c**) ($[M+H]^+$ 313 *m/z* and $[M-H]^-$ 311 *m/z*). Compound **4b** (X-30 amu; Rt 14.1 min) was assumed to be formed by reduction of the nitro group *para* to the *N*-diethylamine moiety via the hydroxylamine intermediate **4a**, which was not observed. The hydroxylamine metabolite (**4c**; X-14 amu; Rt 11.2 min) was assumed to be formed by nitro group reduction *ortho* to this moiety. The *para*-hydroxylamine (**1a**) and *para*-amine (**1b**) metabolites of **1** have identical UV spectra (Patterson *et al.*, 2007) and retention times which differ by 0.6 min. In contrast, **4b** and **4c** have a relatively large difference in retention time of 2.9 min and also have different absorbance spectra (**4b** λmax 250 nm; **4c** λmax 250 and 400 nm); for details of UV spectra see supplementary data. Hence these metabolites have been classified as products of *para*- and *ortho*-nitro reduction respectively.

The lipophilic metabolite **4j** (Rt 18.5 min) had a higher mass ($[M-H]^-$ 367 *m/z*) but similar UV spectrum to the parent compound **4** (Rt 15.5 min). Sequential fragmentation of this metabolite using ion trap mass spectrometry (positive mode) resulted in the following daughter ions: MS^2 308, MS^3 267 and MS^4 253 *m/z* (corresponding to elimination of $-C_2H_6O_2$; $-C_2H_3N$; and -O respectively). This

metabolite (**4j**) was identified as the *O*-acetylated metabolite of **4** and this was confirmed by independent synthesis.

Metabolite **4f** ($[M+H]^+$ 299 *m/z*, $[M-H]^-$ 297 *m/z*) with a distinctive UV spectrum, λ_{max} 365 nm (supplementary data) was identified as the product of *N*-dealkylation of the diethylamine moiety. This was confirmed by independent synthesis. An additional metabolite **4g**, which was poorly resolved from **4f**, had a molecular mass of 296 with an additional change in UV spectrum at 365 nm (supplementary data) and was tentatively identified as an indazolinone product. The primary amine resulting from further *N*-dealkylation of **4f**, 2-amino-*N*-(2-hydroxyethyl)-3,5-dinitrobenzamide, was not observed. However, an additional less lipophilic metabolite **4i** (Rt 9.4 min) with a mass of 292 and a distinctive UV spectrum with λ_{max} at 250 nm and 320 nm, was observed (supplementary data). Sequential fragmentation of this metabolite using ion trap mass spectrometry (positive mode) resulted in the following daughter ions: MS^1 293, MS^2 247 and MS^3 222 *m/z*, corresponding to elimination of $-C_2H_5O$ and $-C_2H_2$ respectively. We have tentatively identified this metabolite as 2,4-dinitro-6,7-dihydro-11*H*-pyridazino[1,2-*a*]indazole-8,11(9*H*)-dione, **4i**.

Similar metabolites were detected when liver S9 was fortified with NADH as co-factor, but most were present at 4-5-fold lower concentrations (data not shown). The only exception was that in the presence of NADH relatively more *O*-acetylated metabolite (**4j**) was detected. No metabolites were observed in the presence of boiled S9.

Thus, in summary there are three main routes of NADPH-dependent metabolism of compounds **1**- **4** in human liver S9: i) aerobic reduction of either nitro group, ii) *N*-dealkylation and iii) *O*-acetylation of the hydroxyethyl side chain. The two livers selected for this current study were chosen since they are representative of cancer drug metabolism in a larger liver bank (Zhou *et al*., 2000 ; Lu *et al.*, 2004; Tang *et al.*, 2004). However, the potential for inter-individual variability in the metabolism of these compounds has not been addressed in these studies.

Metabolic profile in non clinical species

To determine whether any species differences in hepatic S9 metabolic profile exist the compounds were incubated with S9 from three non-clinical species: mouse, rat and dog (table 1). Of the non-clinical species studied, nitro reduction of **1** was greatest in the mouse (3348 ± 1749 mAU*s) and lowest in the dog (589 ± 118 mAU*s). Nitro reduction was also higher in female rat compared with male rat (1994 ± 818 versus 705 ± 377 mAU*s) (table 1). Moreover, there was considerable variability in the amount of *para*-nitro reduced metabolites formed between the two human livers HL5 and HL18 (261 ± 9 and 3338 ± 1064 mAU*s respectively), which may indicate potential inter-individual variability in human nitroreductase enzyme(s) activity for this substrate. The chloromesylate (**2**) was a poor substrate for *para*-nitro reduction in dog liver S9, with 10-fold lower levels of **2a** and **2b** formed, compared with the other species studied (table 1). The *ortho*-nitro reduced metabolites **3d** and **3e** of the dibromo compound **3** were not detected in rat liver S9 (either male or female liver). Additionally, relatively low

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levels of *para*-nitro reduction to form **3b** was observed in rat liver S9 and there was relatively low levels of *N*-dealkylation of compound **3** by female rat liver in comparison with male rat and the other species (table 1). For the diethylamine compound **4**, the same metabolites were formed as observed with human liver S9, with the exception of CD-1 *nu/nu* mouse, which did not support the formation of the nitro reduced metabolite **4c**.

In vitro glucuronidation

Compound **1**, the alcohol metabolite of PR-104, has been reported to undergo *in vivo* glucuronidation in mice (Patel et al., 2007). To evaluate this pathway *in vitro,* the compounds (**1**-**4**) were incubated with liver microsomes fortified with the cofactor UDPGA and detergent. A single more polar metabolite was detected following incubation with each compound. In each case this metabolite had a higher mass than the parent compound (X+176), with $[M+H]^+$ 675, 631, 659 and 503 *m/z* for **1**, **2**, **3** and **4** respectively. The product of UDPGA-dependent metabolism of compound **1m** was confirmed as an *O*-glucuronide of the alcohol side chain by comparison with authentic sample. Formation of the *O*-glucuronide metabolite for all four compounds was observed in human, mouse, rat and dog microsomes, although the amount formed varied across species. In rat and mouse liver microsomes the dinitrobenzamide compounds were relatively poor substrates for glucuronidation compared with human or dog liver (figure 2). The most extensive glucuronidation (66.6 ± 5.2%) was observed for the dibromo compound **3** with dog liver.

Discussion

Four compounds were chosen to study the hepatic metabolism of the dinitrobenzamide hydroxyethyl mustards. Although the diethylamine compound, **4** lacks the mustard moiety, it was included in these studies as a model compound. Principally the lack of an alkylating moiety results in a simpler metabolic profile as intramolecular alkylation of *ortho*-nitro reduced metabolites to form tetrahydroquinoxaline products (Palmer *et al.*, 1995; Helsby *et al.*, 2003) cannot occur. Moreover, this compound was used to elucidate the effect of changes in the *N*-mustard moiety on the metabolic routes of these compounds.

Four metabolic routes were observed: aerobic nitroreduction, *N*-dealkylation, *O*-acetylation and *O*-glucuronidation. Attempts to optimise for every individual metabolic route proved unsuccessful (data not shown) thus the substrate concentration chosen for this study was based on the reported plasma Cmax for compound **1** in mouse and rat (Patel *et al.*, 2007; Patterson *et al.*, 2007). Dinitrobenzamide mustard compounds are substrates for *E coli* nitroreductase (*NfsB*)(Anlezark *et al.*, 1995), in addition the prototype DNBM, SN 23862, also undergoes anaerobic nitroreduction by cell lines which overexpress NAD(P)H P450 oxidoreductase (Wilson *et al.*, 2007). However, this is the first report of nitroreduction of DNBM compounds by hepatic preparations under atmospheric oxygen conditions. Aerobic nitroreduction by liver S9 preparations has been observed previously with the aziridinyl-dinitrobenzamide compound, CB 1954 (Tang *et al.*, 2005), and hence hepatic nitroreduction was not an unexpected finding for this class of compounds.

The least complex analogue studied, the diethylamine (**4**), underwent extensive nitroreduction by human, rat and dog liver S9. Two nitroreduced metabolites were formed, the hydroxylamine and the amine. These metabolites were assumed to be the products of *ortho*- and *para*- nitroreduction respectively as *para*-hydroxylamine and *para*-amine metabolites of **1** have identical UV spectra (Patterson *et al.*, 2007) whereas the hydroxylamine metabolite of **4** has a spectral shift compared with the amine metabolite. In addition, a spectral shift following *ortho*-nitroreduction of related DNBM compounds has been observed previously (Wheeler, 1999) however, the identity of **4c** as the *ortho*-hydroxylamine requires further confirmation by NMR. In contrast, the more complex dibromo mustard analogue, **3** was a substrate for *ortho*-nitro reduction, with characteristic intramolecular alkylation to form a tetrahydroquinoxaline (**3d** and **3e**), in all species except the rat. This may indicate species-dependent enzyme-substrate interactions for the reductase(s) involved in this route. More importantly, the increased complexity of the bromomesylate (**1**) and chloromesylate moiety (**2**) appeared to entirely abrogate *ortho*-nitro reduction in all the species examined. There was no evidence of formation of these metabolites either in the UV chromatogram or by interrogation of the mass chromatogram for the expected *m/z* values of the tetrahydroquinoxaline metabolites of compounds **1** and **2**.

Previous studies have indicated that the steric bulk can influence the *ortho*-nitro reduction of dinitrobenzamides by *E coli* nitroreductase (NTR)(Helsby *et al.*, 2004; Wilson *et al.*, 2007) as *ortho*-nitroreduction of the dichloro DNBM compound, SN 23862, is precluded by a limited binding orientation in the *E coli*

NTR crystal structure due to the steric bulk of the mustard moiety when compared with the smaller aziridinyl moiety (Johansson *et al.*, 2003). *Ortho*-nitro reduction of DNBM results in intramolecular alkylation and formation of "half mustard" compounds and is considered to be an inactivation pathway for these prodrugs. However, extensive metabolic clearance via this route would be detrimental to the systemic availability of the parent prodrug at the site of action, the tumour.

In contrast, reduction of the nitro group *para* to the mustard moiety is a bioactivation pathway which results in the formation of known DNA cross linking cytotoxins. Aerobic *para*-nitro reduction was observed for all four compounds in all species studied. Both compound **3** and **4** underwent six electron nitroreduction to the *para*-amine derivative (**3b** and **4b**), indicating extensive nitro reduction of these compounds. In contrast, both the four electron *para*-hydroxylamine (**1a** and **2a**) and the six electron *para*-amine (**1b** and **2b**) metabolites of compound **1** and **2** were observed, which may indicate a relatively slower rate of nitroreduction for these compounds. However, the amine **1b** is reported to undergo slow autooxidation to the hydroxylamine, **1a** (Patterson *et al.*, 2007) hence the detection of the products of four electron reduction of compounds **1** and **2** may indicate the relative aerobic instability of the *para*-amine metabolite of these compounds.

Although there was some variability in the amount of *para*-nitro reduced metabolites formed across species, in general increasing the steric bulk of the mustard group had little effect on the ability of these analogues to undergo *para*-

nitro reduction. Thus, under aerobic conditions the DNBM analogues can undergo hepatic metabolism to cytotoxic alkylating metabolites and this may result in adducts at the site of formation, *i.e.* the liver. Importantly, these metabolites may not be readily detectable systemically (in plasma) and hence the bioactivation of these compounds in normal (oxic) tissue may be underestimated.

A further pathway of metabolism was observed for the DNBM analogues, namely, *N*-dealkylation. *In vivo N*-dealkylation of the prototype DNBM compound, SN 23862, has been reported previously (Kestell *et al.*, 2000) and this metabolic route results in the formation of an inactive "half mustard" which cannot cross-link DNA and is less cytotoxic. However, the *N*-dealkylation of the DNBM analogues may also release reactive acetaldehydes, such as bromoacetaldehyde which are potent cytotoxins (Khan *et al.*, 1996). An alternative mechanism for *N*-dealkylation of the DNBM is possible via oxidation to the *N*-oxide of the nitrogen mustard followed by nucleophilic attack by hydroxyl ion and elimination of formaldehyde (Tercel *et al.*, 1995).

Thus it is important to minimize metabolic clearance of DNBM via this route to firstly avoid toxicity in normal tissue and secondly to avoid decreased systemic availability of the prodrug. Altering the structure of the mustard group had a dramatic effect on *N*-dealkylation, decreasing the amount of metabolite observed by up to 10-fold for the dibromo compound (**3**) compared with the diethylamine (**4**), and entirely abrogating this route for the more complex bromomeslyate (**1**) and chloromesylate (**2**) compounds. Hence the *in vitro* metabolism data would predict that that both **1** and **2** would undergo minimal dealkylation *in vivo.*

However, the "half mustard" metabolite of **1** resulting from oxidative debromoethylation (**1f**) has been identified in plasma after administration of **1** to mice, although it co-elutes with the *O*-glucuronide metabolite (**1m**)(Patel *et al.*, 2007). Hence, further studies to determine the role of extrahepatic dealkylation of DNBM analogues may be warranted.

The *O*-acetylation of the hydroxyethyl side chain by liver S9 preparations, was a relatively minor route compared with oxidative metabolism. In addition, as the compounds were also *O*-glucuronidated at this position the formation of *O*-acetylated metabolites may be precluded in fully competent hepatocytes *in vivo*. Notably, *O*-glucuronidation was particularly extensive in dog liver compared with the other species. A high rate of glucuronidation by dog liver compared with other species has been reported previously for the HIV drug, bevirimat (Wen *et al.*, 2007). Further studies to elucidate the relative importance of *O*-glucuronidation of the hydroxyethyl side chain *in vivo* are in progress.

In conclusion, increasing the steric bulk of the *N*-mustard moiety abrogated the *ortho*-nitro reduction and *N*-dealkylation routes. This improves the *in vitro* metabolic profile of DNBM compounds with the prediction of a relatively high metabolic stability for compounds **1** and **2**. Indeed, mouse S9 metabolic stability data (t=30 min) indicated <10% loss of prodrug for compounds **1** and **2**, compared with 75% loss of compound **3**. However, structural changes at the mustard moiety had no effect on the aerobic *para*-nitro reduction of these compounds. Although compounds **1**, **2** and **3** undergo bioactivation to form metabolites which are expected to be cytotoxic DNA alkylators, it is postulated

that *para*-nitro reduction may be a relatively minor route of metabolism for these compounds compared with *O*-glucuronidation *in vivo*. Further studies to determine the *in vivo* metabolic profile of these compounds are in progress.

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Supplementary data: UV spectral analysis of the metabolites of SN 29893 (**4**)

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Figure legends:

Figure 1. Typical HPLC chromatograms of NADPH-dependent metabolism of dinitrobenzamide compounds SN 27858 (**1**), SN 29546 (**2**) SN 27686 (**3**) and SN 29893 (**4**) in human liver S9. For metabolite identification and numbering refer to text and figure 2.

Figure 2. Metabolic routes observed for the hydroxyethyl dinitrobenzamides. Metabolite numbering is as indicated in the text. X= $-CH_2Br$, Y= $-CH_2OSO_2CH_3$ (**1**); X= $-CH_2Cl$, Y= $-CH_2OSO_2CH_3$ (**2**). X=Y= $-CH_2Br$ (**3**), X=Y= $-CH_3$ (**4**);

Figure 3. UDPGA-dependent glucuronidation of the dinitrobenzamide compounds by human, mouse, rat and dog microsomes. Glucuronidation is shown as % glucuronide formed relative to parent compound in control microsomal incubations without the cofactor UDPGA. Mean data (± SD) from n=4 incubations.

Table legends:

Table 1. Metabolism of 3,5-dinitrobenzamide 2-mustard analogues (250 μM) by the S9 fraction of livers from various species in the presence of the cofactor NADPH.

[1]All values are mean ± SD peak area at 254 nm (mAU*s) of four replicate incubations. [2]Total *ortho*- or *para*-nitro reduction denotes the sum of the corresponding hydroxylamine and amine metabolites. N.D. – not detected

SN 27858 (1)				
	o-nitroreduction	*p*-nitroreduction (**1a** + **1b**)	*N*-dealkylation	O-acetylation (**1j**)
Human (HL5)	ND	261± 9	ND	227 ± 107
Human (HL18)	ND	3338 ± 1064	ND	369 ± 122
Mouse	ND	3348 ±1749	ND	99 ±70
Rat (male)	ND	705 ± 377	ND	54 ±28
Rat (female)	ND	1994 ± 818	ND	232 ± 93
Dog	ND	589 ± 118	ND	153 ± 44
SN 29546 (2)				
	o-nitroreduction	*p*-nitroreduction (**2a** + **2b**)	*N*-dealkylation	O-acetylation (**2j**)
Human (HL5)	ND	934 ± 602	ND	504 ±208
Human (HL18)	ND	3501± 614	ND	454 ± 338
Mouse	ND	2739 ± 1420	ND	472 ± 208
Rat (male)	ND	3859 ± 984	ND	362 ± 86
Rat (female)	ND	4040 ± 1127	ND	460 ± 271
Dog	ND	113 ± 46	ND	484 ± 215

SN 27686 (3)				
	o-nitroreduction (**3d** + **3e**)	*p*-nitroreduction (**3b**)	*N*-dealkylation (**3f** +**3h**)	O-acetylation (**2j**)
Human (HL5)	892 ± 295	1888 ± 1056	3213 ± 1527	815 ± 248
Human (HL18)	1878 ± 436	3797 ± 740	3508 ± 3935	459 ± 146
Mouse	1131± 1027	1051 ± 1022	552 ± 213	40 ± 60
Rat (male)	ND	396 ± 54	1308 ± 864	ND
Rat (female)	ND	461 ± 345	166 ± 77	205 ± 168
Dog	874 ± 375	965 ± 513	908 ± 379	286 ± 27
SN 29893 (**4**)				
	o-nitroreduction (**4c**)	*p*-nitroreduction (**4b**)	***N*-dealkylation** (**4f** + **4g** + **4i**)	**O-acetylation (**1j**)**
Human (HL5)	2441 ± 1011	1340 ± 283	8373 ± 989	1250 ± 496
Human (HL18)	2559 ± 661	1520 ± 287	7240 ± 858	662 ± 321
Mouse	ND	2140 ± 323	6689 ± 1553	193 ± 11
Rat (male)	2085 ± 404	1698 ± 373	8189 ± 1414	323 ± 70
Rat (female)	1044 ± 423	945 ± 143	4924 ± 1412	198 ± 39
Dog	1303 ± 547	1796 ± 540	8572 ± 1124	182 ± 25

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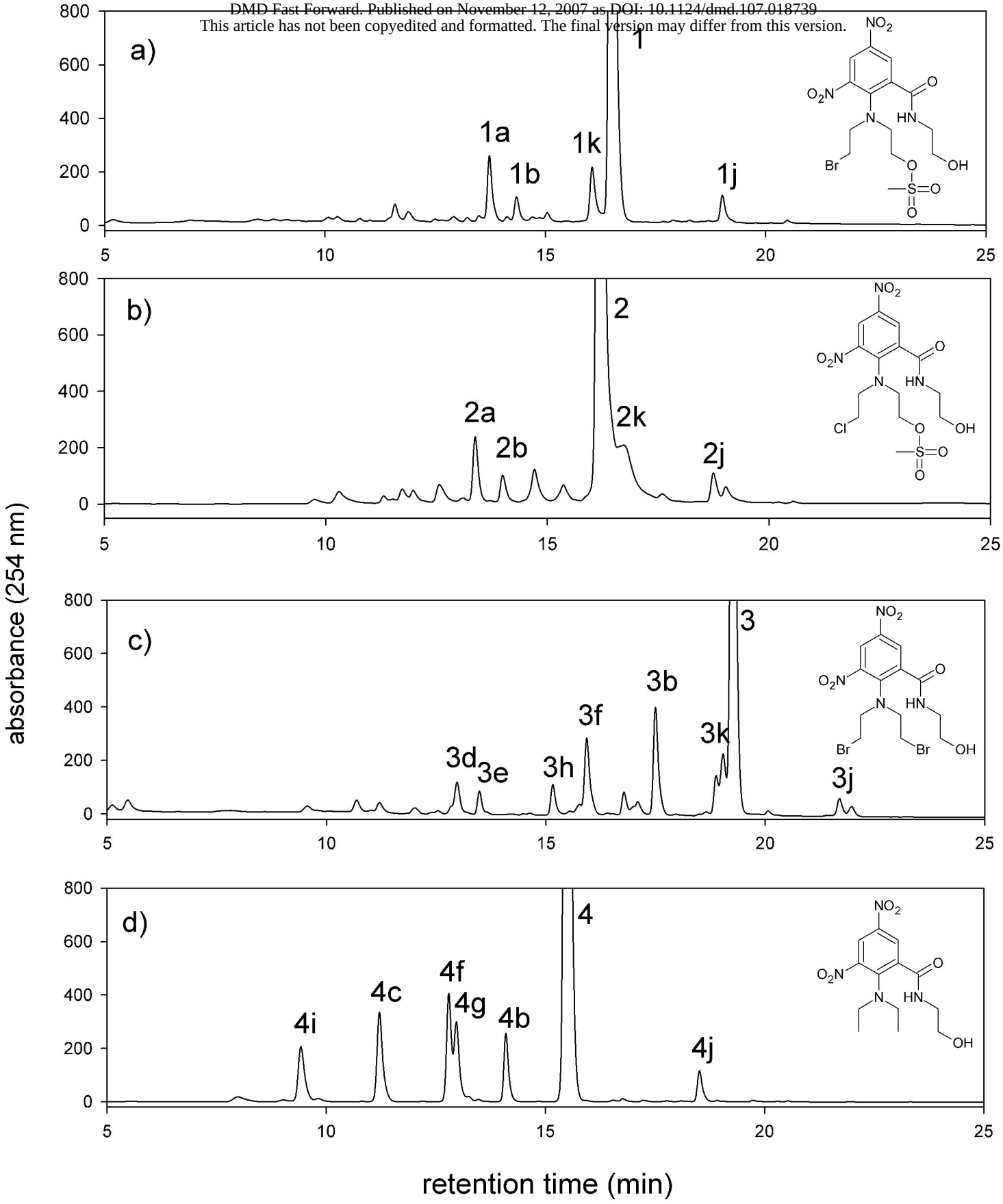

Figure 1

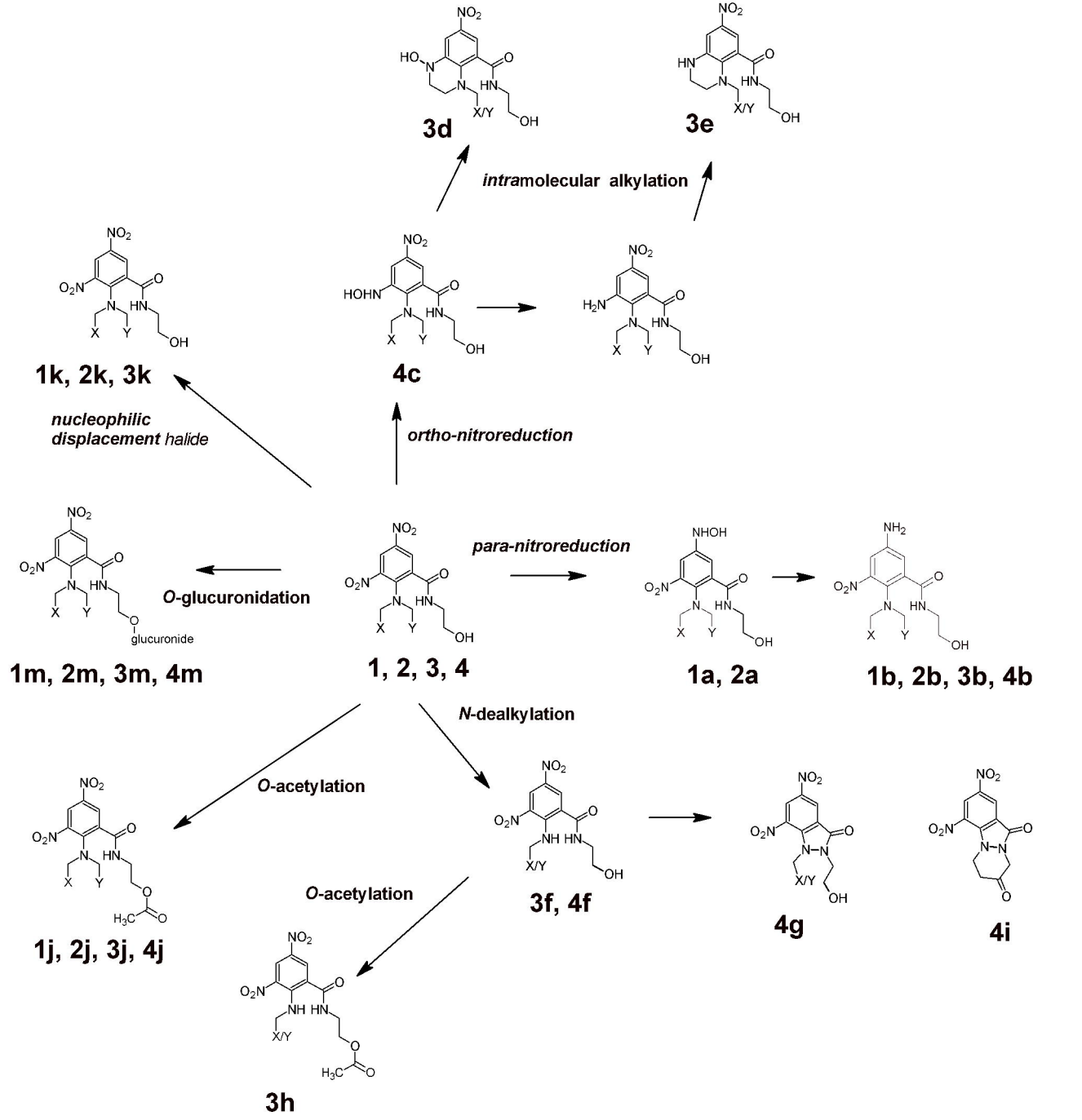
3d
3e
intramolecular alkylation
1k, 2k, 3k
4c
nucleophilic displacement halide
ortho-nitroreduction
O-glucuronidation
1m, 2m, 3m, 4m
1, 2, 3, 4
para-nitroreduction
1a, 2a
1b, 2b, 3b, 4b
N-dealkylation
O-acetylation
1j, 2j, 3j, 4j
3f, 4f
4g
4i
O-acetylation
3h

Figure 2

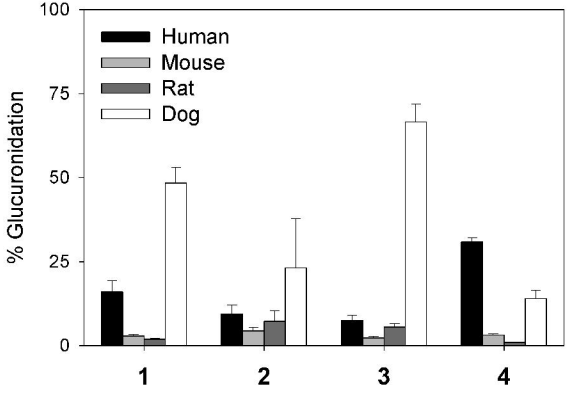

Figure 3