The use of Tris-Lipidation to modify drug cytotoxicity in multidrug resistant cells expressing P-glycoprotein or MRP1

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1 Increasing the lipophilicity is a strategy often used to improve a compound's cellular uptake and retention but this may also convert it into a substrate for an ATP-dependent transporter such as P-glycoprotein or the multidrug resistance-associated protein (MRP1), which are involved in cellular efflux of drugs. Tris-Lipidation of compounds is a convenient way of modifying drug lipophilicity and generating an array of derivatives with diverse properties.

2 To determine the effect of Tris-Lipidation on a drug's cytoxicity in multidrug resistant cells, various glycyl-Tris-mono- (GTP1), di- (GTP2) and tri-palmitate (GTP3) derivatives were prepared of the cancer chemotherapeutic drugs chlorambucil and methotrexate, and of the anti-HIV drug AZT. The cytotoxicity of these derivatives and their parent compounds was determined in the CEM/VLB₁₀₀ cells with increased P-glycoprotein expression, the CEM/E1000 cells that overexpress MRP1 and the parent, drug-sensitive CCRF-CEM cells.

3 Increasing the lipophilicity of AZT increased its cytotoxicity in the sensitive CCRF-CEM parental cell line while decreased cytotoxicity was observed for the methotrexate derivatives. For the chlorambucil derivatives, both increased (GTP1) and decreased (GTP2) cytotoxicity occurred in the CCRF-CEM cells. With the exception of AZT-GTP1, all GTP1 and GTP2 derivatives of chlorambucil, methotrexate and AZT had decreased cytotoxicity in the P-glycoprotein-expressing CEM/VLB₁₀₀ cells while chlorambucil-GTP1, methotrexate-GTP2 and methotrexate-GTP3 were the only compounds with decreased cytotoxicity in the MRP1-overexpressing CEM/E1000 cells.

4 The number of palmitate residues, the position of derivatisation and the type of linkage all may affect the P-glycoprotein and MRP1 substrate properties.

5 Tris-Lipidation may therefore provide a useful way of manipulating the pharmacokinetic properties of drugs.

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- Keywords: Tris-Lipidation; P-glycoprotein; MRP1; drug modification; chlorambucil; methotrexate; AZT; leukaemia cells; multidrug resistance
- Abbreviations: AZT, 3'-azido-3'-deoxythymidine (Zidovudine); BSO, buthionine sulphoximine; GT, glycine-Tris; GTPn, glycine-Tris-palmitate (1, 2 or 3); HPLC, High Performance Liquid Chromatography; MRP1, multidrug resistance-associated protein 1; MTT, 3[-4,5 dimethylthiazo]-2,5 diphenyl tetrazolium bromide; SDZ-PSC-833, Cyclosporin-D derivative produced by Novartis; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Z, benzyloxy-carbonyl

Introduction

The rates of tissue uptake and efflux of a drug are important factors in determining both its specificity and its effective dose. At the cellular level, uptake and efflux are often influenced by the ABC super-family members of ATP-dependent transport proteins. The clinical importance of these transporters is very evident from the role of two family members, P-glycoprotein (Endicott & Ling, 1989) and the multidrug resistance-associated protein (MRP1, Cole *et al.*, 1992) in causing resistance to a wide variety of chemother-apeutic drugs used to treat cancer (Goldstein *et al.*, 1992; Borst *et al.*, 2000). It is therefore important in designing new

drugs, to establish whether they are substrates for these transporters. As more becomes known about the substrate specificity and the tissue and cellular location of the ABC super-family of transporters, it may be possible to engineer the transportability of a drug to enhance its pharmacokinetic properties.

Increasing the lipophilicity of a drug is a common strategy to enhance cellular uptake. However, this approach can be unsuccessful because it also can produce a more effective substrate for the efflux transporters such as P-glycoprotein and MRP1. The balance between the rate of cellular uptake and efflux is probably dictated by the properties of the lipophilic groups. However, because there is little understanding of which of these properties are important, it is

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advantageous to use a chemistry capable of generating and conjugating a diverse array of lipophilic structures.

Liposomal formulations and chemical modifications, such as the addition of fatty acyl groups, are commonly used to increase the lipophilicity of a drug. Fatty acyl groups have usually been introduced through the addition of di- and mono-glycerides. Whilst these conjugates have enhanced biological properties, the chemistry to prepare them is often difficult and problems arise in scale-up because glycerol is an unsymmetrical molecule.

We have previously reported that Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) is easily linked to peptides and other compounds (Whittaker *et al.*, 1993). Tris is accepted for use by the pharmaceutical industry and is relatively inexpensive, non-toxic and readily available. Tris has structural similarities to glycerol but has the advantage of being symmetrical around the central carbon atom. This allows for the easy preparation and purification of mono-, diand tri-glyceride mimics because structural isomers are avoided. Compounds can be readily attached to the amine group, with or without the use of linkers, to give conjugates of the general structure shown in Figure 1. Tri-fatty acyl esters can also be formed using Tris. This is not possible with glycerol. The chemical properties of a drug can be easily



Methotrexate-glycine-Tris-dipalmitate

Figure 1 Structure of Tris-Lipid drug conjugates. The general structure (R = fatty acyl group or OH) and the structure of the Tris-Lipid conjugates of chlorambucil, AZT and methotrexate are presented.

modified by the addition of fatty acyl groups *via* Tris to engineer the desired lipophilicity, solubility, stability, shape and molecular weight. In addition, pro-drugs or new chemical entities can be produced by varying the linker.

Despite the advantages of this approach, little is known about the effect that Tris-Lipidation has on the capacity to be transported by members of the ABC super-family. Pglycoprotein and MRP1 are the best characterized members of this family and they are individually overexpressed in the CEM/VLB₁₀₀ (Beck et al., 1979) and CEM/E1000 sublines respectively, relative to the parental CCRF-CEM human Tcell leukaemia cell line which does not express detectable levels of P-glycoprotein and expresses a low constitutive level of MRP1 typical of the majority of human cell lines (Davey et al., 1995). The drug accumulation characteristics of these cells and the effects of the P-glycoprotein inhibitor, SDZ-PSC-833 and the MRP1 inhibitor, buthionine sulphoximine (BSO) have also been well characterized (Davey et al., 1996). The CEM/VLB₁₀₀ and CEM/E1000 sublines have been used previously to investigate the multidrug resistance characteristics of drugs (Davey et al., 1997, Efferth et al., 2002). They are ideally suited for this as they have similar levels of resistance to many lipophilic and natural product drugs, their multidrug resistance is stable in the absence of continual drug treatment, they are the same size, they have similar growth rates and they are derived from the same parental cell line by selection rather than transfection (Beck et al., 1979; Davey et al., 1995).

To determine the effect that Tris-Lipidation has on a drug's potential to be transported by either P-glycoprotein or MRP1, we have chosen three drugs that are considered to have little interaction with P-glycoprotein or MRP1, the anti-cancer alkylating drug chlorambucil, the anti-cancer folate pathway inhibitor, methotrexate and the nucleoside reverse transcriptase inhibitor, AZT (3'-azido-3'-deoxythymidine; Zidovudine). These were conjugated to various Trispalmitate adducts (Figure 1) and their cytotoxicity against the P-glycoprotein-expressing CEM/VLB₁₀₀ and the MRP1overexpressing CEM/E1000 multidrug resistant sublines was determined relative to the drug sensitive parental CCRF-CEM cells. The effects on cytotoxicity of the P-glycoprotein inhibitor SDZ-PSC-833 and the MRP1 inhibitor BSO (Davey et al., 1995; Grech et al., 1998) were also determined.

Methods

Figure 1 presents the general structure of the drug-glycine-Tris-palmitate conjugates and the structural formulae for conjugates of chlorambucil, methotrexate and AZT.

Benzyloxycarbonyl-GTPn derivatives were synthesized as previously described (Whittaker & Bender, 1992; Whittaker *et al.*, 1993; 1999). This was then conjugated to the various drugs as described below.

Chlorambucil-Tris conjugates

Chlorambucil-Tris conjugates were prepared as previously described (Whittaker *et al.*, 1995). Briefly, the N-hydroxysuccinimide-derived active ester of chlorambucil (Sigma Chemical Co., U.S.A.) was prepared and reacted with GTP1 or GTP2. Products were purified from the reaction mixture by column chromatography and/or HPLC.

Methotrexate-Tris conjugates

Methotrexate was a gift from FH Faulding & Co. Limited, Australia and the Tris conjugates were prepared as previously described (Whittaker *et al.*, 1999). Briefly, modified glutamic acid (carboxyl groups were selectively esterified- α -t-butyl and γ -methyl) was attached to 4-amino-4-deoxy-N¹⁰-methyl pteroic acid (Sigma Chemical Co.) followed by selective methyl ester hydrolysis (Rosowsky *et al.*, 1981). This liberated the γ -COOH site for the attachment of GT, GTP1, GTP2 or GTP3 using a DCC-mediated coupling reaction. The α -ester was subsequently cleaved. This produced conjugates with an amide linkage on the γ -COOH. Conjugates linked through the α -COOH were produced through the addition of GT or GTP1 to the α -COOH following selective removal of the α -t-butyl group. Products were purified by chromatography.

AZT-Tris conjugates

AZT conjugates were prepared as previously described (Whittaker *et al.*, 1995) from AZT produced according to the method of Czernecki & Valery (1991) AZT was treated with succinic anhydride to form AZT-succinate. The active ester was prepared and reacted with either GTP1 or GTP2. Final compounds were isolated by column chromatography.

Cell culture

The CCRF-CEM human leukaemia cell line (Foley *et al.*, 1965), the P-glycoprotein expressing CEM/VLB₁₀₀ subline (Beck *et al.*, 1979) and the MRP1 overexpressing CEM/E1000 subline (Davey *et al.*, 1995) were grown in RPMI-1640 medium (Trace, Sydney, Australia) supplemented with 10% foetal calf serum (Trace), 20 mM HEPES (Trace) and 10 mM NaHCO₃ at 37°C in a humidified atmosphere containing 5% CO₂. The sublines were maintained in the absence of the selecting drug except for treatment for 3 days every 8 weeks. All cultures were free of *Mycoplasma* as determined by *Mycoplasma*-specific culture (Westmead Hospital Microbiology Department, Sydney, Australia).

Cytotoxicity assay

To determine the cytotoxicity of the drugs and their derivatives, cells were plated out in triplicate in 96-well plates at a density of 2.5×10^5 /ml and cultured in the presence of the test compound for 4 days after which cell viability was measured by the MTT assay as previously described (Marks *et al.*, 1992). The IC₅₀ was determined as that concentration that caused a 50% decrease in cell viability. The relative resistance was calculated by dividing the IC₅₀ of a compound in the resistant subline by the IC₅₀ in the sensitive CCRF-CEM cell line. Relative reversal was determined by dividing the IC₅₀ of a compound by the IC₅₀ obtained in the presence of the reversing agent, either 50 μ M BSO (Sigma) or 10 μ M SDZ-PSC-833 (gift from Novartis, Sydney, Australia).

Results

The GTP1 and GTP2 moieties themselves showed little cytotoxicity to the CCRF-CEM cells with an IC_{50} in excess of 20 μ M for both compounds (not shown). However, the conjugation of these compounds to the three drugs, chlorambucil, methotrexate or AZT, dramatically altered the cytotoxicity of these drugs in the CCRF-CEM cells (Figure 2 and Table 1). GTP1 conjugation increased the cytotoxicity of chlorambucil by 10 fold and AZT by 50 fold and decreased the cytotoxicity of methotrexate by greater than 10 fold. Conjugation to GTP2 decreased the cytotoxicity of chlorambucil by twofold, methotrexate by 20 fold and it increased the cytotoxicity of AZT by 50 fold. For methotrexate, the parent drug was more cytotoxic than all the derivatives tested while for AZT the parent drug was less cytotoxic than the GTP1 and GTP2 conjugates.



Figure 2 Effect of drug modification on cytotoxicity in the CCRF-CEM cells. Cells were incubated continuously in drug for 4 days after which the cell viability was determined using the MTT assay as described in the Methods. Assays were performed in triplicate and the mean and standard deviation of each determination are shown. P, Parent drug; α -GT, α -glycine-Tris; γ -GT, γ -glycine-Tris; GTP1, glycine-Tris-monopalmitate; GTP2, glycine-Tris-dipalmitate; GTP3, glycine-Tris-tripalmitate.

 Table 1
 Effect of derivitization on the cytotoxicity of chlorambucil, methotrexate and AZT in multidrug resistant cells

	$IC_{50} \mu M$ (fold-resistance)		
Drug	CCRF-CEM	CEM/VLB ₁₀₀	CÉM/E1000
Chlorambucil	3.4	5.2	2.2
Chlorambucil-GTP1	0.35	14.6 (42)	1.7(5)
Chlorambucil-GTP2	6.8	>200 (>25)	9.3
Methotrexate	0.017	0.045 (3)	0.015
Methotrexate- α -GT	36	164 (5)	48
Methotrexate-y-GT	0.57	1.2 (2)	0.52
Methotrexate-y-GTP1	0.21	1.2 (6)	0.27
Methotrexate-y-GTP2	0.4	2.0 (5)	0.7 (2)
Methotrexate- γ -GTP3	94	99	202 (2)
ΔΖΤ	5000	5000	6000
AZT-GTP1	93	80	80
AZT-GTP2	100	227 (2)	101

Cells were incubated continuously in drug for 4 days after which the viability was determined using the MTT assay and the fold resistance () calculated as described in Methods.

The effect of Tris-Lipidation on drug cytotoxicity was determined in the P-glycoprotein expressing CEM/VLB₁₀₀ cells relative to that in the CCRF-CEM cells. Figure 3 shows that the CEM/VLB₁₀₀ cells were 42 fold more resistant to chlorambucil-GTP1 than the parental CCRF-CEM cells. The CEM/VLB₁₀₀ cells were also greater than 25 fold resistant to the GTP2 derivative. The P-glycoprotein inhibitor, SDZ-PSC-833 reversed the resistance 14 fold for chlorambucil-GTP1 and greater than 20 fold for chlorambucil-GTP2 in the CEM/VLB₁₀₀ cells which provided further evidence that these derivatives were probably interacting with P-glycoprotein. AZT-GTP2 also appears to interact with P-glycoprotein since the CEM/VLB₁₀₀ cells were 2 fold more resistant than the CCRF-CEM cells and SDZ-PSC-833 reversed this resistance (Figure 3 and Table 1).

The CEM/VLB₁₀₀ cells were 6 fold more resistant to methotrexate- γ -GTP1 and 5 fold more resistant to methotrexate- γ -GTP2 compared to the CCRF-CEM cells while the CEM/VLB₁₀₀ cells were not resistant to methotrexate- γ -GTP3 (Table 1). This suggests that if the addition of - γ -GTP1 and - γ -GTP2 to methotrexate promotes interaction with P-glycoprotein, the addition of a further palmitate residue reverses this effect. We also examined whether or not the addition of GT (with no palmitate(s) attached) to the α - or γ -carboxyl groups of methotrexate affected cytotoxicity. Addition of both the - α -GT and - γ -GT increased the resistance in the CEM/VLB₁₀₀ cells 5 and 2 fold respectively (Table 1).

The effect of Tris-Lipidation on drug cytotoxicity in the MRP1-overexpressing CEM/E1000 cells, relative to that in the CCRF-CEM cells, was determined. Of the derivatives tested, chlorambucil-GTP1 had the highest relative resistance (5 fold) in the CEM/E1000 cells and this was reversed (7 fold) by BSO treatment (Figure 4). Relative to the CCRF-CEM cells, methotrexate-GTP2 and -GPT3 were also less cytotoxic in the CEM/E1000 cells (Table 1).

It is also worth noting that the CEM/E1000 cells were not resistant to the parent drug, methotrexate under these conditions of 4 day drug exposure (Table 1). Further, methotrexate resistance was not detected with shorter drug exposure times of 1, 4 or 24 h (data not shown).

Discussion

Tris-Lipidation allows the preparation of a range of lipophilic derivatives of a wide range of therapeutic agents. The method is simple and very versatile. Varying the number and/or type of fatty acyl group attached, as well as the nature of the linkage to the drug, can produce a vast array of different properties. Tris-Lipidation has already been applied to a range of applications including the delivery of genes (Cameron et al., 1999), vaccines (Reilly et al., 1991; Walker et al., 2000) and therapeutic agents (Whittaker et al., 1995). The versatility of Tris-Lipidation was also evident in our study. Compared to chlorambucil, the GTP1 derivative was 10 fold more cytotoxic in the parental CCRF-CEM cells while the GTP2 derivative had half the cytotoxicity of chlorambucil. Derivatization also had a dramatic effect on chlorambucil's cytotoxicity in the P-glycoprotein-expressing CEM/VLB₁₀₀ cells which were 42 fold resistant to the GTP1 derivative and >25 fold resistant to the GTP2 derivative.

Increasing the lipophilicity of AZT by adding GTP1 and GTP2 groups caused increased cytotoxicity (greater than 50 fold) and this has previously been reported for other cell lines (Wells *et al.*, 1999). The strategy of increasing the lipophilicity is often used to increase the uptake and efficacy of a compound. Our results show that while increasing the lipophilicity by conjugation with Tris-palmitates increased the cytotoxicity of AZT, this was not the case for the other two drugs (Figure 2). The GTP1 conjugate of chlorambucil was more cytotoxic, however, chlorambucil-GTP2 was less cytotoxic than the parent drug. All methotrexate-conjugates were less cytotoxic.

Processes other than cellular uptake and retention are also important in determining the cytotoxicity of a compound. This includes the intracellular modification by conjugation to compounds including glutathione, glucuronate, sulphonate and polyglutamate. In the case of methotrexate, the γ carboxyl group is the site of polyglutamylation, a process important in cellular drug retention and enzyme binding. Modification of the γ -carboxyl group would therefore reduce the cytotoxicity and our results are consistent with this as all methotrexate-y-conjugates were less cytotoxic than the parent compound (Figure 2). This confirms the earlier observations of Wells et al. (1999). Our results also show that modification to the α -carboxyl group is more disruptive than changes to the γ -carboxyl group since the α -GT conjugate was far less cytotoxic than the γ -GT conjugate of methotrexate (Figure 2). This demonstrates that the site of conjugation is also important in determining drug cytotoxicity.

The GTP2 derivative was less cytotoxic than the GTP1 derivative for all three parent compounds and for methotrexate, the GTP3 derivative was less cytotoxic than the GTP2 derivative. This trend of decreasing cytotoxicity with increasing number of palmitate groups could be because an increase in the number of palmitate residues may reduce the rate of transport into cells. Alternatively the increased lipophilicity may reduce solubility and consequently decrease availability to the cells. Using fluorescent labelled conjugates, we have previously observed that the GTP1 conjugates enter



Figure 3 Effect of Tris-Lipidation and SDZ-PSC-833 on cytotoxicity. The CCRF-CEM cells and CEM/VLB₁₀₀ cells were incubated continuously in the drug derivative for 4 days either in the presence or absence of 10 μ M SDZ-PSC-833 after which the cell viability was determined by the MTT assay as described in the Methods. Assays were performed in triplicate and the mean and standard deviation of each determination are shown.

cells more rapidly and possibly *via* a different transport route than GTP2 and GTP3 conjugates (Bender *et al.*, 1994). Alternatively, it could be speculated that differences in molecular configuration between the GTP1 and GTP2 conjugates may alter the susceptibility of the drug-GTPn bond to degradation and this would affect cytotoxicity. The stability of the linkage between the drug and the adduct is probably important in determining the drug's cytotoxicity. In the case of the AZT, the Tris-palmitate moieties are attached *via* an ester linkage which is more labile due to cellular esterases than the amide link used for the chlorambucil and methotrexate derivatives. Once inside the cell, the AZT derivative would be quickly converted to free AZT which is not as likely to be actively effluxed from the cell. This is consistent with the dramatic 50 fold increase in cytotoxicity of the AZT derivatives over the parent compound compared to that for the chlorambucil and methotrexate derivatives (Table 1).

P-glycoprotein and MRP1 cause resistance to lipophilic natural product drugs. Therefore altering the lipophilicity of a drug may affect the interaction with these multidrug transporters. This has been reported previously for the anthracyclines (Lampidis et al., 1997). While there is no clear relationship between the degree of lipophilicity and Pglycoprotein and MRP1 substrate properties, the addition of GTPn to the drugs described above did generally reduce their cytotoxicity, particularly in the P-glycoprotein-expressing CEM/VLB₁₀₀ cells. The exception was the addition of GTP3 to methotrexate. No resistance to this derivative was observed in the CEM/VLB100 cells. However, this was associated with a dramatic decrease in cytotoxicity (greater than 2000 fold increase in IC_{50} in all cells suggesting either that methotrexate-GTP3 may have difficulty entering the cell or that its limited solubility reduces its bio-availability in this cell culture system.

Chlorambucil-GTP1, methotrexate-GTP2 and methotrexate-GTP3 were the only compounds to be less cytotoxic in the MRP1-overexpressing CEM/E1000 cells and this was minor compared to their decreased cytotoxicity in the Pglycoprotein-expressing CEM/VLB₁₀₀ cells. The increase in sensitivity to chlorambucil-GTP1 associated with the BSO treatment of the CCRF-CEM cells (Figure 4) was consistent with the endogenous level of MRP1 expression in these cells (Davey *et al.*, 1995).

From these data it is difficult to establish which properties determine the P-glycoprotein and MRP1 substrate characteristics of a compound. If it is assumed that a decrease in cytotoxicity of a compound in the multidrug resistant cell lines compared to that in the sensitive CCRF-CEM cells indicates that compound is a substrate for the relevant multidrug resistant transporter, then there is evidence that the position of the Tris-Lipidation and the number of palmitate residues are important in determining the substrate properties. Attachment of GT alone (no palmitate attached) to the α -carboxyl group improved the P-glycoprotein substrate properties of methotrexate (5 fold resistant) while attachment to the γ -carboxyl group had a reduced effect (2 fold resistant, Table 1). This also suggests that interfering with the polyglutamylation of methotrexate, which occurs on the yposition, has little effect on its P-glycoprotein substrate properties. The further addition of one or two palmitates to the methotrexate-y-GT increased the P-glycoprotein substrate properties while addition of the third palmitate produced poor substrate properties. Therefore the number of palmitate residues appears to influence the substrate properties. It is interesting to note that although methotrexate-GTP3 appears to be a poor P-glycoprotein substrate, it was a potential substrate for MRP1. This suggests that changing the degree



Figure 4 Effect of BSO on Chlorambucil-GTP1 cytotoxicity. The CCRF-CEM cells and CEM/E1000 cells were incubated continuously in chlorambucil-GTP1 for 4 days either in the presence or absence of 50 μ M BSO after which the cell viability was determined by the MTT assay as described in the Methods. Assays were performed in triplicate and the mean and standard deviation of each determination are shown.

of Lipidation may be a way of altering a drug's specificity for members of the ABC super-family of transporters.

Based on this assumption that increased resistance in the CEM/VLB_{100} cells equates to increased P-glycoprotein substrate properties, Table 1 shows that methotrexate has low, but significant P-glycoprotein substrate activity. Methotrexate is not usually considered to be a P-glycoprotein substrate. However, there have been reports (Bebawy *et al.*, 1999; Gifford *et al.*, 1998; de Graaf *et al.*, 1996) that suggest it is and our data support this.

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There are, however, reports suggesting that methotrexate is an MRP1 substrate (Hooijberg et al., 1999; Bakos et al., 2000) but resistance in MRP1-expressing cells, is only evident after short drug exposure times. Our data supports the view that methotrexate is not a substrate for MRP1. Table 1 shows that the MRP1-overexpressing CEM/E1000 cells were not resistant to methotrexate and we have further shown that there is no methotrexate resistance detected after shorter drug exposure times of 1 and 4 h (data not shown). Since little polyglutamation occurs within 4 h, it is unlikely that polyglutamation accounts for the difference between our results and the other reports (Hooijberg et al., 1999; Bakos et al., 2000). One explanation for these differences is that our studies were using whole cells selected for resistance whereas the evidence supporting methotrexate as a substrate for MRP1 comes from transport studies using isolated inside-out membrane vesicles and transfected cells.

Although we have made the assumption that resistance to a compound in a resistant cell equates to transport of that compound, transport is but one of several reasons for resistance. At the very least, our studies have highlighted the profound effects that Tris-Lipidation can have on a drug's cytoxicity, irrespective of the resistance mechanisms operating. Thus we have demonstrated the utility of derivatising drugs by Tris-Lipidation and the diverse effects this has on drug cytotoxicity in sensitive and multidrug resistant cells. The extent of the diversity was unexpected. As more becomes known about the substrate specificity and tissue distribution of the ABC transporters, it will be this diversity that may allow us to manipulate the pharmacokinetic properties of drugs by Tris-Lipidation.

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