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Glucuronidation as a mechanism of intrinsic drug resistance in colon cancer cells: contribution of drug transport proteins

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Abstract

We have recently shown that drug conjugation catalysed by UDP-glucuronosyltransferases (UGTs) functions as an intrinsic mechanism of resistance to the topoisomerase I inhibitors 7-ethyl-10-hydroxycamptothecin and NU/ICRF 505 in human colon cancer cells and now report on the role of drug transport in this mechanism. The ability of transport proteins to recognise NU/ICRF 505 as a substrate was evaluated in model systems either transfected with breast cancer-resistance protein 1 (*Bcrp1*), multidrug-resistance protein 2 (*Mrp2*) or *Mrp3*, or overexpressing MRP1 or P-170 glycoprotein. Results from chemosensitivity assays suggested that NU/ICRF 505 was not a substrate for any of the above proteins. In drug accumulation studies in human colon cancer cell lines NU/ICRF 505 was taken up avidly and retained in cells lacking UGTs (HCT116), whereas, following equally rapid uptake, it was cleared rapidly from cells displaying UGT activity (HT29) as glucuronide metabolites. HT29 cells were shown to express MRP1 and 3, but not P-170 glycoprotein, MRP2 or breast cancer-resistance protein. The major glucuronide of NU/ICRF 505 inhibited ATP-dependent transport of estradiol 17-β-glucuronide in Sf9 insect cell membrane vesicles containing MRP1 or MRP3, while co-incubation of HT29 cells with the MRP antagonist, MK571, significantly restored intracellular concentrations of NU/ICRF 505. These data lead us to conclude that the presence of a glucuronide transporter is essential for glucuronidation to represent a major *de novo* resistance mechanism and that UGTs will contribute more as a primary resistance mechanism when the parent drug (e.g. NU/ICRF 505) is not itself recognised by transport proteins.

Keywords: NU/ICRF 505; SN-38; Drug resistance; Glucuronidation; Drug transport proteins; Human colon cancer cells

1. Introduction

Human colorectal cancer (CRC) accounts for 10–12% of all new cases of cancer reported world-wide and ranks as the second leading cause of cancer related death in the Western world [1,2]. The disease is characterised by poor responsiveness to drug treatment [3,4], however, the mechanisms underlying *de novo* drug resistance remain unclear [5–7].

Glucuronidation catalysed by the UGT (EC 2.4.1.17) super family of drug metabolising enzymes has recently been identified in human colon cancer cell lines as a novel clearance mechanism to the topo I inhibitors 7-ethyl-10-hydroxycamptothecin (SN-38) and the *N*-tyrosine anthraquinone conjugate NU/ICRF 505 (see Fig. 1) [8]. By identifying the UGT isoform responsible for metabolising both compounds in HT29 CRC cells (UGT1A9) and selectively inhibiting drug metabolism with the UGT1A9 selective substrate propofol [9], glucuronidation has been

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Abbreviations: CRC, colorectal cancer; UGT, UDP-glucuronosyltransferase; topo, topoisomerase; SN-38, 7-ethyl-10-hydroxycamptothecin; MRP, multidrug-resistance protein; C-4-G, NU/ICRF 505 C-4-*O*-β-glucuronide; tyr-G, NU/ICRF 505 tyrosyl-*O*-β-glucuronide; MDCK cells, Madine–Dabry canine kidney II cells; BCRP, breast cancer-resistance protein; $E_2$17βG, estradiol 17-β-D-glucuronide; P-gp, P-170 glycoprotein.

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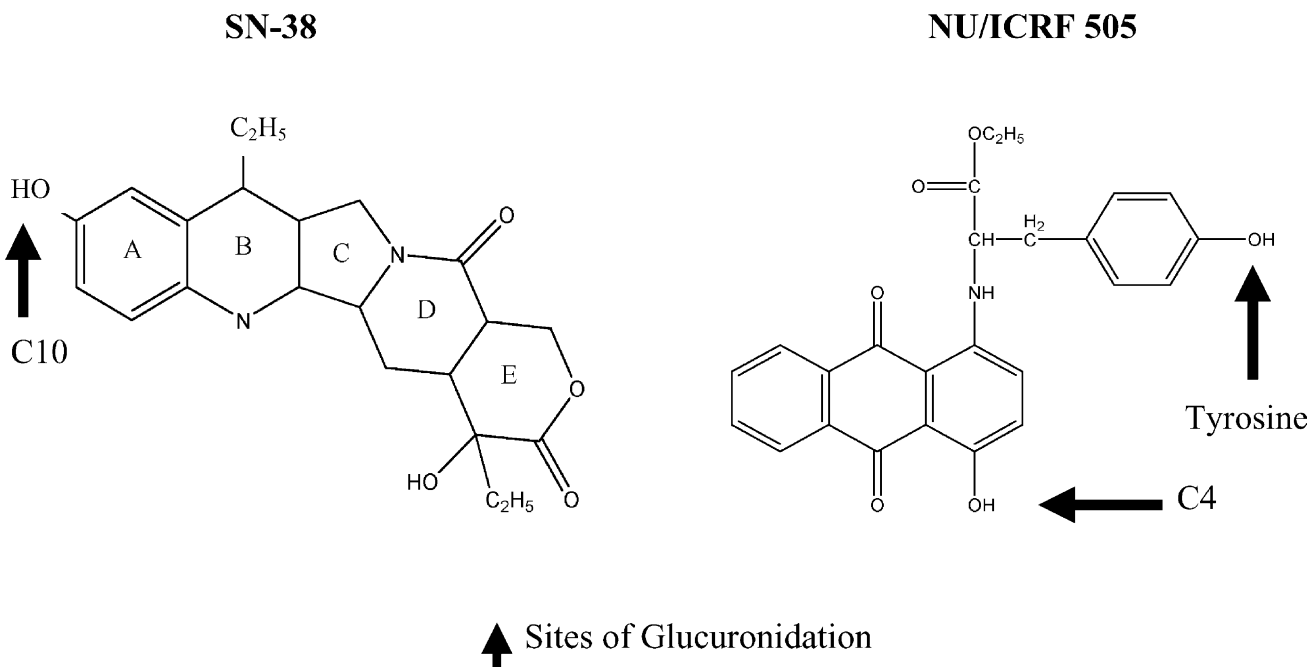

Fig. 1. Molecular structures of SN-38 and NU/ICRF 505 and sites of glucuronidation.

reported to be a novel mechanism of intrinsic drug resistance in colon cancer cells accounting for at least 5-fold resistance to NU/ICRF 505 and 2-fold resistance to SN-38 [10].

An increasingly large body of evidence suggests that as a defence to toxicological insult by anticancer drugs, co-ordinated induction of a drug metabolising enzyme occurs together with a drug transport protein [11–14]. Indeed, studies with the alkylating agent chlorambucil suggest that the presence of MRP1 is essential for the expression of drug resistance mediated by the phase II conjugating enzyme glutathione *S*-transferase (GSTA1-1) [15], due to the fact that the chlorambucil conjugate accumulates intracellulary and prevents further detoxification of the parent drug by product inhibition of GSTA1-1 [16]. Therefore, we have investigated the contribution and role of drug transport proteins in the glucuronidation mechanism of intrinsic drug resistance in colon cancer cells.

2. Materials and methods

2.1. Drug standards

NU/ICRF 505 and its hydrolysis product NU/ICRF 505/M were synthesised and chemically characterised as previously reported [17,18]. The anthraquinone ring C-4-*O*-β-glucuronide (C-4-G) and tyrosyl-*O*-β-glucuronide (tyr-G) of NU/ICRF 505 were biosynthesised, purified and structurally characterised as previously reported [8], and these were then used as standards in quantitative HPLC analysis. The high affinity MRP antagonist MK571 was a kind gift from Merck Frosst (Merck Frosst Canada and Co) [19].

2.2. Cell lines

HT29 human colon adenocarcinoma cancer cells were originally from the ATCC while HCT116 human colon cancer cells were originally from the ECACC. The A2780 human ovarian cancer cell line and its multidrug resistant counterpart 2780^{AD} were kindly provided by Drs. T.C. Hamilton and R.F. Ozols, Fox Chase Cancer Center, Philadelphia, USA. 2780^{AD} was cultured in the presence of 2 μM doxorubicin, which was removed 1 week prior to experimentation. The above cell lines were grown as monolayers as previously described [20]. 2008 human ovarian cancer cells stably expressing MRP1 (2008/MRP1 cells) were generated, cultured and characterised as reported in detail [21]. Adherent, spontaneously immortalised embryo (MEF3.8) and ear (KOT52) fibroblast cell lines were derived from *Mdr1a/b*$^{-/-}$*Mrp1*$^{-/-}$ triple knockout mice [22]. The drug resistant subline of MEF3.8 termed T6400 was selected by continuous exposure to topotecan and the drug resistant subline of KOT52 termed D320 was selected by continuous exposure to doxorubicin [22]. A series of stable clones (termed MRP3 #38 and MRP3 #77) that overproduce MRP3 were generated using kidney fibroblasts obtained from the *Mdr1a/b*$^{-/-}$*Mrp1*$^{-/-}$ triple knockout mice [23]. As a control, a clone (termed V1) was generated from kidney fibroblasts cells transduced with the empty pBabePURO vector. Madin–Dabry canine kidney II (MDCK) cells that overexpress MRP2 were generated by transfection with pCDNA-neo/MRP2 followed by three successive rounds of subcloning to eventually produce clone 118-14-7, designated as MDCK7.

2.3. Chemosensitivity assays

Drug sensitivity of V1, MRP3 #38 and MRP3 #77, MEF3.8, KOT52, T6400, D320, 2008 and 2008/MRP1 cells was determined using growth inhibition assays under continuous long-term drug exposure. Cells were plated at a density of 500 or 1000 cells per well in triplicate in a 96-well plate. After 24 hr, varying concentrations of NU/ICRF 505 (0, 1–100 μM) were added to each well. Incubations were continued for an additional 72–96 hr, then media were removed and plates were frozen at −80° for at least 4 hr. Total cell number was determined by fluorescence using the CyQuant cell proliferation assay kit (Molecular Probes). Drug sensitivity of A2780 and 2780^{AD} cells to NU/ICRF 505 was determined after a 24-hr drug exposure in 6-well plates [20]. Assays were repeated on three separate occasions and the IC_{50} was defined as the concentration of drug producing 50% growth inhibition compared to non-drug treated controls.

2.4. Vectorial transport assay in MRP2 overexpressing MDCK cells

Cells were cultured for 5 days at an initial density of 2×10^6 cells per transwell permeable growth support (3 μm pore size, 4.7 cm^2 clear polyester filter cup insert obtained from Costar). MDCK and MDCK7 cells were grown in DMEM (GibcoBRL) supplemented with 10% FBS. The transport assay was performed essentially as described by Horio *et al.* [24], using the cell culture medium, but without the serum supplement. Briefly, for the measurement of NU/ICRF 505 transport from the apical side to the basal side, basal medium was replaced with 2 mL of blank media, while the apical medium had 10 μM NU/ICRF 505 added to it, and the cells were incubated at 37°. For transport from the basal side to apical side the above situation was reversed. At each time interval of 0, 1, 2 and 4 hr, 25-μL aliquots of medium were taken from both the apical and basal sides of the transwells for the determination of NU/ICRF 505 concentrations by HPLC as described below.

2.5. Intracellular drug accumulation in HT29 and HCT116 human colon cancer cell lines

Drug uptake studies in the two cell lines were conducted in 12.5 cm^2 tissue culture flasks (Falcon Plastics, Becton Dickinson Labware). Cells were plated at a density of 10^6 cells in 4 mL of RPMI media and allowed 24 hr to attach prior to addition of drug. NU/ICRF 505 was added at a concentration of 10 μM in HCT116 and 20 μM in HT29 and samples collected after drug addition (time 5 min) and at 0.5, 1, 2, 6, 24, 48 and 72 hr. Studies were repeated in a total of three replicate flasks conducted on two to three separate occasions. Separate 12.5 cm^2 flasks were utilised to count cells and intracellular drug concentrations were normalised to 10^6 cells. In separate drug accumulation studies, HT29 and HCT116 cells were co-incubated with NU/ICRF 505 as above plus a range of concentrations of the MRP antagonist MK571 (0, 5, 10 and 20 μM).

2.6. Drug analysis techniques

Media and cell samples from both the vectorial and drug accumulation studies were analysed by HPLC after solid phase extraction [18,25,26].

2.7. Western blot analysis of the breast cancer-resistance protein (BCRP1, ABCG2) in HT29 cells

Logarithmic growth phase adherent HT29 cells were scraped and subsequently lysed in hypotonic lysis buffer, consisting of 100 μM KCl, 2 μM $MgCl_2$, 100 μM Tris–HCl (pH 7.4), 1% SDS, supplemented with protease inhibitors ('Complete', Roche Diagnostics). Lysates were sonicated and stored at −80°. Protein levels were determined using the Lowry method. Proteins were separated on a 7.5% polyacrylamide gel, and subsequently transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell). Proteins were hybridised using the BCRP monoclonal antibody BXP-21 (1:50) [27] and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:1000) (Dako). Subsequently, proteins were visualised using enhanced chemoluminescence (ECL) (Amersham Life Sciences).

2.8. Western blot analysis of MRP1–3 and 5 in HT29 cells

Total cell lysates were prepared in a hypotonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.4), supplemented by a cocktail of protease inhibitors (Roche). Samples containing 30 μg protein were separated on a 7.5% SDS–polyacrylamide gel. Subsequently, gels were blotted overnight in a tank blotting system. MRP1–3 and 5 were visualised using chemoluminescence (Amersham) as described by Scheffer *et al.* [28] using the monoclonal antibodies, MRPr1 (1:1000), $M_{II}6$ (1:250), M_3II-9 (1:250) and M51.1 (1:250), respectively. These antibodies have been subjected to extensive characterisation by us and have been shown to be fully specific for their cognate antigens, as revealed by a lack of cross reactivity with other family members [28].

2.9. NU/ICRF 505 glucuronide transport in MRP1 and 3 containing membrane vesicles

Spodoptera frugiperda (Sf9) cells were infected with a recombinant baculovirus containing the cDNA of MRP1 or MRP3 at a multiplicity of infection of 1. After incubation at 27° for 3 days, cells were harvested by centrifugation at 1000 *g* for 5 min. The pellet was resuspended in ice-cold hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.4) supplemented with protease inhibitors

(2 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 10 μM pepstatin) and incubated at 4° for 90 min. The suspension was centrifuged at 100,000 *g* at 4° for 40 min and the pellet was homogenised in ice-cold TS-buffer (50 mM Tris–HCl, 250 mM sucrose, pH 7.4) using a tight-fitting Dounce homogeniser. After centrifugation at 500 *g* at 4° for 10 min, the supernatant was centrifuged at 4° at 100,000 *g* for 40 min. The pellet was resuspended in TS-buffer and passed 20 times through a 27-gauge needle. The vesicles were dispensed in aliquots, frozen in liquid nitrogen, and stored at −80° until use.

The ATP-dependent uptake of [^{3}H]estradiol 17-β-D-glucuronide ($E_2$17βG) into MRP1 and MRP3 containing vesicles was studied following the rapid filtration method. Briefly, membrane vesicles containing 20 μg protein were incubated for 2 min with 1 μM [^{3}H]$E_2$17βG in the absence or presence of 1, 5 or 20 μM NU/ICRF 505 glucuronide (C-4-G) in 50 μL of TS-buffer in the presence of 4 mM ATP or AMP, 10 mM $MgCl_2$, 10 mM creatine phosphate, and 100 μg creatine kinase/mL. The reaction mixture was diluted in 2 mL of ice-cold TS-buffer and immediately filtered through a pure cellulose filter (0.45 μm pore size). The filter was washed twice with 3 mL of ice-cold TS-buffer and the radioactivity retained on the filter measured by liquid scintillation.

3. Results

3.1. Chemosensitivity of drug resistant and drug transport protein expressing cell lines to NU/ICRF 505

The IC_{50} values for NU/ICRF 505 in all the variant cell lines studied, which included lines overexpressing P-170 glycoprotein (P-gp), MRP1 and 3 and BCRP, were not significantly different (Student's two-tailed *t* test) from that of the parental cell lines with resistance factors (RF) ranging from 0.73 to 1.8 (Table 1, compare against the resistance level to conventional cytotoxic drugs or the selecting agent). In addition, vectorial transport studies in MDCK cells also showed there was no significant difference in transmembrane flux in the wild type vs. a subclone stably transfected with MRP2 at all time points studied (MDCK7, Table 1). In preliminary studies, all the above cell lines were demonstrated by HPLC not to catalyse glucuronidation of NU/ICRF 505.

3.2. Drug accumulation in HT29 cells (that catalyse glucuronidation) and HCT116 cells (that do not catalyse glucuronidation)

NU/ICRF 505 was rapidly and avidly taken up into colon cancer cell lines achieving peak concentrations of 3260 ± 100 ng/10^6 cells at 2 hr in HT29 cells and 2760 ± 93 ng/10^6 cells after 24 hr in HCT116 cells (Fig. 2a). NU/ICRF 505 remained at high intracellular concentrations (above 1 μg/10^6 cells) for over 48 hr in HCT116, decreasing only slowly due to enzyme-catalysed hydrolysis of the parent drug to the free amino acid metabolite NU/ICRF 505/M [18]. In striking contrast, NU/ICRF 505 intracellular concentrations fell precipitously in HT29 cells to a level of only 77.4 ng/10^6 cells at 24 hr and by 48 hr the parent drug was no longer detectable in cells (Fig. 2a). However, instead of NU/ICRF 505 or its hydrolysis product appearing in the tissue culture media (as was the case in HCT116 cells), its glucuronides C-4-G and tyr-G (see Fig. 1) accumulated (Fig. 2b) and

Table 1
Chemosensitivity of multidrug resistant and transfectant cell lines to NU/ICRF 505

Parental line	Origin	Resistant line/ transfectant	Phenotype	Resistance level	Reference	NU/ICRF 505 parent line IC_{50} (μM ± SD)	NU/ICRF 505 resistant line/ transfectant IC_{50} (μM ± SD)	Resistance factor (RF)[a]
MEF3.8	*Mdr1a/b*$^{-/-}$*Mrp1*$^{-/-}$ mouse fibroblasts	T6400	Selected with topotecan, increased BCRP1	329-fold to topotecan	[22,34]	21 ± 2	18 ± 1	0.89
KOT52	*Mdr1a/b*$^{-/-}$*Mrp1*$^{-/-}$ mouse fibroblasts	D320	Selected with doxorubicin, increased BCRP1	350-fold to doxorubicin	[22,34]	22 ± 1	21 ± 2	0.95
A2780	Human ovarian cancer	2780^{AD}	Classic MDR, increased P-gp	1700-fold to doxorubicin	[17,20]	4.4 ± 2.7	7.7 ± 1.9	1.75
2008	Human ovarian cancer	2008/MRP1	Stable transfectant MRP1	20-fold to etoposide	[21]	13 ± 2.5	9.5 ± 1	0.73
V1	Mouse kidney fibroblasts	MRP3 #38	Stable transfectant MRP3	6- to 8-fold to etoposide	[23]	29.2 ± 11	30.1 ± 10	1.03
MDCK	Canine kidney	MDCK7	Stable transfectant MRP2	9-fold to doxorubicin		1.3[b] ± 0.2	1.63 ± 0.4	1.27

[a] RF: The resistance factor RF was calculated as the ratio of the mean IC_{50} value (or transmembrane transport ratios) for the resistant or transfected line divided by the mean IC_{50} value (or transmembrane transport ratios) for the respective parent line.

[b] 1.3: Data refer to the results from the vectorial drug transport studies in MDCK cells where transmembrane drug flux was measured in both the basal to apical (b–a) and apical to basal (a–b) directions. The numbers represented in the table are the ratios of (b–a)/(a–b). Since in the MDCK7 cells MRP2 is overexpressed on the apical membrane of cells, if NU/ICRF 505 is transported by this protein this ratio should increase above unity.

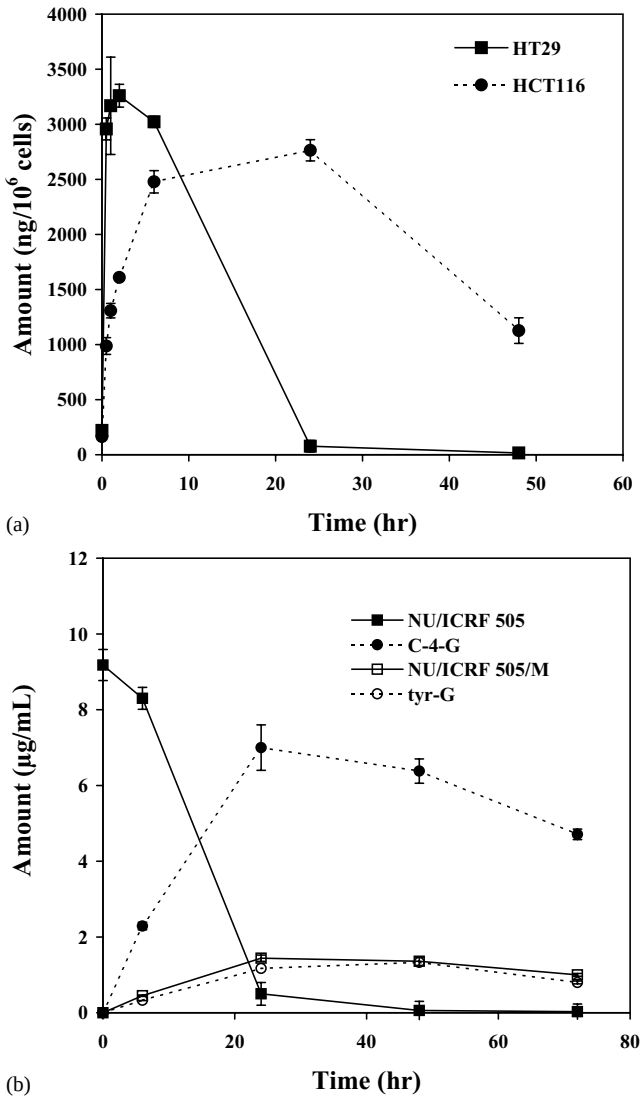

Fig. 2. (a) Concentration time profiles for the intracellular accumulation of NU/ICRF 505 parent compound after exposure of HT29 cells to 20 μM and HCT116 cells to 10 μM drug. Each value represents the mean value ± standard deviation for N = 3–6 replicates. (b) Concentration time profiles of NU/ICRF 505, its hydrolysis product NU/ICRF 505/M and its two glucuronides (C-4-G and tyr-G, see Fig. 1) in the tissue culture medium of HT29 cells after exposure to 20 μM drug. Each value represents the mean value ± standard deviation for N = 3–6 replicates.

accounted for 88.1% of total drug after 24 hr. The two glucuronides were detected in cells at low concentrations (≪50 ng/10^6 cells), while the peak level of C-4-G in the tissue culture media was 7.1 μg/mL.

3.3. Expression of drug transport proteins in HT29 cells

We have previously utilised quantitative (real time) RT–PCR to determine RNA transcript levels of *MDR1*, *MRP1* and *MRP2* in HT29 cells [29]. In the present study, we have extended these findings using monoclonal antibodies and confirmed an absence of MRP2, MRP5 (Fig. 3) and BCRP (Fig. 4) and shown the presence of a weak signal corresponding to MRP1 and a stronger signal corresponding to MRP3.

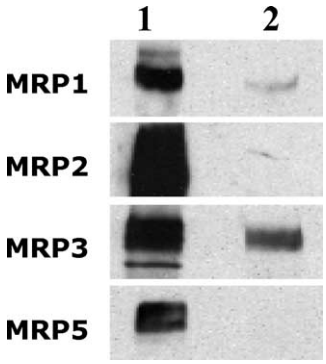

Fig. 3. Western blot analysis of HT29 cells for expression levels of MRP1–3 and 5. Lane 1 contains a positive control transfectant cell line overexpressing a specific MRP, whereas lane 2 contains HT29 cells.

3.4. Effect of the NU/ICRF 505 glucuronide metabolite (C-4-G) on $E_2$17βG transport by MRP1 and 3

More than 50% inhibition of 1 μM $E_2$17βG transport by MRP3 occurred even at the lowest glucuronide concentration studied of 1 μM. The glucuronide also caused inhibition of MRP1-mediated transport of $E_2$17βG, but a concentration of approximately 5 μM was required for 50% inhibition (Table 2).

3.5. Effect of the MRP antagonist MK571 on NU/ICRF 505 drug accumulation in HT29 cells

Co-treatment of HT29 cells with 10 μM NU/ICRF 505 and a range of concentrations of MK571 resulted in a dose-dependent increase in intracellular drug concentrations of parent drug (Fig. 5) from 0 ng/10^6 cells to 70.4 ± 3 ng/10^6 cells. This increase occurred in conjunction with a smaller

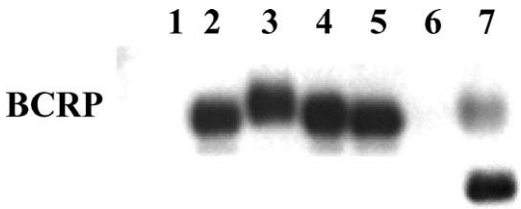

Fig. 4. Western blot analysis of HT29 cells for expression levels of BCRP. Lane 1 contains Igrov1 cells that do not express BCRP as a negative control, lanes 2–5 and 7 contain cell lines that overexpress BCRP and lane 6 contains HT29 cells.

Table 2
Effect of NU/ICRF 505 C-4-glucuronide (C-4-G) on MRP1- or 3-mediated transport of estradiol 17-β-D-glucuronide ($E_2$17βG) into MRP expressing Sf9 membrane vesicles

NU/ICRF 505 C-4-G (μM)	% ATP-dependent uptake of 1 μM $E_2$17βG into MRP expressing Sf9 membrane vesicles	
	MRP3	MRP1
1	35 ± 11	98 ± 2
5	16 ± 7	48 ± 2
20	4 ± 1	18 ± 2

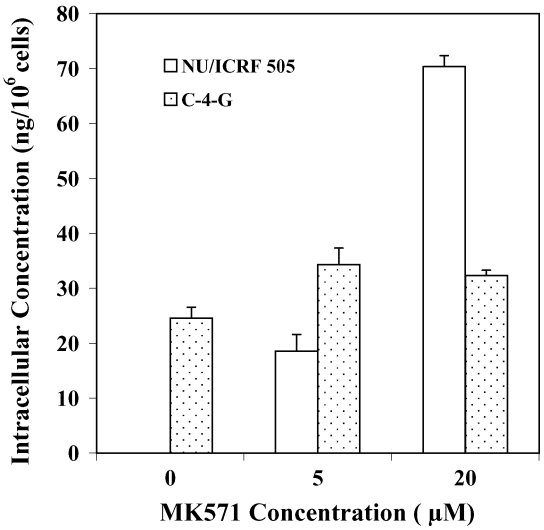

Fig. 5. Effect of increasing concentrations of the MRP1 antagonist MK571 on intracellular concentrations of NU/ICRF 505 and its C-4-O-glucuronide (C-4-G) measured at 24 hr in HT29 cells after exposure to 10 μM drug. Each value represents the mean value ± standard deviation for N = 3 replicates.

but statistically significant increase in intracellular concentrations of the NU/ICRF 505 C-4-G ($P < 0.01$, Student's t test).

4. Discussion

In the present study, the role of ATP-binding cassette (ABC) drug transport proteins [30] in the glucuronidation pathway of *de novo* drug resistance to the topo I inhibitors NU/ICRF 505 and SN-38 [8,10] has been investigated in human colon cancer cells. We were interested to establish if transport proteins functioned in conjunction with, in parallel to or even competed with this mechanism. The first issue addressed was whether or not NU/ICRF 505 parent compound was subject to active transport, prior to screening HT29 cells for ABC protein expression and performing transport studies with the NU/ICRF 505 glucuronide. Utilising a diverse panel of cell lines comprising drug-sensitive and -resistance pairs (see Table 1 and references therein), it was shown that no resistance to NU/ICRF 505 is mediated by either MRP1–3, BCRP and P-gp. NU/ICRF 505 is a highly hydrophobic and electrically neutral molecule; chemical properties that are known to circumvent multidrug resistance in closely related series of anthrapyrazole and anthracycline anticancer compounds [31,32]. SN-38 has been reported by us (and others) to be transported by a number of these transporters including: MRP1 [33,34]; MRP2 [35] and BCRP [11,36,37] but not P-gp [34,38] or MRP3 [21,23].

The high concentrations of NU/ICRF 505 (and SN-38 [29]) metabolites accumulating extracellularly in incubations with HT29 cells suggest active transport of the glucuronides. Previous transport studies with polarised Caco-2 cells have shown that SN-38 glucuronide is unable to cross the biomembrane by passive diffusion [39]. Also, the elevated level of β-glucuronidase activity present in colon cancer cells [40] would promote the hydrolysis of the NU/ICRF 505 glucuronides back to the parent compound and prevent accumulation of these metabolites in the tissue culture medium unless a high affinity efflux pump was operative, as exemplified in the present study where treatment of HT29 cells with the MRP inhibitor MK571 resulted in a greater increase in intracellular concentrations of the parent drug than the glucuronide metabolites. A screen of HT29 cells for ABC transport proteins revealed that MRP3 was present at high levels followed by a weaker signal due to MRP1. P-pg, MRP2 and 5 and BCRP were not present at detectable levels of protein expression. Recent studies using an RNase protection assay have also detected the transcript for MRP4 expressed in HT29 cells [41], but this transport protein has been linked more to the physiological export of nucleotides, steroids and bile salts rather than multidrug resistance and exhibits a low affinity for glucuronide metabolites [42–44]. Our transmembrane transport studies with MRP expressing vesicles demonstrated that the major glucuronide of NU/ICRF 505 was able to inhibit $E_2$17βG transport in both MRP1 and MRP3 membranes but appeared to be most effective against MRP3. However, the affinity of MRP1 for $E_2$17βG is high (K_m, 2 μM) whereas the affinity of MRP3 for $E_2$17βG is moderate (K_m, 18–25 μM) [45,46]. Therefore, both proteins may participate in the transport of NU/ICRF 505 glucuronide. Further studies with radioactively labelled NU/ICRF 505 glucuronide will be required to determine directly the apparent K_m of this substrate for these transporters.

MK517 is a leukotriene LTD4 receptor antagonist and a high affinity and specific inhibitor of MRP1, with a K_i of 0.6 μM for the inhibition of LTD4 transport [47]. MK571 has previously been shown to completely ablate 5-carboxyfluorescein efflux and reverse colchicine resistance in HT29 cells [48]. However, in the present studies MK571 had only a small (but significant) effect on drug accumulation of NU/ICRF 505 and its glucuronides in HT29 cells, although these studies were performed in whole cells where MK571 has only limited membrane permeability [47]. Therefore, both MRP1 and MRP3 remain candidates as the NU/ICRF 505 glucuronide transporter; nonetheless, other unidentified proteins cannot be completely ruled out [49]. While SN-38 glucuronide has been previously reported to be a moderate substrate for MRP1 and a high affinity substrate for MRP2 [38], we have yet to establish whether or not MRP3 recognises this metabolite.

The drug accumulation studies described above with NU/ICRF 505 and previously reported by us for SN-38 [29] lead us to conclude that a different set of dynamics operates for each of the two compounds in HT29 cells. Initially, NU/ICRF 505 is avidly taken up into cells and accumulates at high concentrations due to a lack of active

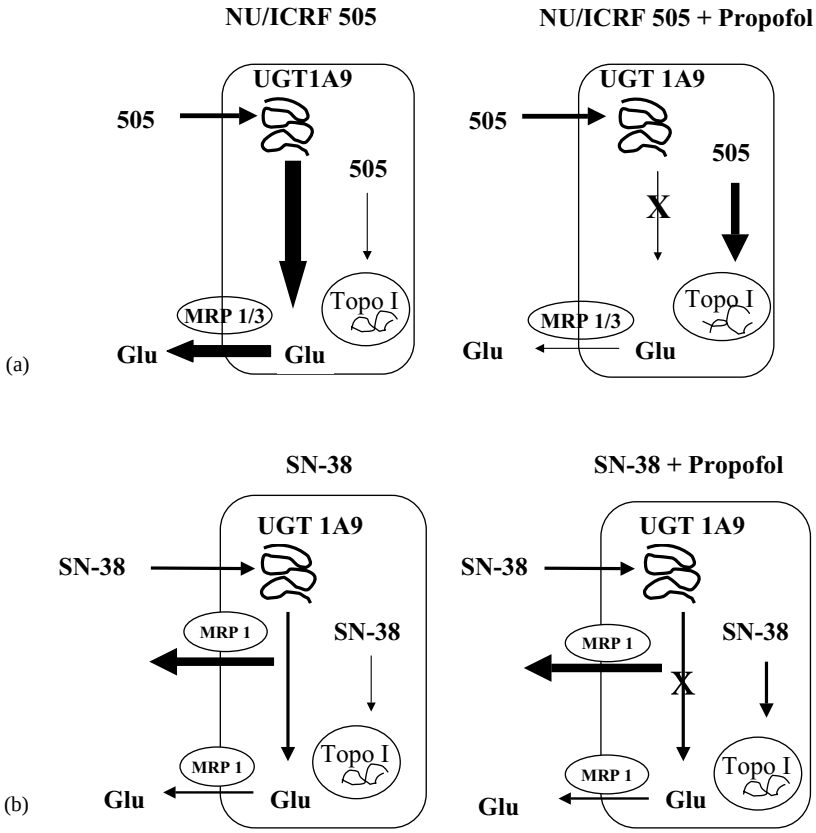

Fig. 6. Role and contribution of drug transport proteins to the glucuronidation mechanism of intrinsic drug resistance in HT29 human colon cancer cells. (a) Schematic representation for NU/ICRF 505 and (b) schematic representation for SN-38. Propofol is a specific inhibitor of glucuronidation catalysed by the UDP-glucuronosyltransferase isozyme UGT1A9.

efflux of the parent compound (see Fig. 6a). Then, the parent drug is continuously, but slowly, metabolised inside cells by UGT1A9 until the entire extracellular compartment is exhausted and replaced by the two glucuronides. High affinity active efflux of the resultant glucuronides by MRP1 and/or 3 is proposed as a major driving force in this model preventing an accumulation of product that could potentially cause UGT enzyme inhibition [16]. In the absence of parent drug efflux glucuronidation functions as the first line of defence, but clearly requires the participation of a glucuronide efflux pump. Consequently, inhibition of UGT1A9 with propofol produces a 32-fold increase in intracellular drug levels of NU/ICRF 505 and a 5-fold increase in drug activity [10]. By contrast, SN-38 is only poorly taken up into cells, at two orders of magnitude below that of NU/ICRF 505, despite sharing similar chemical characteristics. Recognition of the parent drug by MRP1 for active efflux [33,34] coupled to *in situ* chemical degradation of SN-38 lactone in tissue culture media [50] probably both contribute to limit overall drug accumulation (Fig. 6b). Once taken up into cells, SN-38 is metabolised by UGTs but due to less efficient active efflux of the SN-38 glucuronide, glucuronidation only functions as a minor pathway of drug clearance. Studies have shown that SN-38 is a better substrate for ABC proteins than the SN-38 glucuronide [38,51]. The more limited net clearance of the drug by glucuronidation against significant efflux of the parent drug means that inhibition of UGT with propofol results in only a modest increase in intracellular drug and a 2-fold increase in drug activity [10]. Hence, while active drug transport of the parent drug can occur in parallel to drug clearance *via* glucuronidation, it is in a sense competing with and reducing the contribution of UGTs in intrinsic drug resistance. In summary, we now demonstrate that the presence of an efficient glucuronide transporter is an essential component in the glucuronidation mechanism of drug resistance and that the contribution of glucuronidation to intrinsic drug resistance is more significant when the parent drug (NU/ICRF 505) is not recognised by transport proteins.

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