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Enhanced clearance of topoisomerase I inhibitors from human colon cancer cells by glucuronidation

Jeffrey Cummings[a,*], Gary Boyd[a], Brian T. Ethell[b], Janet S. Macpherson[a], Brian Burchell[b], John F. Smyth[a], Duncan I. Jodrell[a]

[a]*Imperial Cancer Research Fund, Medical Oncology Unit, Western General Hospital, Edinburgh EH4 2XU, UK*
[b]*Molecular and Cellular Pathology, Ninewells Hospital & Medical School, Dundee DD1 9SY, UK*

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Abstract

As part of a program to identify novel mechanisms of resistance to topoisomerase I (topo I) inhibitors, the cellular pharmacology of 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of clinically used irinotecan (CPT-11) and NU/ICRF 505, an anthraquinone–tyrosine conjugate, has been investigated in two human colorectal cancer (CRC) cell lines. Two novel metabolites of NU/ICRF 505 (M1 and M2) and a single metabolite of SN-38 (M1) were detected by high performance liquid chromatography in the culture medium of HT29 cells but were absent in HCT116 cells. Identities of all three metabolites were established by a combination of biochemical and physicochemical techniques. M1 of SN-38 was the C10-(β)-glucuronide of the parent lactone while M1 of NU/ICRF 505 was the C4-*O*-glucuronide and M2 the tyrosine-*O*-glucuronide, both of the parent compound. Drug transport studies revealed that by 24 hr HT29 cells had effectively cleared 82.5% of NU/ICRF 505 (10 μM) into the culture medium as the two glucuronides. In contrast, intracellular concentrations of NU/ICRF 505 were maintained in HCT116 cells in the absence of glucuronidation at a level 550 times greater than in HT29 cells. HT29 cells cleared 40.9% of SN-38 (1 μM) as the glucuronide to the culture medium, while the parent drug was maintained at a level 2-fold greater in HCT116 cells. Enhanced drug clearance due to glucuronidation may contribute to intrinsic drug resistance of human CRC.

Keywords: SN-38; NU/ICRF 505; Drug uptake; Glucuronidation; Drug clearance; Human colon cancer cells

1. Introduction

The topo I remains an important focus in new anticancer drug development. Camptothecin (CPT) is the classic inhibitor of topo I and until recently has acted almost exclusively as the template in rational design of new inhibitors [1,2]. Several water-soluble analogues of CPT are currently undergoing clinical evaluation as anticancer drugs [3,4]. Among these is CPT-11 (irinotecan), a prodrug for release by carboxylesterases of the extremely potent topo I inhibitor SN-38 [5]. CPT-11 offers the potential of a major development in the treatment of chemoresistant human CRC having demonstrated significant clinical activity in phase II clinical trials [5,6].

Increasingly, a variety of non-CPT-11 based compounds are being identified as topo I inhibitors with the promise of distinct pharmacological properties [7–9]. NU/ICRF 505 is the lead member of a novel class of anthracenyl–peptide topo inhibitors [10,11]. The compound has been demonstrated to be non-cross-resistant in a CPT resistant cell line [12].

Human CRC is recognised as being intrinsically resistant to most cancer chemotherapeutic drugs [6] while the nature of the underlying mechanisms responsible for *de novo* drug resistance remain poorly characterised [13,14]. In the present study, the metabolic fate of SN-38 and NU/ICRF 505 has been investigated in two human colon cancer

* Corresponding author. Tel.: +44-131-332-2471; fax: +44-131-332-8494.
E-mail address: j.cummings@icrf.icnet.uk (J. Cummings).

Abbreviations: Topo I, topoisomerase I; CPT-11, camptothecin; SN-38, 7-ethyl-10-hydroxycamptothecin; HPLC, high performance liquid chromatography; DMSO, dimethylsulphoxide; SPE, solid phase extraction; EI, electron impact ionisation mass spectrometry; UDP-GA, UDP-glucuronic acid; LC/MS, high performance liquid chromatography with mass spectrometry; ESI, electrospray ionisation mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; UGT, UDP-glucuronosyltransferase; M1, metabolite 1; M2, metabolite 2; MRP2, canalicular multispecific organic anion transporter (cMOAT); ABC, ATP binding cassette; MRP, multidrug resistance-associated protein; P-gp, P-170 glycoprotein; BCRP, breast cancer resistance protein; t_R, HPLC retention time.

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cell lines. Utilising a combination of biochemical and physicochemical techniques, human colon cancer cells are shown to metabolise SN-38 and NU/ICRF 505 into glucuronides resulting in enhanced drug clearance.

2. Materials and methods

2.1. Materials

NU/ICRF 505 and its hydrolysis product NU/ICRF 505/M were produced as previously described [11,15]. The anthraquinone ring C4-*O*-glucuronide of NU/ICRF 505 and C10-*O*-glucuronide of SN-38 were biosynthesised in incubations with HT29 cells and then purified by the sample preparation technique described below. SN-38 was a gift from Rhone-Poulenc Rorer and its hydrolysis product known as the hydroxy acid was prepared by alkaline hydrolysis.

2.2. Cell lines

The HT29 human colon adenocarcinoma cancer cell line was from the American Tissue Culture Collection and the HCT116 human colon carcinoma cell line was from the European collection of cell culture and both were cultured under standard conditions.

2.3. Drug incubations

SN-38 (1 μM) and NU/ICRF 505 (10 μM) were incubated with cell lines in 12.5 cm^2 tissue culture flasks (Falcon Plastics, Becton Dickinson Labware, USA) as described in detail [15].

2.4. Drug analysis techniques

Quantitative determinations of the two topo I inhibitors and their metabolites in tissue culture media and cell sonicate specimens were performed by gradient elution, reversed phase HPLC with solid phase extraction (SPE) sample preparation essentially as previously described [15,16]. An equally high level of recovery was obtained for both NU/ICRF 505 and SN-38 (80–100%).

2.5. Incubation with β-glucuronidase and microsomes prepared from HT29 human colon cancer cells

NU/ICRF 505 metabolites extracted from the culture media of HT29 cells were incubated with 200 units of *Escherichia coli* β-glucuronidase (type VII) or *Aerobacter aerogenes* sulphatase (type VI, Sigma, USA) in 1 mL of 0.1 M sodium phosphate buffer pH 7.0 in a shaking water bath at 40°. At regular time intervals 100 μL samples were withdrawn and analysed directly by HPLC.

2.6. LC/MS (EI)

Electron impact spectra were obtained for NU/ICRF 505 metabolites during chromatography. The mass spectrometer was a Waters Alliance system set at the following parameters: source temperature (ion volume) 200°, nebulizer temperature 80°, expansion region temperature 85°, spectra acquisition rate (s^{-1}), gain 10 and mass scan range 100–700.

2.7. LC/MS (ESI)

Metabolites of NU/ICRF 505 were also analysed by HPLC with mass spectrometry (LC/MS) using electrospray ionisation. Chromatographic conditions were again as above. The mass spectrometer was a Micromass Platform II single quadruple instrument operating in positive electrospray mode. Source parameters were as follows: capillary voltage 2.8 kV, HV lens 0.6 V, cone voltage 50 V, skimmer lens offset 5 V, and source temperature 140°. MS parameters were LM resolution 15.5, HM resolution 16.0, ion energy 0.5, ion energy ramp 0.0 and multiplier 650.

2.8. Nuclear magnetic resonance spectroscopy (NMR)

NMR spectra for purified samples of NU/ICRF 505 and NU/ICRF 505 C4-*O*-glucuronide were obtained using a Brucker 360 MHz spectrometer by D. Reid, Chemistry Department, University of Edinburgh and his assistance is gratefully acknowledged.

3. Results

3.1. Detection of drug metabolites of NU/ICRF 505 and SN-38 in the tissue culture media of HT29 human colon cancer cells

After a 24 hr incubation with HT29 cells, HPLC analyses of tissue culture media revealed that NU/ICRF 505 had been converted to two novel chromatographic peaks while the presence of the C10-glucuronide metabolite of SN-38 was confirmed by LC/MS (data not shown). In incubations with the HCT116 human colon cancer cell line there was no evidence of production of the SN-38 glucuronide metabolite or the two novel metabolites of NU/ICRF 505.

3.2. Biochemical characterisation of the novel metabolites of NU/ICRF 505 produced by HT29 cells

Extracts from the tissue culture media of HT29 cells dosed with NU/ICRF 505 were treated with either β-glucuronidase or sulphatase. Only in the case of β-glucuronidase was a significant affect on chromatographic profiles observed (Fig. 1). With time, the two novel metabolite peaks disappeared and there was a concomitant increase in the peak area of the parent drug peak. Microsomal fractions

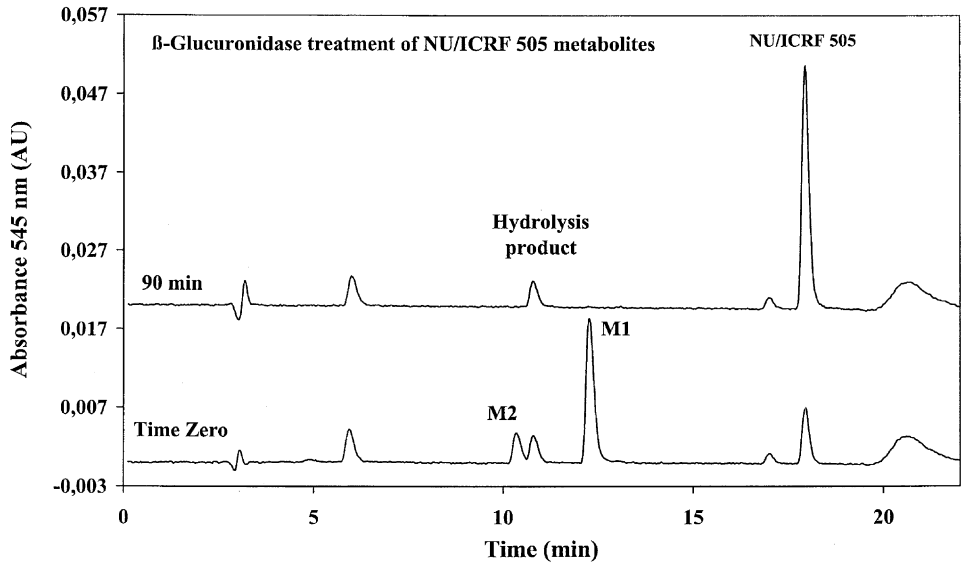

Fig. 1. The effect of 200 units of *E. coli* β-glucuronidase on the chromatographic profile of NU/ICRF 505 chromatographic peaks. A total of 10 μM of NU/ICRF 505 was incubated with HT29 cells for 24 hr to generate metabolites. Several extracts of tissue culture media were pooled and then incubated with β-glucuronidase. At time zero of the enzyme incubation concentrations were: NU/ICRF 505, 840 ng/mL; M2, 387 ng/mL; hydrolysis product, 258 ng/mL; and M1, 2.132 ng/mL. At 90 min, concentrations were: NU/ICRF 505, 3.560 ng/mL and hydrolysis product, 261 ng/mL.

prepared from HT29 cells were capable of converting NU/ICRF 505 into these two metabolite peaks only when enriched with UDP-GA acid.

3.3. Mass spectrometry of the novel metabolites of NU/ICRF 505

LC/MS was performed initially using harsh ionisation (EI) to induce fragmentation and then followed by soft ionisation (ESI) to preserve molecular ions (Table 1). Both of the novel metabolites yielded identical EI spectra with the largest fragment (*m/z* 431) corresponding to the molecular weight of the parent drug. ESI spectra yielded molecular ions for the two novel metabolites $[MH]^+$ of 608, corresponding to direct addition of one molecule of glucuronic acid to intact NU/ICRF 505 (Fig. 2).

3.4. Nuclear magnetic resonance (NMR) of the novel metabolites of NU/ICRF 505

There are two possible sites available for direct conjugation of NU/ICRF 505 with glucuronic acid: the C4 hydroxyl group present in the ring system and the tyrosine hydroxyl group. NMR analysis of the parent compound showed that the protons present on both these groups were significantly downfield shifted: δ 13.60 for the C4 ring OH and δ 8.26 for the tyrosine OH (see Table 1). NMR analysis of the major metabolite species (metabolite 1 (M1), retention time 12.3, Fig. 1) revealed the absence of the signal corresponding to the C4 ring OH, while a signal corresponding to the tyrosine OH was still present (Table 1). Accordingly, the identity of M1 was ascribed to the anthraquinone ring C4-*O*-glucuronide, while that of metabolite 2 (M2) ascribed to the tyrosine-*O*-glucuronide (see Fig. 3).

3.5. Comparative metabolism and disposition of SN-38 and NU/ICRF 505 in human colon cancer cell lines

After a 24 hr incubation in excess of 99% of the initial concentration of NU/ICRF 505 (4.31 μg/mL) was exhausted from the tissue culture medium, taken up into cells and metabolised, effectively clearing the drug from the HT29 cell line (5 ± 2 ng/10^6 cells). The resultant glucuronides accumulated in the tissue culture medium at high concentrations (C4-*O*-glucuronide, 1010 ± 26 ng/mL and tyrosine-*O*-glucuronide, 326 ± 2 ng/mL, mean $\pm$ SD, Table 2) and were

Table 1
Summary of physicochemical characterisation of the metabolites of NU/ICRF 505

t_R (min)	EI/MS $[M]^+$	ESI/MS $[MH]^+$	NMR	Identity
10.2 (M2)	431	608	ND	Tyrosine-*O*-glucuronide
10.9	403	404	ND	Hydrolysis product
12.3 (M1)	431	608	δ 10.65, ring NH_2; δ 8.63, Tyr OH; δ 8.34, ring H; δ 7.93, anthraquinone ring	Ring (C4)-*O*-glucuronide
17.8	431	432	δ 13.60, C4-ring OH; δ 10.67, ring NH_2; δ 8.4, ring H; δ 8.26, Tyr OH; δ 7.99, anthraquinone ring	Parent drug

ND: not done.

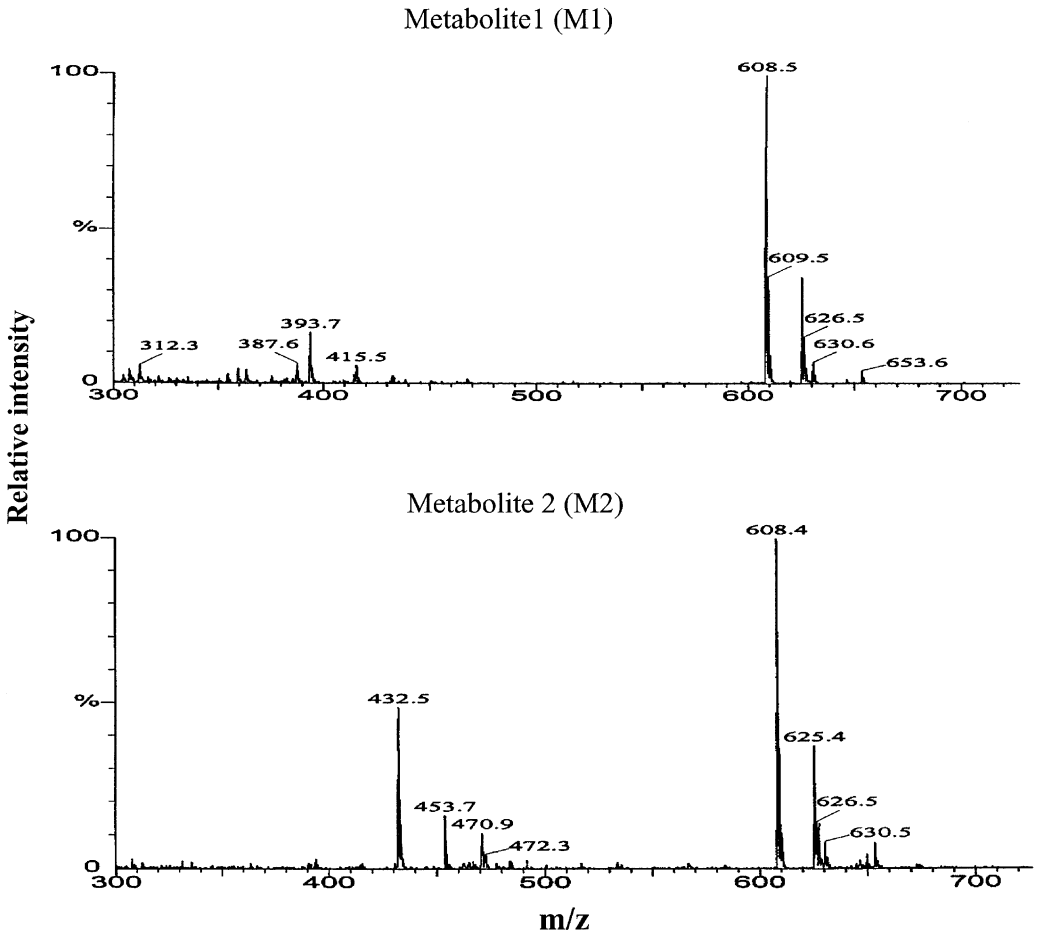

Fig. 2. ESI spectra of the two novel chromatographic peaks (M1 and M2) generated by incubation of NU/ICRF 505 with HT29 cells (see Fig. 1). LC conditions and MS parameters are described in Section 2. Both peaks yielded molecular ions of $[MH]^+$ 608, corresponding to the direct addition of glucuronic acid to the parent drug NU/ICRF 505.

not detectable in cells. In the absence of glucuronidation, intracellular drug concentrations in HCT116 cells were maintained at levels, three orders of magnitude higher than in HT29 cells (2760 ± 95 ng/10^6 cells, Table 2).

A lower level of glucuronidation occurred in HT29 cells with SN-38 (40.9%) and again, there was an accumulation of the glucuronide in the tissue culture medium (152 ± 53 ng/mL, Table 2). There was a smaller but significant increase (2-fold) in intracellular concentrations of SN-38 lactone in HCT116 cells in the absence of glucuronidation (1.03 ± 0.25 ng/10^6 cells in HT29 vs. 2.17 ± 0.47 ng/10^6 cells in HCT116, $P < 0.01$, Student's t-test).

Table 2
Comparative metabolism and disposition of 10 μM NU/ICRF 505 and 1 μM SN-38 in human colon cancer cell lines 24 hr after drug incubations

Cell line	HT29 (μg/mL or 10^6 cells ± SD)					HCT116 (μg/mL or 10^6 cells ± SD)		
	IC	EC				BC	EC	
	Parent drug	Parent drug	C4-glucuronide	Tyrosine-*O*-glucuronide	Hydrolysis product	Parent drug	Parent drug	Hydrolysis product
NU/ICRF 505								
Concentration	0.005 ± 0.002	0.035 ± 0.015	1.01 ± 0.026	0.326 ± 0.002	0.246 ± 0.017	2.76 ± 0.095	2.68 ± 0.27	2.30 ± 0.12
%		2.2	62.5	20.2	15.2		53.8	46.2
	HT29 (ng/mL or 10^6 cells ± SD)					HCT116 (ng/mL or 10^6 cells ± SD)		
	Parent drug	Parent drug	Hydroxy acid	C10-glucuronide		Parent drug	Parent drug	Hydroxy acid
SN-38								
Concentration	1.03 ± 0.25	50 ± 1.01	170 ± 11	152 ± 53		2.17 ± 0.47	99 ± 13	266 ± 18
%		13.4	45.7	40.9			27.1	72.9

IC: intracellular concentration and is restricted to only the parent compound, EC: extracellular concentrations of parent compound, glucuronide metabolites and hydrolysis products (free acid for NU/ICRF 505 and hydroxy acid for SN-38). Mean ± SD from $n = 3$ to 5 replicates.

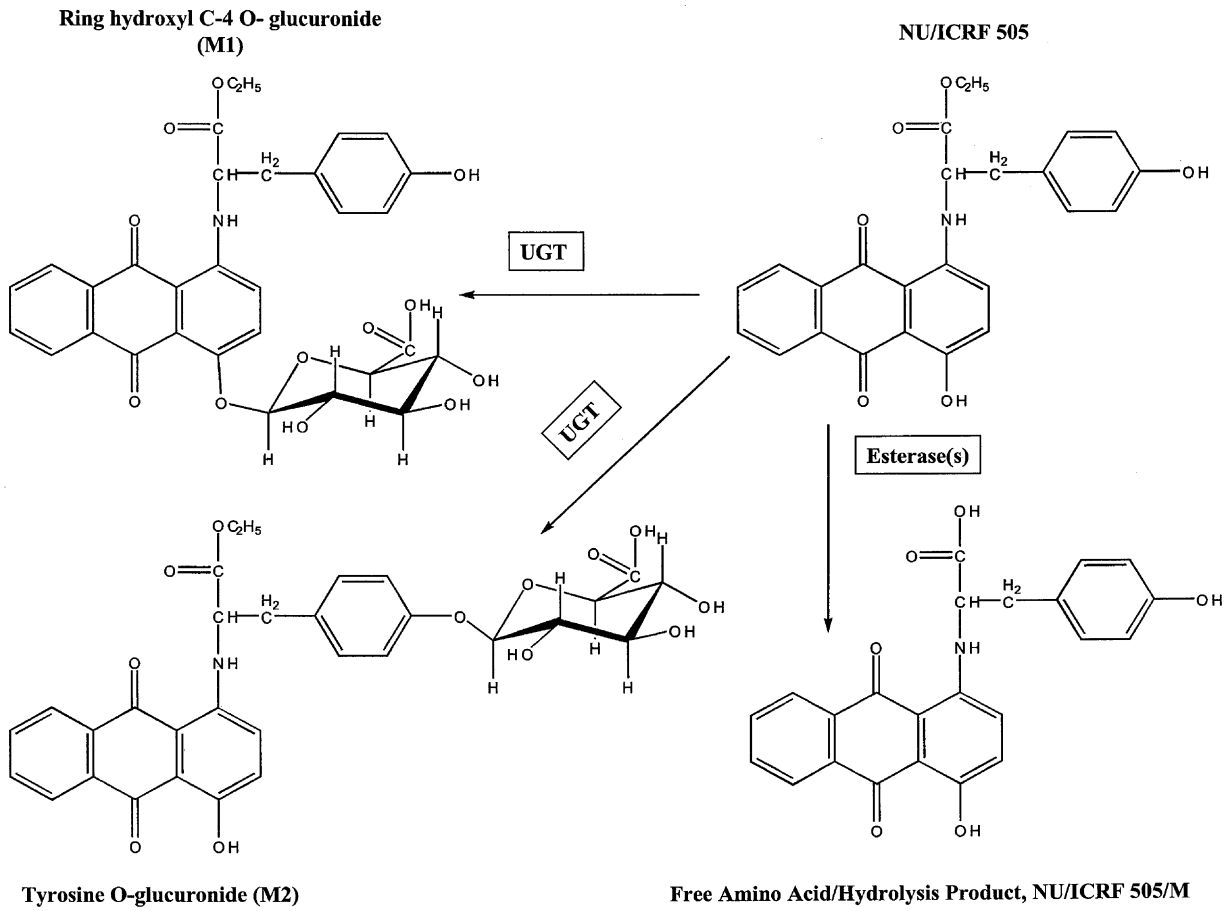

Fig. 3. Pathways of metabolism of NU/ICRF 505 in HT29 human colon cancer cells.

4. Discussion

The topo I inhibitors display a striking cell cycle (S-phase) dependency for the induction of cell death [17] requiring active DNA replication [18,19]. In common with other S-phase selective agents, prolonged exposure to lower concentrations is more effective than shorter exposures to higher concentrations, both *in vitro* and *in vivo* [3,20]. However, the toxic lesion generated by topo poisons, termed the cleavable complex, is reversible and has a short half-life due to proteolytic destruction [21,22]. Thus, accelerated drug clearance of a topo I inhibitor from target cancer cells is likely to significantly lower the activity of the drug.

Glucuronidation has recently been implicated in acquired resistance to SN-38 in a lung cancer cell line (PC-7/CPT) selected by continuous exposure to CPT-11 associated with co-ordinate up-regulation of several UDP-glucuronosyltransferase (UGT) isozymes (UGT 1A1, 1A3 and 1A6) [23]. Co-incubation of these cells with a general UGT substrate 4-methylumbelliferone (4-MU) partially reversed the 8.6-fold resistance to SN-38 but by only a small factor of 1.46-fold [23,24]. However, the presence of the SN-38 glucuronide metabolite in the first instance or inhibition of its formation by 4-MU were not confirmed.

Colon cancer cells (S1) and MCF-7 breast cancer cells also appear capable of glucuronidating SN-38, as well as mitoxantrone and epirubicin [25]. Here, glucuronides were detected by TLC but apart from treatment with β-glucuronidase were not subjected to further characterisation. The level of metabolism in the S1 colon cell line was higher than that of the MCF-7 breast cancer cell line, prompting the authors to suggest that glucuronidation may be important in intrinsic drug resistance in colon cancer [25]. In addition, glucuronidation has also been implicated in intrinsic drug resistance to mycophenolic acid in a panel of human colon cancer cell lines including HT29 cells [26].

In the present study, human colon cancer cells (HT29) have been shown, for the first time, to catalyse glucuronidation of topo I inhibitors: the CPT-11 analogue SN-38 and the novel anthraquinone–tyrosine conjugate NU/ICRF 505. An array of biochemical and physicochemical techniques were employed to firmly establish the molecular identity of the glucuronide metabolites. As a consequence of drug metabolism, intracellular concentrations of both

compounds were significantly reduced after 24 hr, establishing a link between glucuronidation and enhanced drug clearance. In a separate study, a relationship has been reported between intracellular drug concentrations of topo I inhibitors and cytotoxicity in HT29 cells [27], thus completing the loop between glucuronidation and drug resistance.

Effective clearance of water-soluble negatively charged glucuronide metabolites from cells imply active transport. Indeed, directional transport studies in Caco-2 cells have recently shown that SN-38 glucuronide cannot cross the plasma membrane by passive diffusion alone [28]. Multiple drug transporters, including MRP2 have been demonstrated to accept SN-38 glucuronide as a substrate [29–31]. The transport protein(s) responsible for active efflux of the glucuronides of NU/ICRF 505 remains unknown, but their identities are presently under investigation.

Two different agents were investigated representing diverse chemical classes and it is clear that the dynamics of drug clearance were influenced by structure. Accumulation of SN-38 (in the absence of glucuronidation) was two orders of magnitude less than NU/ICRF 505. Two factors probably account for this difference. First, extensive *in situ* chemical degradation of SN-38 lactone in tissue culture media occurs. The resultant negatively charged hydroxy acid form of SN-38 is only poorly taken up into cells passively and requires participation of an active transport mechanism [27,31]. Second, after SN-38 does enter into cells, there are a larger number of members of the ATP binding cassette (ABC) super family of transporters that recognise SN-38 for active efflux from cells including MRP1 and 2, P-170 glycoprotein (P-gp) and breast cancer resistance protein (BCRP) [25,31–33]. NU/ICRF 505 is chemically stable in tissue culture media and is not a substrate for ABC transporters [15]. In conclusion, glucuronidation is now presented as a unique (and potentially exploitable) disposition mechanism that may operate with a large number of anticancer compounds in colon cancer cells.

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