Glucuronidation of SN-38 and NU/ICRF 505 in Human Colon Cancer and Adjacent Normal Colon

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Abstract. Background: Glucuronidation represents a novel mechanism of intrinsic drug resistance in colon cancer cells. To safely reverse this mechanism in vivo, it is essential to identify which isoforms of UDP-glucuronosyltransferases are responsible for catalysing this drug metabolism in tumour tissue. Materials and Methods: LC-MS was applied to measure rates of glucuronidation of two anticancer compounds (SN-38 and NU/ICRF 505) by patient colon cancer biopsies and paired normal colon. Results: Three independent lines of enquiry indicated that, in the tumour specimens, SN-38 was glucuronidated primarily by UGT1A1, the isozyme generally recognised as being responsible for hepatic detoxification of this compound, while with NU/ICRF 505 two candidate isoforms emerged – UGT1A8 and/or UGT1A10 – both of which are not normally expressed in the liver. Conclusion: These data suggest that tumour selective modulation of this drug resistance mechanism in patients may be feasible with NU/ICRF 505 but more difficult to realise with SN-38.

De novo drug resistance is recognised as contributing significantly to the poor response rates of colorectal cancer (CRC) to chemotherapy (1). Nonetheless, the underlying mechanisms responsible for drug insensitivity remain poorly characterised (2-4). Glucuronidation, catalysed by the UDP-glucuronosyltransferases (EC 2.4.1.17, UGT) superfamily of drug metabolising enzymes, has recently been identified in vitro in human CRC cell lines as a novel intrinsic drug resistance mechanism operative against the topoisomerase I (topo I) inhibitors 7-ethyl 10-hydroxy-camptothecin (SN-38, the active metabolite of clinically administered irinotecan) and the novel N-tyrosine conjugate of anthraquinone, NU/ICRF 505 (5-8). Selective inhibition of this drug metabolism pathway with the UGT1A9 isozyme specific substrate propofol demonstrated that glucuronidation could account de novo for, at least, five-fold resistance to NU/ICRF 505 and two-fold resistance to SN-38 and provided proof of principle that this mechanism can be effectively reversed by UGT aglycones, at least in vitro (7). However, in man specific hepatic UGT isoenzymes contribute significantly to the detoxification and elimination of SN-38 via the bile (9-13). Thus, in order to consider glucuronidation a possible exploitable mechanism of drug resistance amenable to reversal in the clinic with modulators, it is essential to establish which isoform(s) of the enzyme are responsible for metabolising anticancer drugs in CRC.

The UGT superfamily consists of 18 gene products that are subdivided into two major families: the UGT1 family comprising nine different proteins encoded by a single genetic locus on chromosome 2 (UGT1A) and the UGT2 family (including the UGT2A and UGT2B sub-families), also comprising nine different proteins but encoded by individual genes (with the exception of UGT2A1 and 2) located on chromosome 4 (14, 15). Differential expression of individual UGTs occurs in different tissues (16-18) and certain isoforms can be predominately expressed by a single organ (19). Therefore, the concept of tissue (or possibly even tumour) selective target protein expression is not unprecedented. Individual UGT isoforms can also exhibit a high degree of substrate specificity (20-23), while a large pool of natural products, food additives and proprietary medicines is available from which to screen candidates as non-toxic, high affinity, isozyme specific inhibitors (24).

Abbreviations: CRC, colorectal cancer; UGT, UDP-glucuronosyltransferases; topo I, topoisomerase I; SN-38, 7-ethyl 10-hydroxy-camptothecin; C4-G, NU/ICRF 505 C4-O-β-glucuronide; Tyr-G, NU/ICRF 505 tyrosyl-O-β-glucuronide; SN-38 C10-G, SN-38 C10-O-β-glucuronide; LC-MS, high-performance liquid chromatography with mass spectrometry; UDPGA, UDP-glucuronic acid; SIR, single ion recording.

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In this report, the glucuronidation of SN-38 and NU/ICRF 505 was studied by human colon cancer biopsies and adjacent normal colon specimens in situ, with the aim of providing insights into the identity of the UGT isoenzymes responsible for catalysing this drug metabolism activity.

Materials and Methods

Drug standards. NU/ICRF 505, its hydrolysis product NU/ICRF 505/M, the anthraquinone ring C4-O-β-glucuronide (C4-G) and tyrosyl-O-β-glucuronide (Tyr-G) of NU/ICRF 505 were synthesised and purified to homogeneity as described in full previously (25-27). SN-38 was a gift from Aventis (Vitry, France). Purified SN-38 C10-O-β-glucuronide (C10-G) was synthesised as described above for the glucuronides of NU/ICRF 505 (27). 1-Naphthol glucuronide was from Sigma, Poole, UK, and morphine 3 and 6 glucuronides were from Ultrafine Chemicals, Salford, UK. A standard of propofol glucuronide was generated by in vitro incubation of propofol with V79 Chinese hamster fibroblasts transfected with human UGT1A9, as previously described in detail (28).

Cell lines. The HT29 human colon adenocarcinoma cancer cell line, which was used as a positive control for glucuronidation activity, was from the ATCC (Manassas, VA, USA), while the HCT116 human colon cancer cell line, used as a negative control, was from the ECACC (European collection of cell culture, Salisbury, UK), both being cultured as monolayers as previously described (29).

Transfected cell lines expressing UGT isoforms. V79 Chinese hamster fibroblasts and HEK293 fibroblasts stably expressing full-length human UGT cDNAs were as previously described (30, 31). Micromosomes prepared from SF-9 insect cells transfected with human UGT1A7 and 1A10 were purchased from Oxford Biomedical Research (Oxford, MI, USA).

Clinical specimens and sample preparation. Nineteen colon cancer biopsies, each from a different patient including nine with paired adjacent normal colon specimens (0.2-0.5), were kindly provided by Professor Malcolm Dunlop, Western General Hospital, Edinburgh, UK. After resection, all the colon specimens were –80°C until utilised. Written informed patient consent and local committee ethical approval were both obtained to conduct the experimental investigations described in this report.

Glucuronidation activity of clinical specimens determined by LC-MS. Crude lysates of the clinical specimen fragments (approximately 100 mg) were prepared by hand homogenisation in pre-chilled 0.25 M sucrose, 5 mM HEPES buffer pH 7.4 (50 µl per 10 mg of tissue) in a loose fitting 1-ml glass homogeniser and aliquoted into 100-µl fractions. Any remaining tissue was broken up further by sonication (MSE Soniprep 150, Sanyo Gallenkamp, Loughborough, UK) by two 5-sec bursts with 1 min resting on ice between bursts. The protein concentration was measured according to the Lowry method.

Glucuronidation activity was measured by incubating the clinical specimen lysates (or expressed cell sonicates) at 37°C with the substrate and co-factor over a period of up to 2 h in order to obtain a kinetic rate of glucuronidation by measuring glucuronide formation by LC-MS (21, 28, 32). Quantitation of the glucuronides was achieved by calibrating the LC-MS with authentic glucuronide standards and running a full 7- to 10-point calibration curve with each assay of patient samples. The range of the calibration curves extended from 30 pg to 20 ng on the column and each glucuronide yielded a linear calibration curve over this range with regression correlation coefficients ($r^2$) always better than 0.975. The reactions were stopped by the addition of methanol to precipitate the protein followed by direct injection, ensuring quantitative recovery of the glucuronides from the incubation mixtures. Substrate concentrations were: propofol, 500 µM; 1-naphthol, 500 µM; morphine, 500 µM; NU/ICRF 505, 150 µM and SN-38, 250 µM.

The kinetics of inhibition of NU/ICRF 505 glucuronidation at 10- and 150-µM substrate concentrations were also measured in the human colon cancer biopsies by adding increasing concentrations of propofol (0, 20, 50 100 150, 250 and 500 µM). The IC50 for enzyme inhibition was defined as the concentration of propofol necessary to induce a 50% reduction in glucuronidation activity.

The experiments were repeated on two to four separate occasions and glucuronidation activity was calculated as pmol glucuronide formed/min/mg of protein (pmol/min/mg).

Results

preferential formation of the two different glucuronides of NU/ICRF 505 by individual UGT isoforms. NU/ICRF 505 is metabolised to two different glucuronides: C4-G and Tyr-G (6). Utilising a panel of individual cDNA expressed UGT isoforms, it was demonstrated that each active isoform displayed a reproducible and characteristic preference towards the formation of the two different glucuronides (Figure 1A-E), where UGT1A1 generated both in similar proportion, UGT1A9 and 1A7 showed selectivity for the tyrosine glucuronide, UGT1A10 showed selectivity for the ring C-4 glucuronide and UGT2B7 exhibited complete specificity for C4-G.

Relative levels of production of the two different glucuronides of NU/ICRF 505 by the clinical specimens. The pattern of glucuronide formation observed in incubations of both colon cancer biopsies and normal paired colon specimens with NU/ICRF 505, while likely to be a composite measure, produced a profile almost identical to that of UGT1A10. In this case, the C4-glucuronide was generated in large excess of Tyr-G, by a factor of 5.1±1.7 SD in the tumour biopsies and by 5.7±1.0 SD in the normal colon specimens compared to 4.6 for UGT1A10.

Glucuronidation activities present in human colon cancer biopsies and paired normal colon specimens. Glucuronidation activity was detected in 18 of the 19 colon cancer biopsies and all nine adjacent paired normal colon specimens, but with marked inter-patient variations, ranging from <1 to 300 pmol/min/mg depending on the substrate utilised. These rates of activity were in the same range as that observed with single UGT isozymes expressed in the cells in culture and, more significantly, were in the same range as that...
observed in HT29 cells where the UGTs were responsible for the expression of intrinsic drug resistance to anticancer drugs (7, 8).

The profiles of glucuronidation activity determined in the nine paired colon cancer and normal colon specimens towards the three different UGT probe substrates are shown in Figure 2A-C. Interestingly, in four out of nine patients higher rates of glucuronidation activity were measured in the tumour compared to the normal colon (patients 84, 95, 99 and 271, with the exception of patient 271 with propofol), while in the remaining five patients higher rates were recorded in the normal colon versus the tumour (patients 87, 94, 106, 125 and 461). Only low rates of activity were detected with the UGT2B7 substrate morphine (normally <1 pmol/min/mg, Figure 2A), in keeping with previous reports of more modest activity of the UGT2 family in the gastrointestinal tract (17, 33, 34), while by far the highest level of activity was recorded with 1-naphthol (Figure 2B). In addition to UGT1A6, which we have shown not to be capable of metabolising both SN-38 and NU/ICRF 505, UGT1A1 will accept 1-naphthol as a substrate (31). Intermediate activity was recorded with the UGT1A9 probe substrate propofol (1-10 pmol/min/mg, Figure 2C) (21). By comparison, rates of glucuronidation

Figure 1. Preferential formation of the two different glucuronide metabolites of NU/ICRF 505 (Tyr-G, 6.4 min and C4-G, 7.1 min) by cloned UGT isoforms. cDNA expressed cell sonicates or microsomes (100-200 µg of protein) were incubated with 150 µM of NU/ICRF 505 for 2 h and the formation of glucuronide metabolites determined by single ion recording (SIR) LC-MS. For the purposes of comparison, all five chromatograms were normalised to the most abundant chromatographic peak: A, UGT1A10; B, UGT1A7; C, UGT2B7; D, UGT1A9; E, UGT1A1.
activity with the anticancer drugs as substrates where comparatively high: 5-25 pmol/min/mg for the C-4 glucuronide of NU/ICRF 505.

Correlation between anticancer drug and isozyme probe substrate glucuronidation. When the tumour and normal colon profiles of glucuronide formation determined with NU/ICRF 505 and SN-38 were compared against that of the three probe substrates, interesting correlations emerged (Table I). The profile of formation of the C4-G of NU/ICRF 505 in the different colon cancer samples correlated strongly with the pattern of glucuronide formation obtained with propofol (see Table I). No other highly significant correlations were evident, suggesting that the isozyme responsible for metabolising propofol was also responsible for generating the major glucuronide metabolite of NU/ICRF 505. There was also a very strong correlation between the formation of SN-38 C10-G, NU/ICRF 505 Tyr-G and 1-naphthol, again suggesting that the same isozyme(s) were responsible for metabolising all three different compounds. In the normal colon a more complex picture emerged where formation of C4-G correlated with that of propofol glucuronide and Tyr-G; formation of Tyr-G correlated with that of propofol glucuronide, 1-naphthol glucuronide and C4-G, and formation of SN-38 glucuronide correlated with that of Tyr-G, 1-naphthol glucuronide and morphine 3-glucuronide (see Table I).

Kinetics of inhibition of NU/ICRF 505 glucuronide formation by propofol in human colon cancer biopsies. The kinetics of inhibition of NU/ICRF 505 glucuronide formation in the colon cancer biopsies by the UGT1A9 substrate propofol was investigated. Substrates of this isozyme often show cross reactivity with UGT1A7 and UGT1A10 (33), due to close sequence homology (35).

The IC\textsubscript{50} for inhibition of NU/ICRF 505 glucuronide formation by cloned UGT1A9 was 99 \textmu M for Tyr-G and 461 \textmu M for C4-G (Figure 3). UGT1A9 showed a strong preference for the formation of Tyr-G (see Figure 1). In HT29 cells, where propofol has been demonstrated to effectively inhibit NU/ICRF 505 metabolism (5, 36), the IC\textsubscript{50} for inhibition of Tyr-G was 94 \textmu M, consistent with UGT1A9 catalysing this metabolism. However, the IC\textsubscript{50} for inhibition of C4-G was 143 \textmu M, indicative of participation of other closely related UGTs in the metabolism of this drug, such as UGT1A7, 1A10 or even 1A8 which were not investigated in this study. In the colon cancer biopsies, up to a concentration of 500 \textmu M propofol was without effect on the formation of Tyr-G, powerful evidence against the involvement of the UGT1A7-10 series of isozymes.

Propofol effectively prevented the formation of C4-G in six out of eight biopsies investigated with an IC\textsubscript{50} ranging from 172-372 \textmu M, indicating participation of the UGT1A7-10 series, but with either a significant contribution or the exclusive participation of isozymes other than UGT1A9. In patient biopsies 271 and 461, propofol was without effect,
highlighting a significant inter-patient variation in UGT isozenzyme profiles or perhaps even the presence of a polymorphism, which has been reported for each member of this series (37-41).

**Discussion**

In the present study, a functional approach was utilised in an attempt to provide insights into the identity of the UGT isoforms responsible for metabolising anticancer drugs in colon cancer, utilising LC-MS and *in situ* incubations with patient biopsies. Three different strategies were pursued. Use was made of the fact that NU/ICRF 505 is converted into two different glucuronides (27) and that individual UGT isoforms produce a unique signature in their preference for the formation of each metabolite. A comparison was then made to the pattern of NU/ICRF 505 glucuronide formation observed in the incubations with colon tumour and normal tissue specimens. The next approach was to screen patient samples with UGT probe substrates and then correlate the activity profiles to those obtained with the two anticancer compounds. Third, the identity(ies) of UGT isoform(s) catalysing anticancer glucuronidation in the colon cancer biopsies was further clarified by studying the effect of an inhibitor of NU/ICRF 505 metabolism.

Data from a series of previous *in vitro* based analytical studies utilising cloned UGT isozymes demonstrated that only a limited number of isoforms are capable of metabolising both compounds, thus limiting the potential number of candidates in the present study. SN-38 is metabolised predominately by UGT1A1, 1A7 and 1A9 and to a lesser extent 1A10 (7, 10, 42-44); while, NU/ICRF 505 is metabolised predominately by UGT1A9 and 1A1, and to a lesser extent 1A10 (7).

Though pattern recognition data supported UGT1A10 as the isoform solely responsible for NU/ICRF 505 metabolism in both the tumour and normal samples, the probe substrate data indicated a far more complex picture. UGT1A10 was originally reported to be expressed predominantly in the colon (19), although it is now clear that this isoform is present throughout the gastrointestinal tract as well as the biliary canal (16, 17). UGT1A10 has also been demonstrated to be very active in the metabolism of the anticancer and immunosuppressant agent mycophenolic acid, while most other UGTs investigated had relatively little activity towards this drug (20). This protein is, therefore, a strong candidate as the UGT isoform primarily responsible for the expression of intrinsic resistance to mycophenolic acid in human colon cancer cells, including HT29 (45, 46) where intrinsic resistance to NU/ICRF 505 and SN-38 was first reported (5, 27). However, recent reports on the glucuronidation of mycophenolic acid suggested a more complicated picture implicating isoforms such as UGT1A9 and UGT2B7 (47, 48).

In the studies with probe substrates, corroborative evidence was obtained that a member of the UGT1A7-1A10 homologous series of isoforms (19) was responsible for the formation of the C4-G major metabolite of NU/ICRF 505 and SN-38 was first reported (5, 27). However, recent reports on the glucuronidation of mycophenolic acid suggested a more complicated picture implicating isoforms such as UGT1A9 and UGT2B7 (47, 48).

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**Table I.** *P* values for the degree of correlation between the patterns of glucuronidation activity measured in a panel of biopsies obtained using NU/ICRF 505 (C4-G and Tyr-G) and SN-38 (SN-38 C10-G) as substrates compared to that obtained using 3 different UGT isozyme probes as substrates.

<table>
<thead>
<tr>
<th>Probe (Isozyme selectivity)</th>
<th>Propofol (1A9, 7, 10)</th>
<th>Naphthol (1A6, 1)</th>
<th>Morphine (2B7)</th>
<th>C4-G</th>
<th>Tyr-G</th>
<th>SN-38 C10-G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human colon cancer biopsies</td>
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<tr>
<td>C4-G</td>
<td>0.0001*</td>
<td>0.06</td>
<td>0.03</td>
<td>-</td>
<td>0.17</td>
<td>0.02</td>
</tr>
<tr>
<td>Tyr-G</td>
<td>0.39</td>
<td>0.004</td>
<td>0.04</td>
<td>0.17</td>
<td>-</td>
<td>0.0004</td>
</tr>
<tr>
<td>SN-38 C10-G</td>
<td>0.07</td>
<td>0.002</td>
<td>0.02</td>
<td>0.02</td>
<td>0.0004</td>
<td>-</td>
</tr>
<tr>
<td>Adjacent normal colon specimens</td>
<td></td>
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<tr>
<td>C4-G</td>
<td>0.007*</td>
<td>0.03</td>
<td>0.09</td>
<td>-</td>
<td>0.00005</td>
<td>0.03</td>
</tr>
<tr>
<td>Tyr-G</td>
<td>0.006</td>
<td>0.002</td>
<td>0.03</td>
<td>0.00001</td>
<td>-</td>
<td>0.01</td>
</tr>
<tr>
<td>SN-38 C10-G</td>
<td>0.04</td>
<td>0.002</td>
<td>0.003</td>
<td>0.03</td>
<td>0.01</td>
<td>-</td>
</tr>
</tbody>
</table>

*Two data sets of glucuronidation activity obtained with two different substrates (e.g., SN-38 and 1-naphthol) consisting of 18 values for the tumour biopsies and nine values for the normal colon were graphed on an X,Y scatter plot using the computer programme Excel. The regression correlation coefficient was first determined and then the level of significance of the correlation calculated using Excel, and these latter data are presented in Table I.
Likewise, in the colon tumour specimens there was also a strong correlation between SN-38 C10-G formation and 1-naphthol. Since UGT1A6 does not accept either SN-38 or NU/ICRF 505 as a substrate, UGT1A1 may possibly emerge as the lead candidate. While there are numerous reports that implicate UGT1A1 in the hepatic metabolism and biliary clearance of SN-38 (9-11, 13, 50-52), the present study is one of the first reports implicating UGT1A1 in the metabolism of the drug and possible expression of intrinsic drug resistance, by colon cancer tissue. However, the fact that the same isoform may be responsible for both systemic drug detoxification and drug resistance might indicate that tumour selective modulation of this pathway would be more difficult to achieve. Nonetheless, selective modulation of SN-38 resistance mediated by glucuronidation may be possible in other tumour types. Recent evidence suggested that UGT1A10 is responsible for the expression of drug resistance to SN-38 in human lung cancer (44, 53).

The data generated using propofol as an inhibitor of NU/ICRF 505 glucuronidation by the tumour biopsies adds further support to the conclusion that one or more members of the UGT1A8-10 trio produce C4-G but not Tyr-G. However, the IC\textsubscript{50} values for inhibition of C4-G by propofol revealed two interesting observations. The first was that there appeared to be a significant inter-patient variation in the spectrum of isoforms expressed in the colon cancer biopsies. The second was that the isoform(s) present behaved differently to that of UGT1A9 in their response to enzyme inhibition. Thus, by a process of elimination, UGT1A10 and 1A8 remain as the main candidates responsible for the formation of the major metabolite of NU/ICF 505 in colon cancer tissue. Although, the pattern recognition data tends to favour UGT1A10, in the absence of analogous data on this isoform, UGT1A8 cannot be discounted. Therefore, it is not possible at this stage to distinguish between UGT1A8 and UGT1A10. However, since both these isoforms are not expressed in the liver (17, 19, 49), selective modulation of NU/ICF 505 glucuronidation (which is predominantly through C4-G) by colon tumour may be possible without adversely affecting systemic detoxification in the liver. The data generated with the probe substrates in the normal colon specimens suggested that multiple forms of UGTs catalyse the metabolism of both SN-38 and NU/ICRF 505, further supporting the contention that tumour-specific modulation of this drug may be achievable.

In summary, it is suggested that, in human colon cancer, SN-38 is glucuronidated predominantly by UGT1A1 while NU/ICRF 505 is mainly metabolised by UGT1A8 and/or UGT1A10.
References


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