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Cytotoxicity of rhein, the active metabolite of sennoside laxatives, is reduced by multidrug resistance-associated protein 1

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Anthranoïd laxatives, belonging to the anthraquinones as do anthracyclines, possibly increase colorectal cancer risk. Anthracyclines interfere with topoisomerase II, intercalate DNA and are substrates for P-glycoprotein and multidrug resistance-associated protein 1. P-glycoprotein and multidrug resistance-associated protein 1 protect colonic epithelial cells against xenobiotics. The aim of this study was to analyse the interference of anthranoids with these natural defence mechanisms and the direct cytotoxicity of anthranoids in cancer cell lines expressing these mechanisms in varying combinations. A cytotoxicity profile of rhein, aloe emodin and danthron was established in related cell lines exhibiting different levels of topoisomerases, multidrug resistance-associated protein 1 and P-glycoprotein. Interaction of rhein with multidrug resistance-associated protein 1 was studied by carboxy fluorescein efflux and direct cytotoxicity by apoptosis induction. Rhein was less cytotoxic in the multidrug resistance-associated protein 1 overexpressing GLC4/ADR cell line compared to GLC4. Multidrug resistance-associated protein 1 inhibition with MK571 increased rhein cytotoxicity. Carboxy fluorescein efflux was blocked by rhein. No P-glycoprotein dependent rhein efflux was observed, nor was topoisomerase II responsible for reduced toxicity. Rhein induced apoptosis but did not intercalate DNA. Aloe emodin and danthron were no substrates for MDR mechanisms. Rhein is a substrate for multidrug resistance-associated protein 1 and induces apoptosis. It could therefore render the colonic epithelium sensitive to cytotoxic agents, apart from being toxic in itself.

Keywords: anthranoids; multidrug resistance; apoptosis; colon; carcinogenesis

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The first panel consists of GLC4, GLC4/ADR, GLC4/VM, GLC4/AMSA and GLC4/P-gp. GLC4 is a human small cell lung carcinoma cell line and, by ongoing incubation, GLC4/ADR is its at this moment 345-fold in vitro acquired doxorubicin resistant subline. Resistance in the GLC4/ADR is due to MRP1 overexpression and to a reduced Topo II activity (Zaman et al, 1993; De Jong et al, 1991). GLC4/ADR is cultured with 1.2 μM doxorubicin twice weekly. In the in vitro acquired 3-fold ansamycin-resistant subline GLC4/AMSA and the 20-fold teniposide-resistant subline GLC4/VM of GLC4, resistance is due to reduced Topo IIβ and Topo IIα expression respectively without overexpression of MRP1 or P-gp (Withoff et al, 1996). The GLC4/P-gp subline has a 325-fold resistance to vincristine compared to GLC4. GLC4/P-gp was obtained after infection of GLC4 cells with an MDR1-gene-carrying retrovirus (Hendrikse et al, 1999). GLC4/P-gp cells were cultured twice weekly with 50 nM vincristine sulfa te to retain the transfect MDR1 gene.

The second panel of cell lines includes A2780, an ovarian carcinoma cell line, and 2780/AD its 100-fold in vitro acquired doxorubicin resistant P-gp overexpressing subline (Rogan et al, 1984). 2780/AD cells are cultured with 2.0 μM doxorubicin twice weekly. In addition two unrelated cell lines representing extreme inherent drug resistance and drug sensitivity, respectively, are used: the Caco-2 cell line, derived from a colon carcinoma (Fogh et al, 1977), and the N-Tera 2/D1 (Tera), an embryonal carcinoma cell line derived from a testicular tumour (Andrews et al, 1984).

All cell lines are routinely cultured in RPMI medium with 10% FCS. For the cell lines cultured in the presence of drugs, cells were cultured in drug-free medium for at least four passages before the start of the experiments.

Screening of anthranoid laxatives by cytotoxicity profile

The microculture tetrazolium (MTT) assay was used for determination of drug cytotoxicity as described earlier (Timmer-Bosscha et al, 1989). In 96-well plates (Nunc, Life Technologies, Paisley, UK), in 0.1 ml medium consisting of 40% HAM’s F12, 40% DME and 20% FCS different numbers of cells were used for each cell line in order to assure linearity of the assay. For GLC4, GLC4/AMSA, GLC4/VM and for Caco-2 (in 0.2 ml) 3750 cells, for GLC4/ADR and GLC4/P-gp 10 000 cells, for A2780 1250 cells, and for 2780/AD 5000 cells per well were incubated with increasing concentration that caused 50% cell kill (IC50) was determined in AMSA and GLC4/VM and for Caco-2 (in 0.2 ml) 3750 cells, for GLC4/ADR and GLC4/P-gp 10 000 cells, for A2780 1250 cells, and for 2780/AD 5000 cells per well were incubated with increasing concentrations of rhein, aloe emodin or danthron. Plates were kept at 37°C in a humidified atmosphere with 5% CO2. After 4 days incubation, 20 μl of MTT solution (3 mg MTT ml-1 phosphate buffered saline (PBS), (136 mM NaCl, 2.5 mM KCl, 6.5 mM Na2HPO4,2H2O, 1.5 mM KH2PO4, pH 7.2) was added. After 3.75 h incubation the plates were centrifuged (15 min, 180 g), supernatant was aspirated, 200 μl 100% DMSO added and extinction read at 520 nm (Timmer-Bosscha et al, 1989). The mean concentration that caused 50% cell kill (IC50) was determined in at least three independent experiments each performed in quadruplicate. The cytotoxicity assay with aloe emodin and danthron did not discriminate between GLC4 and GLC4/ADR or between A2780 and 2780/AD. Therefore these drugs were not studied further in the other cell lines.

The role of drug efflux mechanisms, modulation of cytotoxicity

In the presence or absence of MRP1-inhibitor MK571 (50 μM) rhein cytotoxicity was determined in GLC4 and GLC4/ADR. To determine the glutathione-dependency of rhein cytotoxicity (Müller et al, 1994), GLC4 and GLC4/ADR cells were preincubated with the glutathione synthesis inhibitor BSO, at a concentration of 25 μM 24 h before testing, which was shown before to reduce cellular glutathione to levels below 0.1 μg glutathione mg-1 cellular

MATERIALS AND METHODS

Chemicals

Rhein (9,10-dihydro-4,5-dihydroxy-9,10-dioxo-2-anthracenecarboxylic acid), aloe emodin (1,8-Dihydroxy-3-(hydroxymethyl)-anthraquinone), danthron (1,8-Dihydroxyanthraquinone), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide), DL-buthionine-(S,R)-sulphoximine (BSO) and protease XXIV were purchased from Sigma (St Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, foetal calf serum (FCS), Dulbecco’s modified eagle (DME) and Ham’s F12 (HAM) media were obtained from Life Technologies (Paisley, UK), dimethyl sulphoxide (DMSO) and acidine orange from Merck (Darmstadt, Germany). Rhein, aloe emodin and danthron were dissolved in DMSO. Doxorubicin was purchased from Pharmacia Upjohn (Woerden, The Netherlands) and vincristine sulphate-TEVA from Abic Ltd (Netanya, Israel). Carboxy fluorescein diacetate was obtained from Molecular Probes (Leiden, The Netherlands). Agarose I for electrophoresis was purchased from Amresco (Solom, Ohio) and ethidium bromide from Serva (Heidelberg, Germany). Dr Ford-Hutchinson, Merck Sharp, Canada kindly provided MK571.

Cell lines

Table 1 presents the cell lines used with their specific characteristics, according to which they were selected for the experiments.
Cytotoxicity of rhein is reduced by MRP1.

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Characteristics of different cell lines used in the experiments

| Relative doxorubicin-resistance | MDR-related mechanism involved in resistance | PS3 status |
|---------------------------------|---------------------------------------------|------------|
| GLC4                            |                                             | Wild-type  |
| GLC4/ADR*                       | 345, MRP, Topo IIa                        | Mutant     |
| GLC4/AMSA*                      | 5, Topo IIβ                               | Mutant     |
| GLC4/VMP*                       | 8, Topo IIα                               | Mutant     |
| GLC4/P-gp*                      | 12, P-gp                                   | Mutant     |
| A2780                           | 1                                          |            |
| 2780/AD*                        | 100, MRP, MRP, P-gp                        | Deletion   |
| Caco-2                          | 345, MRP, MRP                             | Wild-type  |
| Tera                            |                                             | Wild-type  |

*Relative to GLC4 (Withoff et al., 1996; Hendrikse et al., 1999), at the time of the experiments. Relative to A2780 (Rogan et al., 1984). Stephens et al., 2001.

The role of the drug efflux mechanisms, flow cytometric detection of carboxy fluorescein (CF) retention by rhein

Carboxy fluorescein diacetate (CFDA) is a nonfluorescent compound, which permeates the plasma membrane, and upon cleavage of the ester bonds by intracellular esterases, it is transformed into the fluorescent anion CF, which is a specific substrate for MRP1. The efflux of CF can be blocked specifically by the MRP1-inhibitor MK571 (Van der Kolk et al., 1998). The effect of rhein coinoculation on cellular CF efflux was studied in GLC4 and GLC4/ADR cells. Of each cell line 5 × 10⁵ cells were incubated in RPMI medium for 20 min at 37°C with CF 0.5 μM or vehicle only, or combined with 15 μM or 60 μM rhein, or with 50 μM MK571 as a control. Cells were washed with ice-cold RPMI medium and then resuspended in RPMI medium 37°C without CF but with rhein 15 or 60 μM or MK571 and kept for 1 h at 37°C to allow for CF efflux or CF efflux-blocking. Efflux was stopped by pelleting the cells and adding ice-cold RPMI medium. Fluorescence of CF was analysed with a FAC-Star flow cytometer (Becton and Dickinson, Sunnyvale, CA, USA) equipped with an argon laser. The CF fluorescence of 10,000 events was logaritically measured as a signal at an excitation wavelength of 488 nm through a 450 nm band-pass filter. The logaritically acquired signals were converted into linear values and expressed as relative fluorescence units using the WinList 2.0 program (Verity Software House, Inc., Topsham, ME, USA). Results are expressed as CF retention by the cells relative to the values found for CFDA plus MK571 coinoculation. All experiments were performed in triplicate.

Direct cytotoxicity of anthranoid laxatives, induction of apoptosis by rhein

To study the role of apoptosis susceptibility in anthranoid cytotoxicity, 10⁶ cells of GLC4, GLC4/ADR, Caco-2 and Tera cell lines were incubated with 100 μM rhein, and plated in PETRI dishes (6 cm diameter) in RPMI 1640 medium with 10% FCS. The GLC4 and GLC4/ADR were chosen because of different, probably efflux related, rhein induced cytotoxicity. As controls the Caco-2 a (colon) carcinoma cell line with a very low susceptibility to drug-induced apoptosis and Tera (embryonal carcinoma cell line) with a high propensity to go into apoptosis were used. In the Caco-2 cell line MRP1, P-gp and MRP2 (Buchler et al., 1996) are expressed (Stephens et al., 2001). In the Tera cell line no efflux pumps were detected (unpublished data). Besides, Caco-2 has a deleted and Tera a wild type p53 genotype, which may play a major role in their different drug sensitivities. After 6, 24, 48, 72 and 96 h acridine orange (1 mg ml⁻¹) was added to distinguish apoptotic from vital cells by fluorescence microscopy (Olympus IM) (Evans and Dive, 1993). All experiments were performed in triplicate. Relative apoptosis induction was calculated: the per cent apoptotic cells after incubation with 100 μM rhein at a certain time point is divided by the per cent apoptotic cells in an untreated control sample at the same time point.

Direct cytotoxicity of anthranoid laxatives, intercalation of DNA

Intercalation of anthranoids as a possible mechanism of action was studied by the unwinding of supercoiled plasmid DNA. Intercalation of a drug in supercoiled DNA leads to unwinding and this can be visualised by a reduced migration of plasmid DNA in an agarose gel (Hazelhurst et al., 1995). Supercoiled dimer of plasmid PBR322 was prepared from Escherichia coli strain HB101. Vials containing 3 μl PBR322, 9 μl tris-HCI-d-natrium-EDTA (TE) and 10 μl rhein, or aloe emodin, danthron or doxorubicin at different concentrations were incubated for 15 min at 37°C, 2 μl loading buffer was added to each vial and samples were put on a 1% agarose gel in tris-HCI-boric-acid-d-natrium-EDTA (TBE). Samples were run for 20 h at 20 V and stained afterwards with ethidium bromide (0.5 μg ml⁻¹). DNA bands were visualised by transillumination with UV and photographed using the Imagemaster with Fuji Thermal film (Pharmacia Biotech, Roosendaal, The Netherlands). All experiments were performed at least in triplicate.

Statistical analysis

The paired Student’s t-test was used for statistical analysis. Only P-values < 0.05 were considered significant.

RESULTS

Screening of anthranoid laxatives by cytotoxicity profile

The MTT assay was performed with three anthranoid laxatives in the GLC4, GLC4/ADR, A2780 and 2780/AD cells. For aloe emodin or danthron no difference in cytotoxicity was observed between GLC4 and GLC4/ADR or between A2780 and 2780/AD. Rhein however, was more toxic for the GLC4 than for GLC4/ADR and no difference in cytotoxicity could be demonstrated between GLC4 and the MDR1-transfected subline GLC4/P-gp (Table 2), whereas a distinct difference was seen in the cytotoxicity of vincristine (0.9 ± 0.2 and 283.2 ± 45.1 respectively, P < 0.002).

To define Caco-2, one of the control cell lines in apoptosis induction, it was incubated with rhein in the MTT assay. The rhein IC50 was 64.3 ± 11.6 μM (mean ± s.e.m.) which is comparable to GLC4/ADR.
The role of drug efflux mechanisms, modulation of cytotoxicity

Blockage of the MRP1-pump by coincubation with MK571 increased rhein toxicity in GLC4/ADR to the same level as that in GLC4 (Table 3, Figure 2). After preincubation with BSO, followed by a 4 day, continuous, drug incubation there was no difference in rhein cytotoxicity in GLC4 or GLC4/ADR (Table 3), while doxorubicin cytotoxicity in GLC4/ADR increased (IC_{50} 6.8 ± 1.9 and 5.0 ± 5.3 µM (mean ± s.e.m.) in GLC4/ADR and GLC4/ADR+BSO respectively, P<0.01). Experiments were repeated with 4 h drug incubation followed by a 4 day drug-free culture. Doxorubicin cytotoxicity increased during the 4 h incubation in GLC4/ADR after preincubation with BSO (IC_{50} 32.6 ± 5.7 and 8.4 ± 2.6 µM (mean ± s.e.m.) in GLC4/ADR and GLC4/ADR+BSO respectively, P<0.01). However, due to poor solubility of rhein, no discriminating cytotoxic effect of this drug could be tested in this experiment.

The role of drug efflux mechanisms, flow cytometric detection of CF retention by rhein

To test whether rhein was also able to functionally affect the MRP1 pump, GLC4 and GLC4/ADR cells were loaded with the fluorescent MRP1 substrate CF with and without 15 or 60 µM rhein or 50 µM MK571 and efflux was allowed for 1 h. In GLC4 and GLC4/ADR CF efflux was blocked in the presence of MK571. To correct for inter-experimental fluctuations in the FACS analyses CF cell concentrations in the presence of MK571 were defined as 100% retention and served as controls. Figure 3 shows the relative retention of CF after incubation with rhein 15 µM or 60 µM compared to controls in both GLC4 and GLC4/ADR. CF efflux was blocked by coincubation with rhein at 60 µM in GLC4 (P<0.05) and in GLC4/ADR (P<0.002). There was a trend for higher CF efflux, and

Table 2: Cytotoxicity profile of aloe emodin, danthron and rhein in the panel of GLC4-cell lines, GLC4/ADR, GLC4/AMSA, GLC4/VM and GLC4/P-gp and the panel of A2780 and 2780/AD cell lines as measured by MTT-assay, expressed as relative sensitivity compared to the parent cell line GLC4 and A2780 respectively (± s.e.m.)

| Cell line       | Aloe emodin | Danthron | Rhein     |
|-----------------|-------------|----------|-----------|
| GLC4            | 1.00 ± 0.15 | 1.00 ± 0.27 | 1.00 ± 0.06 |
| GLC4/ADR        | 1.26 ± 0.16 | 1.06 ± 0.27 | 1.61 ± 0.10 |
| GLC4/AMSA       | ND          | ND       | 1.25 ± 0.12 |
| GLC4/VM         | ND          | ND       | 1.18 ± 0.15 |
| GLC4/P-gp       | ND          | ND       | 1.07 ± 0.22 |
| A2780           | 1.00 ± 0.16 | 1.00 ± 0.21 | 1.00 ± 0.11 |
| 2780/AD         | 0.80 ± 0.11 | 0.71 ± 0.07 | 0.53 ± 0.04 |

*P<0.001 compared to GLC4, *P<0.05 compared to A2780, ND not done. IC_{50} GLC4: 23.4 ± 3.6 µM; IC_{50} A2780: 17.7 ± 2.8 µM; IC_{50} GLC4: 23.1 ± 6.2 µM; IC_{50} A2780: 22.8 ± 4.9 µM; IC_{50} GLC4: 41.4 ± 2.5 µM; IC_{50} A2780: 57.2 ± 6.3 µM.

Table 3: Effect of MK571 and BSO preincubation on rhein cytotoxicity in GLC4 and GLC4/ADR as measured by MTT-assay, expressed as relative sensitivity compared to the parent cell line GLC4 (± s.e.m.)

| Cell line       | Rhein     | Rhein+MK571 coinubation | Rhein+BSO coinubation |
|-----------------|-----------|-------------------------|-----------------------|
| GLC4            | 1.00 ± 0.09 | 0.99 ± 0.18               | 0.91 ± 0.16            |
| GLC4/ADR        | 1.50 ± 0.02 | 1.05 ± 0.13               | 1.54 ± 0.21            |

*P<0.005 compared to GLC4. *P<0.05 compared to GLC4/ADR. *P<0.05 compared to GLC4+BSO.

Figure 2: Survival curve of GLC4, GLC4/ADR, GLC4+MK571 and GLC4/ADR+MK571 after 4 days incubation with rhein in the MTT-assay. Increased toxicity (decreased survival) of rhein at IC_{50} in the GLC4/ADR cell line after inhibition of the MRP1-pump by MK571 (mean±s.e.m.). (P<0.05 compared to GLC4/ADR without MK571).

Figure 3: Cellular retention of CF in GLC4 and GLC4/ADR at different concentrations of rhein determined by flow cytometry. Retention of CF in GLC4 and GLC4/ADR in the presence of MK571 is defined as 100% retention and served as controls. CF retention in the presence of 15 µM or 60 µM rhein is expressed as relative to controls. In GLC4 complete retention of CF could be achieved at 60 µM rhein. In GLC4/ADR 72% CF retention is accomplished (mean±s.e.m.), *P<0.05 compared to CF in GLC4, **P<0.002 compared to CF in GLC4/ADR, ***P<0.05 compared to CF + Rhein 60 µM in GLC4/ADR.
thus less retention, in the GLC4/ADR cell line compared to GLC4 at 15 ($P=0.16$) and 60 μM rhein ($P=0.21$).

**Direct cytotoxicity of anthranoid laxatives, induction of apoptosis by rhein**

Figure 4 shows the relative rhein induced apoptosis. Apoptosis induction is found in the Tera cell line at 24 h and 96 h and a trend for apoptosis induction is also seen at 6 h ($P=0.053$). In the relative apoptosis resistant Caco-2 cell line at 48 h a significant induction of apoptosis is found. In GLC4/ADR after 96 h apoptosis was found while in GLC4 no significant induction was observed.

**Direct cytotoxicity anthranoid laxatives, intercalation of DNA**

No intercalation of either rhein, aloe emodin or danthron could be demonstrated at concentrations of 1, 10, 100 and 10,000 μM, whereas with doxorubicin a shift of plasmid DNA was already seen at concentrations of 1 and 10 nM.

**DISCUSSION**

Chronic use of anthranoid laxatives, such as senna, is considered to increase the risk of colorectal cancer (Siegers et al, 1993). In this study two routes which could be involved in this increase are studied, an effect of anthranoid laxatives on cellular efflux pumps reducing the physiological defence mechanisms of the colon epithelium and/or a direct cytotoxic effect of these agents on human cells.

With the use of related cell line panels this study demonstrates that the cytotoxicity profile of the active anthranoid laxative metabolite rhein is not homologue to that of doxorubicin in the panels of cell lines chosen. In the P-gp overexpressing cell line 2780/AD, the doxorubicin resistant subline of A2780, an increased sensitivity of 2780/AD for rhein was observed. This seems not to be related to increased expression of the P-gp drug efflux pump as in GLC4/ADR no increase in rhein cytotoxicity was found compared to GLC4. Although the mechanism of this differential sensitivity of A2780 and 2780/AD for rhein is unclear, one could speculate that it could be due to the antimitochondrial activity of rhein (Verhaeren, 1980; Bironaite et al, 1992). Increased sensitivity to mitochondrial inhibitors has been shown in another doxorubicin resistant cell line (Bironaite et al, 1992) and the phenomenon of enhanced toxicity in 2780/AD has been observed before for a number of resistance modifying agents (Schuurhuis et al, 1990).

As colorectal tumours are known to exhibit high levels of the P-gp protein (Fojo et al, 1987) and are intrinsically resistant to drugs involved in MDR it could be interesting to investigate whether a resistance modifying effect of rhein exists in human P-gp overexpressing colon carcinomas. Based on our study in cell lines the effect is not expected to be mediated through P-gp. Besides P-gp, MRP1 is found in normal colonic epithelium (Chaman et al, 1996) and colorectal tumours (Filipits et al, 1997). This drug efflux pump most likely protects colonic epithelial cells against xenobiotics induced damage (Shen et al, 1996) and is involved in the extrusion of several, structurally unrelated cytotoxic drugs including anthracyclines (Paul et al, 1996). In this study, we have demonstrated for the first time that rhein is a substrate for the MRP1 drug efflux pump. Cytotoxicity of rhein was reduced in the MRP1-overexpressing cell line GLC4/ADR compared to GLC4. The influence of alterations in topoisomerase II levels on this phenomenon was ruled out by experiments showing similar cytotoxicity of rhein in GLC4 compared to GLC4/AMSA and GLC4/VM, with reduced levels of topoisomerase IIβ and Iiz respectively. The degree of rhein resistance does not seem very high. However, in the same cell line only a 4.4-fold resistance is found for vincristine (Cole et al, 1994), a well established MRP1-substrate of which the cytotoxicity is not affected by topoisomerase reduction.

Modulation experiments revealed a complete reversal of resistance of the GLC4/ADR cell line to rhein by coincubation of the
cells with rhein and MK571, a leukotriene receptor antagonist (Paul et al, 1996) and MRP1 blocker.

Apart from in vitro and ex vivo experiments efflux experiments with the fluorescent MRP1 substrate CF also showed that rhein is a substrate for MRP1. Rhein was, in a comparable concentration to MK571 (Van der Kolk et al, 1998), a blocker of CF efflux.

Transport function for several drugs of MRP1 is dependent on cellular glutathione levels (Versantvoort et al, 1995). Cellular depletion of glutathione can be achieved by preincubation with BSO, a substance that specifically inhibits glutathione synthesis in cells (Meister, 1991). It was previously shown that preincubation of GLC4 and GLC4/ADR cells with 25 μmol BSO for 24 h reduced GSH levels effectively (Meijer et al, 1987). In this study we confirmed this by the increased toxicity of doxorubicin in GLC4/ADR cells. However, no effect of BSO preincubation on rhein toxicity was found. Although MRP1 has repeatedly been reported either to transport glutathione conjugates itself or to activate an endogenous glutathione-conjugate pump (Müller et al, 1994), it is not clear whether glutathione conjugation or co-transport are always needed for MRP1 activity (Paul et al, 1996; Broxtermann et al, 1995).

Recently, it was shown that cellular glutathione depletion did not affect MRP1-dependent extrusion of the acetoxy methyl ester of calcine or free calcine either (Holló et al, 1996). In contrast to our study, in an in vitro study with rat hepatocytes increased rhein cytotoxicity was found after 5 h preincubation with 0.5 mM BSO (Bironaite and Öllinger, 1997). Although it cannot be excluded that the 20-fold higher BSO concentrations used in that study compared to our study is toxic in itself for rat hepatocyte cells, evaluation of any short-lasting (4 h) effect of 24 h BSO preincubation could not be determined in our experimental design, as the solubility of rhein did not allow high enough concentrations to induce cytotoxicity.

Induction of carcinomas by anthranoid laxatives have been demonstrated in animal studies (Mori et al, 1985) and mutagenic and genotoxic effects were described in bacterial strains (Westendorf et al, 1990; Brown and Brown, 1976). Mutations occurred predominantly in those strains sensitive to frame-shifting mutagens (Brown and Dietrich, 1979), which suggest that anthranoids might achieve DNA-damage probably by intercalation. In the present study no DNA-intercalating capacity of rhein, aloe emodin or danthon would be demonstrated in the bacterial plasmid DNA pBR322.

Anthranoid laxatives have been shown to induce apoptosis of colonic epithelial cells in man within 6 h after ingestion (Van Gorkom et al, 2001). Animal studies also demonstrated increased apoptosis 6 h after danthon administration (Walker et al, 1988). In our in vitro study, although little apoptosis was found in the GLC4 and GLC4/ADR cell lines rhein induced apoptosis in the control cell lines. These different effects are more likely to be cell type than resistance related. In the apoptosis sensitive cell line Tera the highest apoptosis induction was found. Caco-2, a colon carcinoma cell line which is relatively insensitive to apoptosis-induction by doxorubicin, readily underwent apoptosis induction after 48 h incubation with 100 μmol rhein. This concentration is below the concentration reached in the colonic lumen after therapeutic doses of anthranoid laxatives used as a bowel preparation for diagnostic procedures (Schörkhuber et al, 1998). Single doses of 0.15 g sennosides corresponding to 0.5 mmol rhein anthrone in approximately 2 litre colonic contents yield concentrations of about 250 μmol.

In conclusion, this study demonstrates that rhein, the active metabolite of sennoside laxatives, is a substrate for the MRP1 drug efflux pump and is a cytotoxic agent capable of inducing apoptosis. This cytotoxicity is neither topoisomerase II related nor a result of DNA intercalation. The capacity to extrude the anthranoid laxative may be important to protect the epithelium. Evaluation of colonic MRP1 levels in chronic users of anthranoid laxatives can be of interest. It may well be that the risk to develop colon cancer in anthranoid laxative users is negatively related to MRP1 expression. In addition also mutations or polymorphisms in MRP1 may play a role.

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REFERENCES
Andrews PW, Damjanov I, Simon D, Banting GS, Carin C, Dracopoli NC, Fogh J (1984) Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera 2. Differentiation in vitro and in vitro. Lab Invest 50: 147 – 162
Bironaite D, Öllinger K (1997) The hepatotoxicity of rhein involves impairment of mitochondrial functions. Chem Biol Interact 103: 35 – 50
Bironaite DA, Cenas NK, Anusevicius ZJ, Medentsev VK, Akimenko VK, Usanov SA (1992) Fungal quinone pigments as oxidizers and inhibitors of NADH:ubiquinone reductase. Arch Biochem Biophys 297: 253 – 257
Breuninger LM, Paul S, Gaughan K, Miki T, Chan A, Aaronson SA, Kruh GD
Brown JP (1980) A review of the genetic effects of naturally occurring flavonoids. Mutat Res 75: 213 – 39
Brown JP, Dietrich PS (1979) Mutagenicity of anthraquinone and benzanthrone derivatives in the Salmonella/microsome test: Activation of anthraquinone glycosides by enzymic extracts of rat cecal bacteria. Mutat Res 66: 9 – 24
Broxtermann HJ, Giaccone G, Lankelma J (1995) Multidrug resistance proteins and other drug transport-related resistance to natural product agents. Curr Opin Oncol 7: 532 – 540
Buchler M, Konig J, Brom M, Kartenbeck J, Spring H, Horie T, Keppler D (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. J Biol Chem 271: 15091 – 15098
Chaman Y, Sumizawa T, Takebayashi Y, Niwa K, Yamada K, Haraguchi M, Furukawa T, Akiyama S-I, Aikou T (1996) Expression of the multidrug-resistance-associated protein (MRP) gene in human colonic, gastric and non-small cell lung carcinomas. Int J Cancer 66: 274 – 279
Cole SPC, Sparks KE, Fraser K, Lou DW, Grant CE, Wilson GM, Deeleey RG (1994) Pharmacological characterization of multidrug resistant MRP1-transfected human tumor cells. Cancer Res 54: 5902 – 5910
De Jong S, Zijlstra JD, Mulder NH, de Vries EGE (1991) Lack of cross-resistance to MMPI of anthranoid laxatives used as a bowel preparation for diagnostic procedures. Cancer Res 51: 15091 – 15098
Evans DF, Dive C (1993) Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. Cancer Chemother Pharmacol 28: 461 – 464
Defife AM, Batra JK, Goldenberg GI (1989) Direct correlation between DNA topoisomerase II activity and cytotoxicity in adriamycin-sensitive and resistant P388 leukemia cell lines. Cancer Res 49: 58 – 62
Eugene DF, Dive C (1993) Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. Cancer Res 53: 2133 – 2139
Filipits M, Suchomel RW, Deken G, Stiglauer W, Haider K, Depisch D, Pirker R (1997) Expression of the multidrug resistance-associated protein (MRP1) gene in colorectal carcinomas. Br J Cancer 75: 208 – 212
De Jong S, Zijlstra JD, Mulder NH, de Vries EGE (1991) Lack of cross-resistance to MMPI of anthranoid laxatives used as a bowel preparation for diagnostic procedures. Cancer Res 51: 15091 – 15098
Evans DF, Dive C (1993) Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. Cancer Res 53: 2133 – 2139
Filipits M, Suchomel RW, Deken G, Stiglauer W, Haider K, Depisch D, Pirker R (1997) Expression of the multidrug resistance-associated protein (MRP1) gene in colorectal carcinomas. Br J Cancer 75: 208 – 212

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Cytotoxicity of rhein is reduced by MRP1.

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Schuurhuis GJ, Pinedo HM, Broxterman HJ, Van Kalken CK, Kuiper CM, Lankelma J (1990) Differential sensitivity of multi-drug-resistant and -sensitive cells to resistance-modifying agents and the relation with reversal of anthracycline resistance. Int J Cancer 46: 330 – 336

Shen H, Paul S, Breuninger LM, Ciaccio PJ, Laing NM, Helt M, Tew KD, Kruh GD (1996) Cellular and in vitro transport of glutathione conjugates by MRP. Biochemistry 35: 5719 – 5725

Siegers CP, Von Hertzberg-Lottin E, Otte M, Schneider B (1993) Anthranoid laxative abuse - a risk for colorectal cancer? Gastroenterology 34: 1099 – 1101

Steer HW, Colin-Jones DG (1975) Melanosis coli: studies of the toxic effects of irritant purgatives. J Pathol 115: 199 – 205

Stephens RH, O’Neill CA, Warhurst A, Carlson M, Rowland M, Warhurst G (2001) Kinetic profiling of P-glycoprotein-mediated drug efflux in rat and human intestinal epithelia. J Pharm Exp Ther 296: 584 – 591

Stewart CF, Ratain MJ (2001) Topoisomerase interactive agents. In Cancer. Principles & practice of oncology, 6th edition DeVita VT, Hellman S, Rosenberg SA (eds), pp 415 – 459 Philadelphia: Lippincott, Williams & Wilkins

Timmer-Boscha H, Hopsers GAP, Meijer C, Mulder NH, Muskiet FAJ, Martini IA, Uges DRA, de Vries EGE (1989) Influence of docosahexaenoic acid on cisplatin resistance in a human small cell lung carcinoma cell line. J Natl Cancer Inst 81: 1069 – 1075

Van der Kolk DM, de Vries EGE, Koning JA, van den Berg E, Muller M, Vellenga E (1998) Activity and expression of the multidrug resistance proteins MRP and MRP, in acute myeloid leukemia cells, tumor cell lines, and normal hematopoietic CD34+ peripheral blood cells. Cln Cancer Res 4: 1727 – 1736

Van Gorkom BAP, Karrenbeld A, van der Sluis T, Zwart N, de Vries EGE, Kleibeuker JH (2001) Apoptosis induction by sennoside laxatives in man; escape from a protective mechanism during chronic sennoside use? J Pathology 194: 492 – 498

Verhaeren EHC (1980) The uncoupling activity of substituted anthracene derivatives on isolated mitochondria from Phaseolus aureus. Phytochem 19: 501 – 503

Versantvoort CHM, Broxterman HJ, Bagri T, Scheper RJ, Twentymain PR (1995) Regulation by glutathione of drug transport in multidrug resistant human tumor cell lines overexpressing MRP. Br J Cancer 72: 82 – 89

Walker NJ, Bennett RE, Axelsen RA (1988) Melanosis coli. A consequence of anthraquinone-induced apoptosis of colonic epithelial cells. Am J Pathol 131: 465 – 476

Westendorf J, Marquardt H, Piggins B, Dominick M, Schmidt J, Marquardt H (1990) Genotoxicity of naturally occurring hydroxyanthraquinones. Mutat Res 240: 1 – 12

Witthoff S, de Vries EGE, Keith WN, Niemhuis EF, Van der Graaf WTA, Uges DRA, Mulder NH (1996) Differential expression of DNA topoisomerase I Ia and β in P-gp and MRP negative VM26, mAMSA and mitoxantrone resistant sublines of the human SCLC cell line GLC4. Br J Cancer 74: 1869 – 1876

Zaman GJ, Versantvoort CH, Smit JJ, Eijdemans EW, de Haas M, Smith AJ, Broxterman HJ, Mulder NH, de Vries EGE, Baas F, Borst P (1993) Analysis of the expression of MRP1, the gene for a new putative transmembrane drug transporter, in human multidrug resistant lung cancer cell lines. Cancer Res 53: 1747 – 1750

Fogh J, Wright WC, Loveless JD (1977) Absence of Hela cell contamination in 169 cell lines derived from human tumors. J Natl Cancer Inst 58: 209 – 214

Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesman MM, Pastan I (1987) Expression of a multidrug-resistance gene in human tumors and tissues. Proc Natl Acad Sci USA 84: 265 – 269

Gamen S, Anel A, Laiastra P, Alava MA, Martinez-Lorenzo MJ, Piñeiro A, Naval J (1997) Doxorubicin-induced apoptosis in human T-cell leukemia is mediated by caspase-3 acativation in a Fas-independent way. FEBS Lett 417: 360 – 364

Goldstein LJ (1996) MDR1 gene expression in solid tumors. Eur J Cancer 32A: 1039 – 1050

Hazehurst LA, Krakojo AP, Hacker MP (1995) Correlation of DNA reactivity and cytotoxicity of a new class of anticancer agents: azanthracenediones. Cancer Lett 91: 115 – 124

Hendrikse NH, de Vries EGE, Eriks-Fluks E, van der Graaf WTA, Hospers GAP, Willemsen ATM, Vaalburg W, Franssen EJF (1999) A new in vivo method to study P-glycoprotein transport in tumors and the blood-brain barrier. Cancer Res 59: 2411 – 2416

Hollo Z, Homolya L, Hegedüs T, Sarkadi B (1996) Transport properties of the multidrug resistance-associated protein (MRP) in human tumour cells. FEBS Lett 383: 99 – 104

Hu XF, Slater S, Wall DM, Kantharidis P, Parkin JD, Cowman A, Zalberg J (1995) Rapid upregulation of MDR, expression by anthacyclines in a classical multidrug-resistant cell line. Br J Cancer 71: 931 – 936

Kleibeuker JH, Cats A, Zwart N, Mulder NH, Hardonk MJ, de Vries EGE (1995) Excessively high cell proliferation in sigmoid colon after an oral purge with anthraquinone glycosides. J Natl Cancer Inst 87: 452 – 453

Meijer C, Mulder NH, Timmer-Boscha H, Zijlstra JG, de Vries EGE (1987) Role of free radicals in an adriamycin-resistant human small cell lung cancer cell line. Cancer Res 47: 4613 – 4617

Meister A (1991) Glutathione deficiency produced by inhibition of its synthesis and its reversal; applications in research and therapy. Pharmacol Ther 51: 155 – 194

Mori H, Sugie S, Niwa K, Takahashi M, Kawai K (1985) Induction of intestinal tumours in rats by chrysazin. Br J Cancer 52: 781 – 783

Muller M, Meijer C, Zaman GJR, Borst P, Scheper RJ, Mulder NH, de Vries EGE, Jansen PLM (1994) Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. Proc Natl Acad Sci USA 91: 13033 – 13037

Paul S, Breuninger LM, Tew KD, Shen H, Kruh GD (1996) ATP-dependent uptake of natural product cytotoxic drugs by membrane vesicles establishes MRP as a broad specificity transporter. Proc Natl Acad Sci USA 93: 6929 – 6934

Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE (1990) Increased cell division as a cause of human cancer. Cancer Res 50: 7415 – 7421

Rogan AM, Hamilton TC, Young RC, Klecker RW, Ozols RF (1984) Reversal of adriamycin resistance by verapamil in human ovarian cancer. Science 224: 994 – 996

Schorkhuber M, Richter M, Dutter A, Sontag G, Marian B (1998) Effect of anthraquinone-laxatives on the proliferation and urokinase secretion of normal, premalignant and malignant colonic epithelial cells. Eur J Cancer 34: 1091 – 1098