Morphological characterisation of the cell-growth inhibitory activity of rooperol and pharmacokinetic aspects of hypoxoside as an oral prodrug for cancer therapy

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Hypoxoside is the major diglucoside isolated from the corms of the plant family Hypoxidaceae. It contains an unusual E-pent-1-en-4-yne 5-carbon bridging unit with two distal catechol groups to which the glucose moieties are attached. It is non-toxic for BL6 mouse melanoma cells in tissue culture on condition that the fetal calf serum in the medium is heat-inactivated for 1 hour at 56°C in order to destroy endogenous beta-glucosidase activity. The latter catalyses hypoxoside conversion to its cytotoxic aglucone, rooperol, which, when tested as a pure chemical, caused 50% inhibition of BL6 melanoma cell growth at 10 μg/ml. Light and electron microscopy revealed that the cytotoxic effect of rooperol manifested as vacuolisation of the cytoplasm and formation of pores in the plasma membrane. Indications of apoptosis were also found.

Pharmacokinetic studies on mice dosed intragastrically with hypoxoside showed that it was deconjugated by bacterial beta-glucosidase to form rooperol in the colon. Surprisingly, no hypoxoside or rooperol was detectable in the serum. Only phase II biotransformation products (sulphates and glucuronides) were present in the portal blood and bile. In contrast, however, in human serum after oral ingestion of hypoxoside, the metabolites can reach relatively high concentrations.

Rooperol metabolites isolated from human urine were non-toxic for BL6 melanoma cells in culture up to a concentration of 200 μg/ml. In the presence of beta-glucuronidase, which released rooperol from the metabolites, 50% growth inhibition was achieved at a 75 μg/ml metabolite concentration. The supernatant of a human melanoma homogenate could also cause deconjugation of the metabolites to form rooperol.

It can be concluded from these findings that rooperol has promising properties as an oral prodrug for cancer therapy in humans given its complete first-pass metabolism into non-toxic conjugates which may be activated in tumours with high deconjugase activity. Rodent cancer models are, however, not applicable since rooperol metabolites are completely sequestered in the bile.

Up until now, cancer chemotherapy has been a disappointing trade-off between efficacy and toxicity because of the lack of tumour cell selective toxicity. No chemical agent has yet been discovered that selectively eradicates cancer cells without harming normal cells. One approach to the solving of this intractable problem would be the use of a non-toxic prodrug that is selectively activated to become cytotoxic in the immediate vicinity of cancer cells. Although we do not profess to have found such an ideal agent, we wish to report our findings on the plant diglucoside, hypoxoside, which has some features that we believe point in the right direction.

Hypoxoside is the trivial name for (E)-1, 5-bis(4'-β-D-glucopyranosyloxy-3'-hydroxyphenyl) pent-4-en-1-yne (CAS Reg. No. 83643-94-1), which is a norlignan diglucoside isolated from the corms of Hypoxis plants of the family Hypoxidaceae.[1,2] It is a pale yellow water-soluble crystalline compound[3] and is readily converted to the more lipophilic aglucone, rooperol, by beta-glucosidase action.[1,2,4] This conversion is shown schematically in Fig. 1.
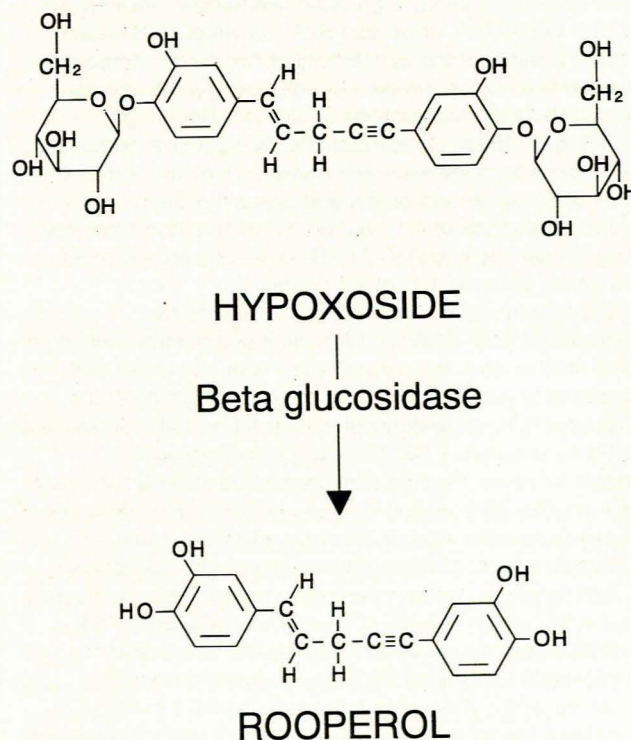

HYPOXOSIDE

Beta glucosidase

ROOPEROL

Fig. 1. The inactive diglucoside, hypoxoside, is deconjugated by beta-glucosidase to form the cytotoxic and lipophilic aglucone, rooperol.

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We have isolated hypoxoside from the corms of members of the family Hypoxidaceae, especially *H. rooperi* and *H. acuminata*.[4] The plants are herbaceous perennials with tuberous rhizomes or corms (up to 10 cm in diameter or length and weighing 2 kg) and abundant adventitious roots that enable them to survive under high-stress conditions such as winter drought and fire. Traditional uses in folk medicine have been reported, *inter alia* for the treatment of cancers, testicular tumours, prostate hypertrophy and urinary disease.[5] Members of the Hypoxidaceae family are found mainly in the southern hemisphere, especially in Africa.

A high-performance liquid chromatography (HPLC) purified sample of hypoxoside (compound NSC 61783) was submitted to the National Cancer Institute (NCI), Bethesda, USA, for evaluation (M. R. Boyd — Research report: *In vitro* screening data review checklist, compound NSC: 613783, Experiment ID: 8909NS63, Date tested: 10.4.89) using the new investigational *in vitro* disease-orientated primary antitumour screen.[6] The compound was found to inhibit the growth of all 60 human cancer cell lines tested. The mean concentration giving a 50% inhibition of growth was 8,0 µg/ml. One non-small-cell lung cancer cell line (NCI-H522) was about 14 times more sensitive than the average for all the cell lines tested and showed 50% inhibition of growth at 0,6 µg/ml and complete inhibition at 1,8 µg/ml. Sub-panels of non-small-cell lung cancer and melanoma cells showed a statistically significant measure of differential sensitivity in respect of 50% growth inhibition compared with the other sub-panels tested.

HPLC-purified hypoxoside was also evaluated by the Huntingdon Research Centre, England, by means of an *in vitro* clonogenic assay employing five tumour cell lines of human origin (P. F. Uphill and S. A. Crowther — Research report: A study of the cytotoxicity of two novel compounds against five human tumour cell lines using a clonogenic assay. Huntingdon Research Centre Ltd, Report INI 26/851646, 1986). Concentrations giving 50% inhibition (ID_{50}) of colony formation with cells derived from carcinomas of the colon, uterus and breast and with a melanoma cell line were in the range of 4,1 - 8,2 µg/ml, while a non-small-cell lung cancer cell line (ATCC HTB 53; A-427) proved most sensitive, giving an ID_{50} of 1,1 µg/ml.

Experiments at our laboratories confirmed that hypoxoside was cytotoxic for murine and human melanoma cells lines *in vitro*, but surprisingly it was found that this cytotoxicity was dependent on the deconjugation of the diglucoside by an endogenous, heat-labile, beta-glucosidase in fetal calf serum (FCS) to form the toxic aglucone, rooperol.[7] When the FCS was pre-heated at 56°C for 1 hour or more this enzyme activity was abolished and 100 µg/ml of hypoxoside were without cytotoxic effect but could be activated by the addition of exogenous beta-glucosidase.

Consequently, we surmise that in experiments conducted at the NCI and Huntingdon Research Laboratories, the reported cytotoxicity was caused by the conversion of hypoxoside to rooperol because the researchers were unaware of the endogenous beta-glucosidase in FCS. Therefore, evidence that hypoxoside itself was cytotoxic was erroneous in that the real cytotoxic moiety was the aglucone, rooperol.

In this report we first present morphological data on the cytotoxic effect of rooperol on melanoma cells in culture and then focus on the metabolism of orally ingested hypoxoside in mice, compared with humans. The data support a possible mechanism whereby hypoxoside could act as a latent prodrug for selective cancer therapy in humans.

Materials and methods

Materials

The isolation of hypoxoside, preparation of rooperol and human urinary metabolites of rooperol were done as described earlier.[4] Beta-glucuronidase was purchased from Seravac (South Africa).

B16-F10-BL6 mouse melanoma cells[8] were a gift from Dr I. J. Fidler, Department of Cell Biology, MD Anderson Hospital and Tumor Institute, Houston, Texas; the UCT-Mel 1 human melanoma cells were a gift from Dr L. Wilson of the Department of Clinical Science, University of Cape Town.[9]

C57BL/6J mice came originally from Jackson Laboratories, Ann Arbor, Michigan and were subsequently inbred at the Research Institute for Nutritional Diseases of the MRC in Tygerberg.

Morphological studies

Mouse (BL6) and human (UCT-Mel 1) melanoma cells were cultured in 24 multi-well plates (Falcon) at 37°C in a humidified 5% CO_2 atmosphere in air in an automatically controlled CO_2 incubator (Forma). The culture medium containing 1 ml of McCoy's 5A medium (Flow Laboratories) was supplemented with 10% FCS (Gibco) which had been heated at 56°C for 30 minutes, 100 µg/ml streptomycin, 100 µ/ml penicillin and sodium bicarbonate (2,25 g/l). Twenty-four hours after plating, the cells were exposed to a final concentration of 50 µg/ml of hypoxoside and photographed every 2 hours for 24 hours with an inverted phase-contrast microscope attached to a camera (Nikon).

For transmission electron microscopy, cells were cultured in 25 cm² flasks (Falcon). After 24 hours' exposure to 50 µg/ml hypoxoside they were released with trypsin-EDTA, centrifuged at 500 g for 10 minutes and fixed in 2,5% glutaraldehyde in 0,05M sodium phosphate buffer, pH 7,2 for 4 hours, postfixed in 1% osmium tetroxide for 2 hours, washed in distilled water and stained in 5% aqueous uranyl acetate; this was followed by dehydration through a series of graded ethanol solutions, before cells were cleared in propylene oxide and embedded in an Epon/Araldite mixture. Ultrathin sections were picked up on uncoated copper grids, stained in lead citrate for 1 - 2 minutes and examined with an Hitachi H-300 electron microscope.

For scanning electron microscopy, cells were grown on glass coverslips which had been rinsed in ethanol, dried and pre-incubated for 24 hours in 100% FCS. After 24 hours' exposure to 50 µg/ml hypoxoside, the coverslips with attached cells were gently rinsed in phosphate-buffered saline and fixed for 2 hours at 4°C in 2,5% glutaraldehyde buffered at pH 7,2 in 0,1M phosphate buffer. The coverslips were dehydrated through a graded series of ethanol solutions, critically dried and coated with gold-platinum according to standard practice. Cells were viewed with a Cambridge 180 Stereoscan microscope.

Growth inhibition studies

The effect of human urinary rooperol metabolites on the growth of BL6 melanoma cells was tested in 96 multiwell plates, plated at 5×10^3 cells in 200 µl of McCoy's 5A medium (Flow Laboratories, Ayrshire, Scotland) with 10% of FCS which was heat-inactivated. Exogenous beta-glucuronidase was added to a final concentration of 100 µg/ml. After incubation for 72 hours, viable cells in each well were quantified by an adaption of the tetrazolium assay described by Denizot and Lang[10] as previously outlined.[7]

Pharmacokinetic studies

Hypoxoside (3 mg in 0,2 ml normal saline) was administered intragastrically to adult C57BL/6J male mice 3 hours prior to necropsy. Samples were collected and prepared for HPLC analyses by mixing with guanidine hydrochloride pretreatment reagent (PT) according to the methodology developed by Kruger et al.[11] Urine (20 µl) was mixed with 480 µl PT; 70 µl serum with 430 µl PT; bile was removed from the gall bladder with a 26-gauge needle attached to a 1 ml tuberculin syringe containing 500 µl PT; 1 g faeces was extracted with 12 ml methanol and 100 µl were mixed with 400 µl PT. Heparinised portal blood was collected from 9 mice given 12 mg hypoxoside intragastrically 3 hours prior to necropsy; the plasma was lyophilised, extracted with 5 ml methanol which was evaporated under a stream of nitrogen, the residue dissolved in 50 µl methanol and mixed with 450 µl PT reagent.

Tumour sample

A biopsy (100 mg) of an advanced human melanoma of the lower leg was homogenised in 1 ml of 0,1M sodium acetate buffer pH 5,5 and centrifuged at 3 000 rpm for 10 minutes. Fifty micrograms human rooperol metabolite A, a diglucuronide of rooperol,[4] were added to 0,5 ml of the supernatant and incubated for 20 hours at 37°C. One hundred microlitres of the incubation mixture were analysed by HPLC as described.[11]

Results

Morphological studies

With the use of phase contrast microscopy, destruction of BL6 and UCT-Mel 1 melanoma cells could be visualised over a period of 24 hours after 50 µg/ml of hypoxoside were added to medium containing FCS that was heat-inactivated for only 30 minutes. The earliest signs of aberrant morphology in the BL6 cells occurred after about 12 hours when it was noticed that the majority of cells assumed a flattened appearance with vacuoles forming 'empty spaces' in the cytoplasm of about 10% of the cells. At the same time the chromatin of round and shiny cells undergoing mitosis appeared amorphous, while in control cells without hypoxoside distinct metaphase chromosome plates could be discerned (Fig. 2A v. Fig. 2B; broad arrows). After about 16 hours more than 50% of the cells showed vacuoles in the cytoplasm (Fig. 2C) and after 24 hours most of the cells

attached to the substrate contained large vacuoles and appeared to be disintegrating (Fig. 2D; fine arrows). The rounded cells appeared intact with amorphous chromatin clumps in the centre (Fig. 2D; broad arrows).

Transmission electron microscopy of melanoma cells 24 hours after exposure to 50 µg/ml of hypoxoside showed various features. Most irregular cells showed vacuoles in the cytoplasm (Fig. 3A; arrows) which either appeared to be empty or contained amorphous material (Fig. 3B). Mitochondria appeared to be empty or contained disintegrating cristae (Fig. 3C; arrows). Some cells had a condensed cytoplasm and fragmented nucleus (Fig. 3D) while others had an accentuated irregular outline (Fig. 3E) or a very smooth outline with condensed cytoplasm and chromatin with numerous vacuoles, of which two appeared to cause holes to form in the outer membrane (Fig. 3F).

Scanning electron microscopy showed rounded control human UCT-Mel 1 melanoma cells attached to the glass substrate by thin microvilli, which also covered the surface of the cell (Fig. 4A). Similar cells exposed to hypoxoside, which was deconjugated to form rooperol by the endogenous beta-glucosidase, showed a smooth exterior apparently punctured by holes 0,5 - 2 µm in diameter (Fig. 4B foreground, long arrow), or with detaching cytoplasmic protrusions (Fig. 4B background, short arrows).

Pharmacokinetic studies

HPLC analyses of mouse serum and urine 3 hours after epigastric dosing of 3 mg hypoxoside showed no hypoxoside, rooperol or any new peaks with the characteristic UV absorption spectrum of hypoxoside. Of particular interest, however, was the finding that methanol extracts of faeces showed that hypoxoside had been deconjugated to form rooperol (Fig. 5). Identification of the peaks in Fig. 5 was described by Kruger et al.[4] Bile showed three new fractions with hypoxoside-like UV absorbance spectra (Fig. 6) comparable to those found previously in human serum by Kruger et al.[4] The main peak (B) found in the bile was also present in portal blood of mice dosed epigastrically with 12 mg hypoxoside 3 hours prior to necropsy. No rooperol was detected in the portal blood.

Studies with human metabolites of rooperol

Human urinary metabolites of rooperol comparable to those found in the bile of mice shown in Fig. 6 were isolated with C_{18}-bonded silica[4] and incubated with BL6 mouse melanoma cells in the presence and absence of exogenous beta-glucuronidase. Fig. 7 shows that in a concentration of up to 200 µg/ml the urinary metabolites had no inhibitory effect on cell proliferation, while in the presence of 100 µg/ml of added beta-glucuronidase, 50% growth inhibition occurred at about 75 µg/ml of the metabolites. HPLC analyses of the tissue culture fluid 72 hours after incubation showed that without beta-glucuronidase the metabolite pattern remained unchanged (Fig. 8; upper chromatogram) while in the presence of the enzyme a major shift occurred in terms of the disappearance of the main metabolite peaks and the appearance of new peaks with longer retention times (D, E, F, G and H in the lower chromatogram of Fig. 8).
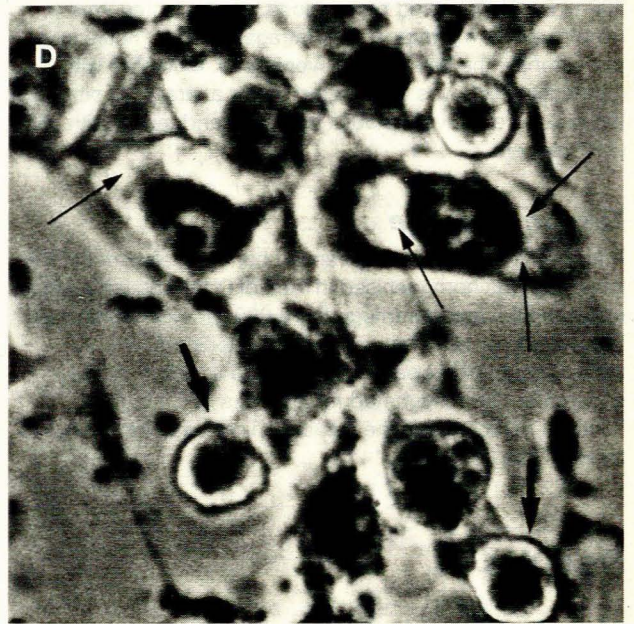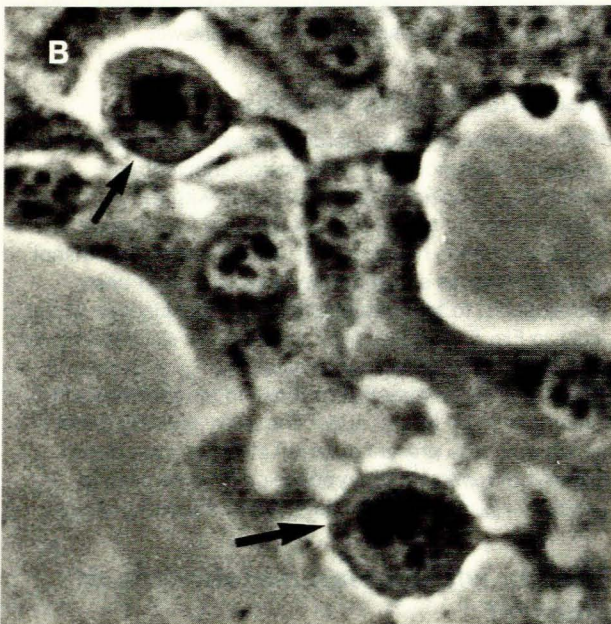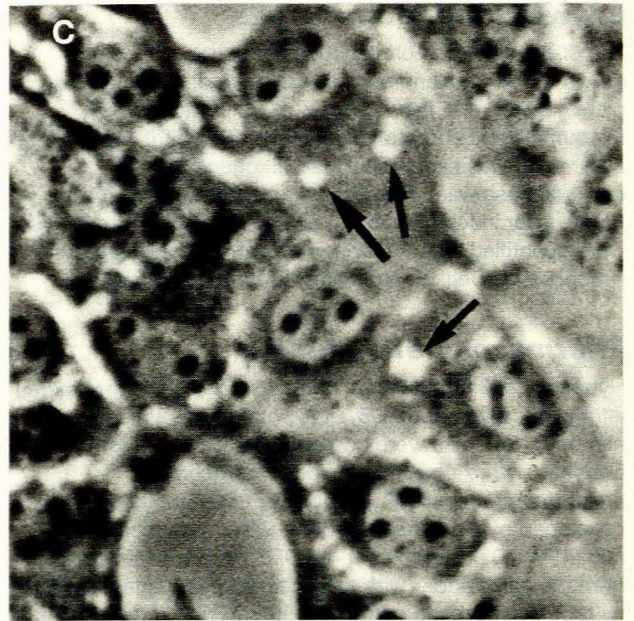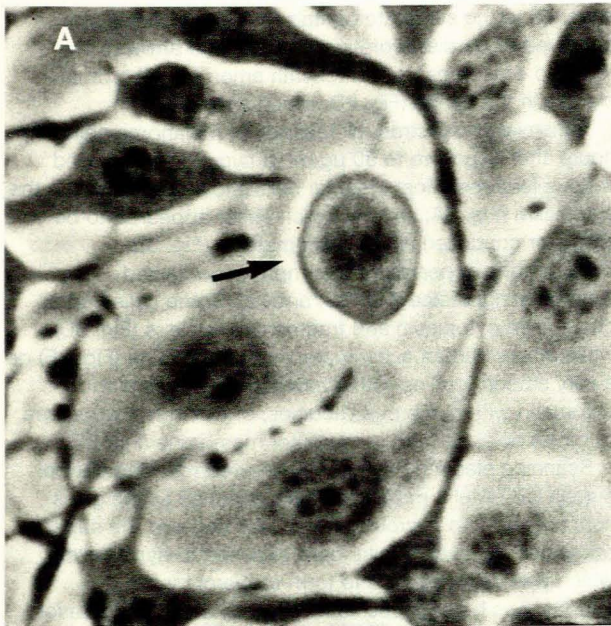
Fig. 2. Phase contrast micrographs of BL-6 mouse melanoma cells exposed to 50 µg/ml hypoxoside in medium with 10% non-heat-inactivated FCS, containing endogenous beta-glucosidase causing increasing amounts of rooperol to be formed,[7] resulting in cell pathology. Normal cells in mitosis contained recognisable metaphase plates (A — broad arrow). After 12 hours, treated cells in mitosis contained amorphous chromatin clumps (B — arrows). Flat cells showed extensive vacuolisation of the cytoplasm (C — arrows) after 16 hours, while after 24 hours most cells contained massive vacuoles (D — fine arrows) and appeared to be disintegrating. Rounded cells had a smooth outline with condensed amorphous chromatin clumps (D — broad arrows).

Discussion

Morphological studies

Morphological studies of the effect of activated hypoxoside on melanoma cells in culture highlight the effect of rooperol, which is progressively released from hypoxoside as a result of deconjugation by endogenous beta-glucosidase.[7] Comparable terminal morphological effects were also seen when rooperol was added directly to the cells, but we believe that the gradual and cumulative effects seen here are more relevant because they pertain to a situation where an inactive drug (hypoxoside) is enzymatically converted into an active drug (rooperol) in the immediate vicinity of cancer cells. This is the situation one would hope to achieve *in vivo* in humans during activation of the metabolites of rooperol.

The morphological effects seem to fall into two categories, i.e. changes in cells undergoing mitosis and in those that do
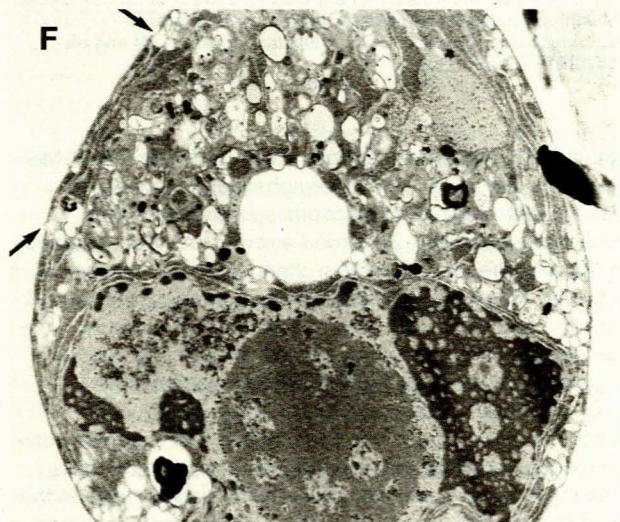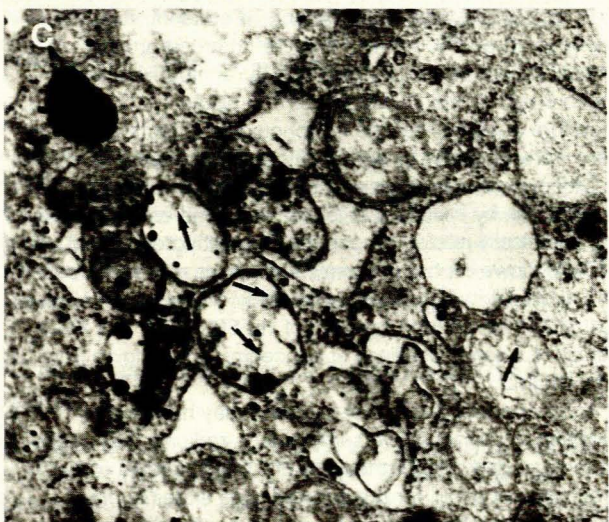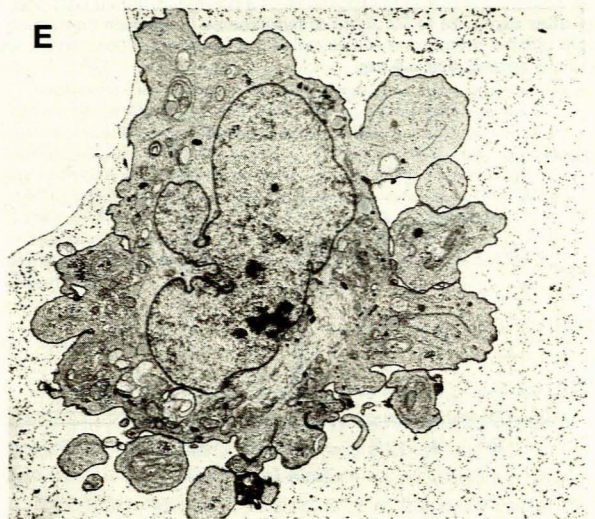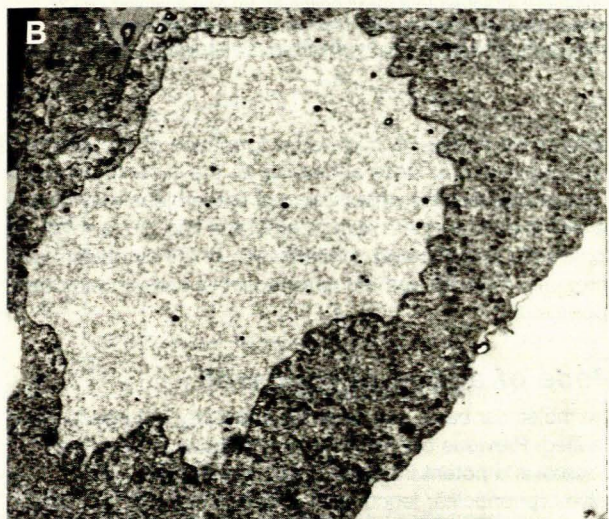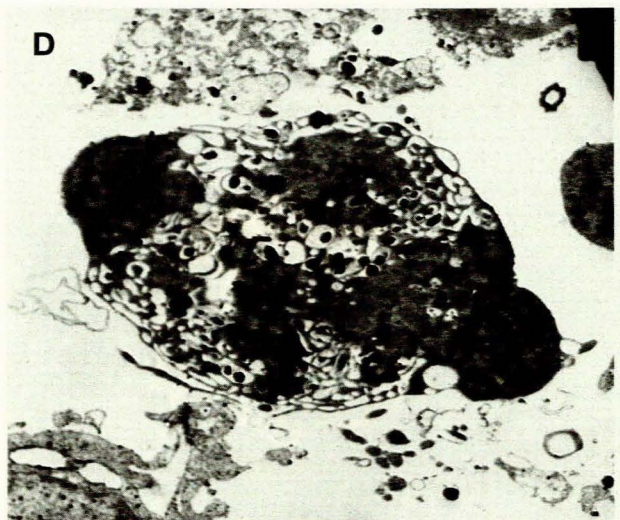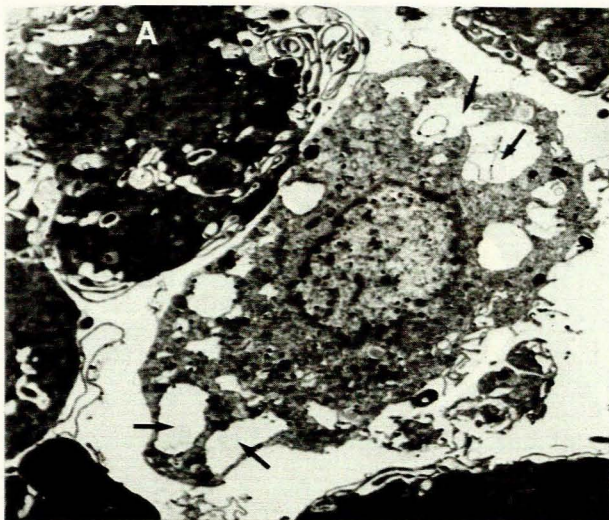
Fig. 3. Transmission electron micrographs of BL6 mouse and UCT-Mel 1 human melanoma cells 24 hours after exposure to 50 µg/ml of hypoxoside in medium containing endogenous beta-glucosidase. Various features are presented; vacuoles in the cytoplasm of a BL6 cell (A — arrows); amorphous material in a vacuole of a BL6 cell (B); disintegrating mitochondria in a BL6 cell (C — arrows); a BL6 cell with condensed cytoplasm and fragmented nucleus (D); an UCT-Mel 1 cell with an irregular outline (E) and a rounded UCT-Mel 1 cell with a smooth outline, condensed cytoplasm and nucleus and two peripheral vacuoles (arrows) which appear to be forming pores in the plasma membrane (F). Figs 3D, E and F suggest that rooperol induced apoptosis.
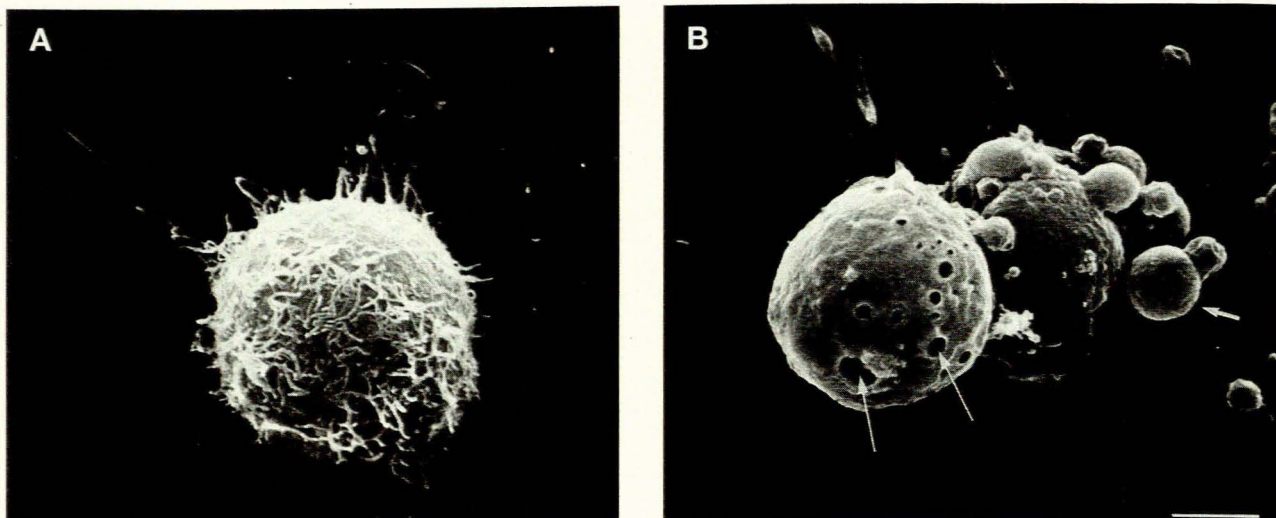
Fig. 4. Scanning electron micrographs of a normal, rounded UCT-Mel 1 melanoma cell attached to a glass coverslip (A) and a similar cell 24 hours after exposure to 50 µg/ml of hypoxoside in medium containing endogenous beta-glucosidase (B). Distinct pores are present in the smooth plasma membrane of the cell in the foreground (long arrow) while the adjacent cell appears to be shedding membrane enclosed spheres (B — short arrows). Bar = 5 µm.
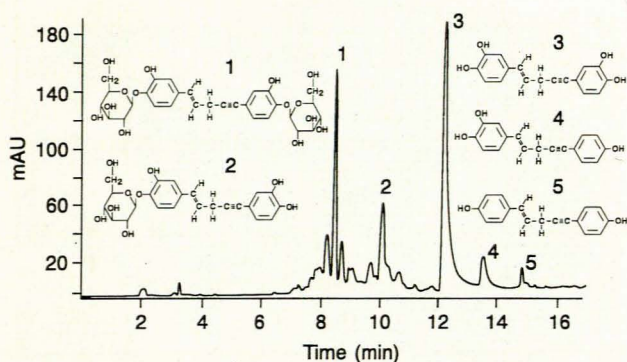

Fig. 5. HPLC chromatogram of a methanol extract of mouse faeces showing a residual trace of hypoxoside (1), hypoxoside monoglucoside (2), rooperol (3), dehydroxyrooperol (4) and bis-dehydroxy-rooperol (5).

not. In both melanoma cell lines studied (BL6 and UCT-Mel 1), cells undergoing mitosis are spherically shaped with clearly visible sets of chromosomes arranged in the metaphase plate (Fig. 2A; broad arrow). Such cells seemed to undergo the first detectable changes in the presence of activated hypoxoside. After 12 hours, chromosome sets could no longer be seen clearly and the rounded cells contained an amorphous mass of chromatin (Fig. 2B). This suggests that rooperol may disturb the mechanisms involved in the maintenance of chromosome structural integrity and segregation during mitosis. Scanning electron microscopy showed rounded UCT-Mel 1 melanoma cells, presumed to have entered mitosis, with distinct holes in the smooth outer membrane (Fig. 4B). This suggests that over and above any effect on the organisation of the chromatin, rooperol also destabilises the outer membrane to the extent that it is severely punctured.

It is important to note that flattened cells, presumably not in mitosis, were also deleteriously affected by rooperol in terms of the extensive, cumulative formation of vacuoles in the cytoplasm and blebs appearing on the outer membrane. It is possible that the detachment of these blebs may have caused the holes in the outer membrane (Fig. 3E; arrows). Some cells also showed condensation of the cytoplasm and chromatin (Figs 3B and 3F) reminiscent of cells undergoing apoptosis.[12]

Mode of action of rooperol

The molecular basis of rooperol cytotoxicity still needs to be clarified. Previous biochemical studies have shown that rooperol is a potent inhibitor of leukotriene synthesis in polymorphonuclear leucocytes at concentrations of 1 µM or less.[13] However, the synthesis of cyclo-oxygenase products, TxB_2 and PGD_2, were inhibited only at concentrations between 10 and 100 µm.[13] Rooperol-induced growth inhibition occurred at concentrations ranging from about 0,6 to 8 µg/ml, which is equivalent to about 1 - 13 µM. It is therefore possible that the morphological effects described here were triggered by an inhibition of leukotriene synthesis.

Other workers have also reported inhibition of cancer cell proliferation by inhibitors of leukotriene synthesis which are not chemically related to rooperol.[14] Nordihydroguiaretic acid (NDGA), however, has a chemical structure related to rooperol and also inhibits leukotriene synthesis.[13] Miller et al.[15] showed that NDGA inhibited the growth of HL-60, K-562 and KG-1 human leukaemia cell lines at concentrations ranging from 5 to 10 µM.

Another possibility is that rooperol may have been oxidised to form reactive semiquinone radicals that could have damaged membranes directly. It was shown earlier[13] that rooperol could form a semiquinone under in vitro oxidative conditions which caused lysis of red blood cells.

Results reported by the NCI suggested that rooperol released from deconjugated hypoxoside in tissue culture affected the NCI-H522 non-small-cell lung cancer cell line at about a 14-fold lower concentration than most of the other lines tested. This suggests that the cytotoxic mechanism of
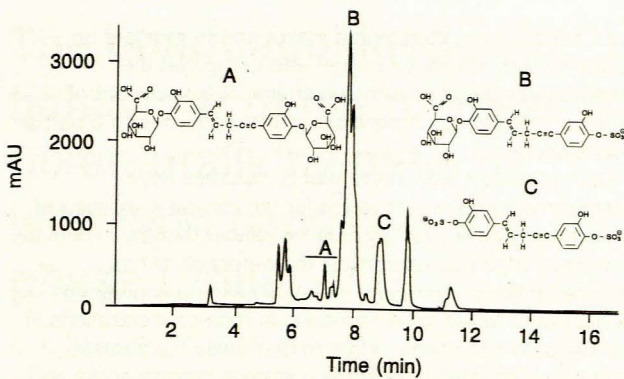
Fig. 6. HPLC chromatogram of mouse bile 3 hours after epigastric dosing of 3 mg hypoxoside. The three peaks (A, B, C) represent conjugated metabolites of rooperol characterised as di-glucuronides (A), mono-glucuronide/monosulphate (B) and disulphates.[4]

Fig. 7. Proliferation of BL6 mouse melanoma cells in the presence of human urinary rooperol metabolites (x——x) and after addition of 100 µg/ml beta-glucuronidase (•——•). The decrease in absorbance at 570 nm was measured using the MTT technique described in the Methods section and represents inhibition of growth. About 75 µg/ml of metabolites caused 50% growth inhibition after 72 hours of incubation.

rooperol may be determined to a large extent by the molecular characteristics of the target cells involved and that it is not a general cytotoxin such as cyanide. Comparative molecular studies of sensitive and very sensitive cell lines are presently being undertaken by us in order to elucidate the exact mode of action of rooperol as a cytotoxic drug.

Pharmacokinetics of hypoxoside in mice

Hypoxoside was not absorbed as such into the bloodstream of mice because it was not detected in serum from the general circulation or from portal blood. However, clear-cut evidence was found that hypoxoside was deconjugated to form rooperol in the caecum and colon of the mouse. There is little doubt that this was due to beta-glucosidase enzymes of bacterial origin because after oral treatment of mice with clindamycin, no rooperol, only hypoxoside was found in the faeces (results not shown).
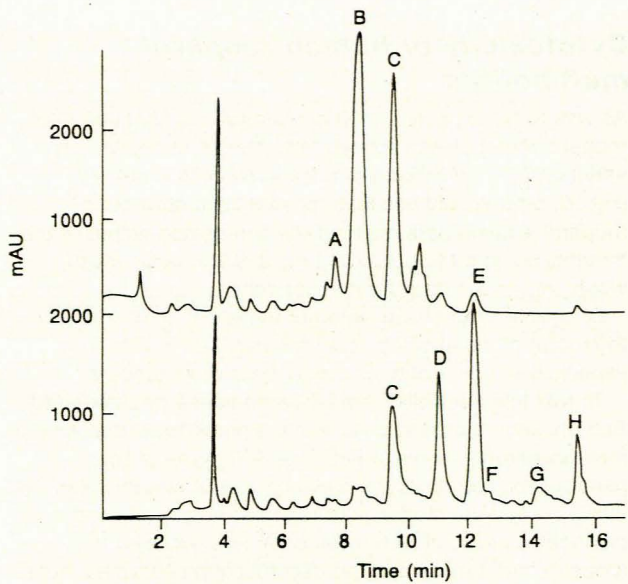

Fig. 8. HPLC chromatogram of human urinary rooperol metabolites 72 hours after incubation without exogenous beta-glucuronidase (upper chromatogram) and with 100 µg/ml exogenous beta-glucuronidase (lower chromatogram). Major changes are the disappearance of peaks A and B and a 50% decrease in peak C and the increase of peaks at retention times (RTs) 11,0 min (D) and 12,1 min (E) which have been shown to be monosulphates of rooperol.[4] Rooperol (RT = 12,6 min, peak F) is a minor peak while dehydroxyrooperol (RT = 14,1 min, peak G) and bis-dehydroxyrooperol (RT = 15,4 min, peak H) are present as described.[4]
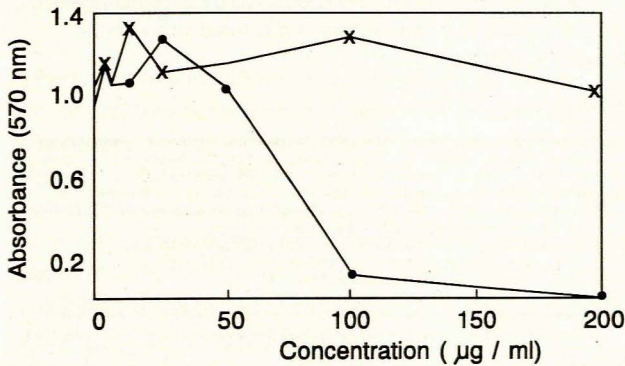
The detection of new peaks in the bile of a mouse with UV absorbance spectra similar to hypoxoside and rooperol indicates that rooperol is absorbed and converted to new conjugates by phase II metabolism as reported by Kruger et al. for human serum.[4] The presence of the main metabolite peak in mouse portal blood suggests that rooperol was also conjugated in the epithelial cells lining the caecum and colon. This interpretation is also supported by the absence of rooperol in blood from the general circulation or from the portal vein of the mouse.

The general conclusion we draw from these data is that in mice, neither hypoxoside, rooperol nor rooperol metabolites enter the general circulation, and that consequently a rodent cancer model would be quite useless in determining any anti-cancer potential of hypoxoside. Indeed, numerous attempts to arrest the growth of various tumours in mice using oral doses of hypoxoside were unsuccessful (data not shown).

The situation in humans is, however, strikingly different. Kruger et al.[4] showed that the metabolites which sequester in the bile of the mouse, appear in the serum of humans in relatively high concentrations.

We conclude from these observations that biochemical mechanisms present in hepatocytes determine to what extent phase II conjugates of xenobiotics are excreted via the biliary or circulatory systems and that in primates these mechanisms allow a certain percentage of rooperol metabolites to enter the systemic bloodstream for excretion via the kidneys. In this respect, humans and rodents are quite distinct.

Cytotoxicity of human rooperol metabolites

As was to be expected, high concentrations (200 µg/ml) of rooperol metabolites (isolated from human urine) had no effect on the proliferation of melanoma cells in culture (Fig. 7), presumably because the cytotoxic potential of rooperol is latent as a result of the conjugated nature of the metabolites imparting a strong molecular charge which would impair the drug's entry into cells.

Of crucial interest was whether the latent cytotoxicity of the rooperol metabolites could be unlocked *in vivo,* especially in the vicinity of cancer cells or in tumours.

To test this possibility, beta-glucuronidase was added to BL6 melanoma cells in culture in the presence of rooperol metabolites. As shown in Fig. 7, at 100 µg/ml of the metabolites, growth of the melanoma cells was inhibited by 90%. In separate studies, not reported here, that used a clonogenic assay of BL6 melanoma cells exposed to rooperol metabolites and the deconjugating enzymes, beta-glucuronidase and aryl sulphatase, a 100% inhibition of colony formation was found and scanning electron microscopy revealed cells with holes in the membranes similar to those shown in Fig. 5B. HPLC analysis of the products of beta-glucuronidase incubation with rooperol metabolites (Fig. 8) showed peaks with retention times longer than that of the metabolites, viz. 11,0 (D) and 12,1 minutes (E). In a separate study,[4] we showed that these peaks represent monosulphates of rooperol and dehydroxyrooperol after removal of glucuronic acid. The presence of peaks at retention times of 14,0 (G) and 15,4 minutes (H) indicate that rooperol was formed, because these peaks are typical of dehydroxy- and bis-dehydroxyrooperol, since they appear when hypoxoside or rooperol metabolites are deconjugated.[4] The absence of rooperol *per se* (Fig. 8, lower chromatogram, F) is due to the duration of incubation of the cells (72 hours), because short-term incubation of rooperol metabolites with beta-glucuronidase reveals rooperol in HPLC profiles (data not shown). It is thought that the absence of rooperol is due to its binding to cellular molecules and/or polymerisation, as suggested before.[13]

After demonstrating that rooperol metabolites could be activated to destroy melanoma cells in the presence of beta-glucuronidase it was of interest to know whether human tumours contained enzymes that could deconjugate the metabolites. Incubation of rooperol metabolites with an aqueous supernatant of a human melanoma resulted in the formation of the same peaks shown in Fig. 8 (lower chromatogram).

Latent cytotoxicity of glucuronides derived from xenosidics in cancer therapy have been described by Connors and Whisson[16] who reported the cure of mice with advanced plasma cell tumours that were treated with aniline mustard, and the relationship between glucuronidase activity and tumour sensitivity. They postulated that aniline mustard glucuronide, formed in the liver of the treated mice, could be cleaved to form the highly toxic hydroxy derivative by high levels of beta-glucuronidase in the tumours and that this sequence of events would produce a local activation of a potent cytotoxic drug within the tumour itself and the observed therapeutic effect. Subsequent to this, Young *et al.*[17] conducted a therapeutic trial of aniline mustard in patients with advanced cancer and compared the therapeutic response with cytochemical assessment of tumour cell beta-glucuronidase activity; they found a partial correlation.

In conclusion, we believe that our studies have established a good motivation for the clinical evaluation of hypoxoside as an oral prodrug for cancer therapy in humans for three important reasons: (*i*) the detection of high concentrations of rooperol metabolites in human serum which were found to be non-toxic at high concentrations in tissue culture; (*ii*) demonstration that these metabolites could be activated to become cytotoxic for melanoma cells in culture in the presence of beta-glucuronidase and that extracts from a human tumour could also deconjugate the metabolites; and (*iii*) the observation that mice were quite unsuitable as test animals for evaluating the *in vivo* potential of hypoxoside as a prodrug for cancer therapy, given the lack of rooperol metabolites in their blood.

REFERENCES

1. Marini-Bettolo GB, Patamia M, Nicoletti M, Galeffi C, Messana I. Research on African medicinal plants — II. Hypoxoside, a new glycoside of unknown structure from *Hypoxis obtusa Busch. Tetrahedron* 1982; **38**: 1683-1687.
2. Drewes S, Hall AJ, Learmonth RA, Upfold UJ. Isolation of hypoxoside from *Hypoxis rooperi* and synthesis of (E) -1,5-Bis (3',4' dimethoxyphenyl) pent-4-en-lyne. *Phytochemistry* 1984; **23**: 1313-1316.
3. Koch HP, Brosch-Starzengruber N. Biopharmaceutical investigation of hypoxoside, a natural antitumour compound from plant origin. *J Biopharmaceutical Sciences* 1991; **2**: 305-318.
4. Kruger PB, Albrecht CF de V, Liebenberg RW, Van Jaarsveld PP. Studies on hypoxoside and rooperol analogues from *Hypoxis rooperi* and *H. latifolia* and their biotransformation in man by using high-performance liquid chromatography with in-line sorption enrichment and diode array detection. *J Chromatogr* 1994; **662**: 71-78.
5. Nicoletti M, Galeffi C, Messana I, Marini-Bettolo GB. Hypoxidaceae. Medicinal uses and the norlignan constituents. *J Ethnopharmacology* 1992; **36**: 95-101.
6. Monks A, Scudiero D, Skehan P, *et al.* Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumour cell lines. *J Natl Cancer Inst* 1991; **83**: 757-766.
7. Theron E, Albrecht CF, Kruger PB, Jenkins K, van der Merwe MJ. ß-glucosidase activity in fetal calf serum renders the plant glucoside, hypoxoside, cytotoxic towards BL-6 mouse melanoma cells. *In Vitro Cell Dev Biol* 1994; **30A**: 115-119.
8. Hart IR. The selection and characterization of an invasive variant of the B16 melanoma. *Am J Pathol* 1979; **97**: 587-600.
9. Hoal-van Helden EG, Wilson EL, Dowdle EB. Characterization of seven human melanoma cell lines: Melanogenesis and secretion of plasminogen activators. *Br J Cancer* 1986; **54**: 287-295.
10. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. *J Immunol Methods* 1986; **89**: 271-277.
11. Kruger PB, Albrecht CF de V, Van Jaarsveld PP. The use of guanidine hydrochloride and ammonium sulfate in comprehensive in-line sorption enrichment of xenobiotics in biological fluids by high performance liquid chromatography. *J Chromatogr* 1993; **612**: 191-198.
12. Kerr JFR. Shrinkage necrosis: A distinct mode of cellular death. *J Pathol* 1971; **105**: 13-20.
13. Van der Merwe MJ, Jenkins K, Theron E, Van der Walt BJ. Interaction and the dicatechols rooperol and nordihydroguaiaretic acid with oxidative systems in the human blood. *Biochem Pharmacol* 1993; **45**: 303-311.
14. Honn KV, Dunn JR. Nafazatrom (Bay g 6575) inhibition of tumor cell lipoxygenase activity and cellular proliferation. *FEBS Lett* 1982; **139**: 65-68.
15. Miller AM, Cullen MK, Kobb SM, Weiner RS. Effects of lipoxygenase and glutathione pathway inhibitors on leukemic cell line growth. *J Lab Clin Med* 1989; **118**: 355-361.
16. Connors TA, Whisson ME. Cure of mice bearing advanced plasma cell tumours with aniline mustard: the relationship between glucuronidase activity and tumour sensitivity. *Nature* 1966; **210**: 866-867.
17. Young CW, Yagoda A, Bittar ES, Smith SW, Grabstald H, Whitmore W. Therapeutic trial of aniline mustard in patients with advanced cancer. Comparison of therapeutic response with cytochemical assessment of tumor cell ß-glucuronidase activity. *Cancer* 1976; **38**: 1887-1895.