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Inhibitory effects of cytotoxic disulfides on membrane Na^+,K^+-ATPase

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Four decyl and phenyl disulfide analogs of 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) (Fig. 1) have been reported to be effective against L1210 and S180 *in vivo* [1]. Inomata *et al.* [1] proposed that the cytotoxicity displayed by these compounds might be the result of the release of the purine portion of the molecules in the cellular milieu. We recently reported on the activity of these analogs against the EMT6 cell line [2]. Our investigation involved the examination of the hypoxic and aerobic cell cytotoxicity of this series of compounds. No differential in activity was observed; however, we did find that glutathione (GSH) was depleted from EMT6 cells after treatment with cytotoxic concentrations of the disulfides. Moreover, when cellular GSH was depleted prior to exposure to these agents, their cytotoxicity was potentiated.

A recent study suggested that the GSH depletion produced by the disulfides was the result of their interaction with the tripeptide [3]. This kinetic study reported that the disulfide–GSH interaction occurs spontaneously in a 1:1 manner and that the rates of the reaction could be enhanced in the presence of GSH-*S*-transferase. The products of the reactions were isolated and identified as the GSH-thiol mixed disulfide, the probable cause for the depletion of GSH from cells treated with the disulfides.

The *in vitro* cytotoxicity displayed by these agents was far greater than that which would be expected due to the release of the purine portion of the drugs [2] as was suggested by Inomata *et al.* [1]. Observations made during the cytotoxicity studies suggested that the membrane may be the critical target of these agents, as cells which were treated with toxic concentrations of compounds 1–4 detached from the culture flask surface and appeared swollen and distorted.* In an attempt to explain their cytotoxic action, the effects of compounds 1–4 on the membrane enzyme Na^+,K^+-ATPase were measured. Inhibition of this enzyme, which mediates active transport across the membrane and is sensitive to thiol active agents [4], has been reported to affect cell replication [5]. A recent report [6] also suggests that porphyrin-induced photosensitization may contribute to cell cytotoxicity through inhibition of Na^+,K^+-ATPase. The enzyme has a number of thiol groups essential for activity [4]. Alkylating agents [7] and nitroimidazoles, which have been found to form complexes with GSH [8], inhibit Na^+,K^+-ATPase [9]. On the basis of these reports, it was anticipated that compounds 1–4, shown recently to react with the thiol group of GSH [3], might inhibit membrane Na^+,K^+-ATPase through thiol interactions, contributing to the death of the cell.

Materials and methods

The decyl and phenyl disulfides of 6-MP and 6-TG (compounds 1–4) were synthesized as previously described [10, 11] and recrystallized prior to use.

These along with *N*-ethylmaleimide (NEM, Sigma Chemical Co., St Louis, MO), dicyclohexylcarbodiimide (BDH Chemicals Canada Ltd., Saskatoon, Sask.), 6-mercaptopurine and thiophenol (Sigma) were dissolved in dimethyl sulfoxide (reagent grade, BDH). Ouabain (Sigma) was dissolved in 95% ethanol:Tris–HCl (10 mM, pH 7.5), 50:50. Acid molybdate, Fiske & Subbarow

	R	R'
1	$-(CH_2)_9CH_3$	H
2	$-(CH_2)_9CH_3$	NH_2
3		H
4		NH_2

Fig. 1. Structures of compounds 1–4.

* Kirkpatrick DL, unpublished observations.

Reducer, phosphorous standard solution and disodium ATP were obtained as a kit from Sigma. All other chemicals and solvents were of reagent grade and were obtained from common chemical sources.

Ion solutions used in the enzyme assay were composed as follows [12]: (A) 1.5 mM $MgCl_2$ in Tris–HCl (50 mM, pH 7.5); and (B) 1.5 mM $MgCl_2$, 120 mM NaCl and 15 mM KCl in Tris–HCl (50 mM, pH 7.5).

The plasma membranes were isolated after trypsinization of EMT6 cells grown in monolayer, following a modified procedure described by Warren [13]. The cells were centrifuged to remove medium and resuspended in cold Tris–HCl (50 mM, pH 7.5). All remaining procedures were carried out at 0–4°. The cells were washed in Tris–HCl and resuspended at a cell concentration of 2.5×10^7 cells/mL in Tris–HCl (50 mM, pH 7.5) containing 5% of a 50 mM $MgCl_2$ solution. The cells were allowed to stand for 8 min and then were sonicated with 40 pulses at 20% duty cycle (Branson sonic dismembrator). The cell homogenate (1.5 mL) was layered over 3.0 mL of Histopague 1077 (Sigma) and centrifuged at 1500 *g* for 30 min. The two layers obtained were separated, and the bottom layer was resuspended in Tris–HCl (10 mM, pH 7.5) and centrifuged at 15,000 *g* for 30 min. The pellet was resuspended in Tris–HCl (10 mM, pH 7.5) and kept on ice until used in enzyme studies. A second, longer method of membrane isolation described by Jorgensen [14] using sucrose gradients of 20–50% provided a preparation of the same purity. Therefore, only the first procedure was used for the described experiments. The protein content of all preparations was determined by the method of Lowry *et al.* [15].

Succinate dehydrogenase activity was determined using the method of Tsai *et al.* [16] and reported as ΔA/mg protein for a marker of mitochondrial contamination.

A modification of the method of Baxter *et al.* [7] was used to measure Na^+,K^+-ATPase activity. Membrane homogenate, 0.10 to 0.50 mg protein, was analyzed for P_i release, while 0.20 mg of protein was used for inhibition studies as described below. Membrane homogenate (0.20 mg protein) in 500 μL Tris–HCl (10 mM, pH 7.5) (control) or 495 μl Tris–HCl (treated) was allowed to sit for 5 min at 37°. Dimethyl sulfoxide, compounds 1–4, 6-MP, thiophenol or *N*-ethylmaleimide (5 μL) was added to treatment tubes to give the final concentrations described in Table 2. The tubes were incubated at 37° for 1 hr. Either 500 μL Tris–HCl (10 mM, pH 7.5) or 400 μL Tris–HCl and 100 μL ouabain (2.5 mM) [5] was added to bring the volume to 1 mL. Dicyclohexylcarbodiimide (40 nM) was added for 15 min immediately following drug treatment in some trials [7]. Ion solutions (A or B) (0.9 mL) were added to duplicate sets of tubes [12], and ATP (1 mM in 100 μL) was added to all tubes. The tubes were incubated with shaking at 30–35° for 30 min. The reaction was terminated by the addition of 100 μL trichloroacetic acid (50%). The samples were centrifuged for 10 min at 15,000 *g*. The supernatant fraction (2 mL) was analyzed for inorganic phosphate (P_i) according to the method of Fiske and Subbarow [17].

The enzyme activity was calculated as micromolar P_i per milligram of protein per hour and Na^+,K^+-ATPase activity is reported as Mg^{2+},Na^+,K^+ ATPase − (Mg^{2+} ATPase and ouabain) activity.

Studies to determine the mechanism of inhibition were carried out using ATP concentrations of 0.5 to 1.0 mM. Concentrations over 1 mM were found to produce substrate inhibition as previously described [18], making the overall determination of mechanism difficult. Both the isolated membrane preparation and purified Na^+,K^+-ATPase (Sigma Grade III: ouabain sensitive, vanadate inhibited) were used in these studies with drug concentrations of 30, 45 and 50 μM, following the method described above.

Results and discussion

EMT6 cells grown in monolayer, treated with the decyl or phenyl disulfides of 6-MP or 6-TG, were found to swell and detach from the surface of the culture flask. An examination of the cells under a microscope during treatment showed this swelling to be preceded by bleb formation, in a manner very similar to that seen when cells were exposed to 2.5 mM ouabain, an inhibitor of Na^+,K^+-ATPase.

Table 1. P_i release versus protein concentration or time of incubation period of membrane preparation

Protein concn (mg)	Incubation time (min)	P_i release (μM)
0.10	20	51*
0.15	20	69
0.20	10	43
0.20	20	87
0.20	30	122
0.20	45	148
0.20	60	186
0.50	20	204

* Mean of duplicate experiments.

The plasma membrane preparation from these cells was analyzed for Na^+,K^+-ATPase activity by measuring the release of P_i versus protein concentration over time. The release of P_i versus protein concentration was found to be linear, and versus time its release was linear up to 30 min (Table 1). The Na^+,K^+-ATPase activity of the membrane preparation (control) was measured to be 489 ± 145 μM P_i/mg protein/hr. Dicyclohexylcarbodiimide [7], used to inhibit contaminating mitochondrial Mg^{2+}-ATPase, showed no effect on the enzyme activity as compared to control values (data not shown). The succinate dehydrogenase activity (mitochondrial enzyme marker) was minimal (0.078 ΔA/mg protein) as compared to the crude

Table 2. Inhibition of Na^+,K^+-ATPase activity after exposure to agents for 1 hr

Agent	Concentration (mM)	% Inhibition*
1	1.0	70 ± 16†
	0.5	65 ± 6
2	1.0	69 ± 2
	0.5	65 ± 9
3	1.0	95 ± 5
	0.5	81 ± 14
4	1.0	75 ± 4
	0.5	55 ± 0
NEM	1.0	47 ± 2
	0.5	39 ± 4
6-MP	5.0	0
TP	5.0	0

* Control Na^+,K^+-ATPase: 489 ± 145 μM P_i/mg protein/hr.

† Mean ± SD of triplicate values from two to four experiments.

preparation (0.53 ΔA/mg protein). The ouabain (2.5 mM) added to either the Mg^{2+},Na^+,K^+ or only the Mg^{2+} preparation inhibited the Na^+,K^+-ATPase to the same extent, providing similar values of 562 ± 67 and 584 ± 25 μM P_i/mg protein/hr for Mg^{2+}-ATPase activity.

The levels of inhibition of Na^+,K^+-ATPase produced by the four disulfide compounds, NEM, 6-MP and TP are listed in Table 2. Of the disulfides, compound 3 affected the enzyme activity to the greatest extent, inhibiting it 95% at 1 mM and 81% at 0.5 mM. NEM, a thiol active agent [4], also inhibited the enzyme activity 47% at 1 mM and 39% at 0.5 mM. Compounds 1, 2 and 4 all displayed lesser degrees of inhibition. These concentrations are similar to those used for *in vitro* cytotoxicity testing which also showed compound 3 to be the most cytotoxic at 0.5 mM [2]. The purine and thiol breakdown products of compound 3, namely 6-MP and TP, were devoid of inhibitory activity up to 5 mM.

A recently reported kinetic study [3], which monitored the reaction between the disulfides and GSH over time in the absence or presence of GSH-*S*-transferase, identified thiol-GSH mixed disulfide as the products of the reaction. Comparing the reaction rates from this previous study to the Na^+,K^+-ATPase inhibition displayed by each compound in this study revealed a direct parallel. Compound 3 reacted with the thiol with the fastest rate of 4174 M^{-1} sec^{-1}. Compounds 1, 2 and 4 displayed much slower rates of 142, 564 and 429 M^{-1} sec^{-1}, respectively. 6-MP and TP, which showed no inhibition of Na^+,K^+-ATPase, did not react with GSH in the previous study. The similarities between the rates of reactions, which resulted in the complexing of the thiol function of GSH, and the inhibition of Na^+,K^+-ATPase suggest that compounds 1–4 also interact with membrane thiol functions. NEM, which also inhibited Na^+,K^+-ATPase, was also reported by Skou [4] to interfere with the essential SH groups of the enzyme. Moreover, two disulfide analogues of thioinosine triphosphate have been reported to bind covalently to SH groups of this enzyme in a competitive manner with ATP [19] and to cause its inhibition in a noncompetitive manner with cations [20].

The inhibition of the enzyme in this study appears to follow mixed-kinetics as both the K_m and V_{max} values change with changing inhibitor concentration [21]. Figure 2 shows the Lineweaver–Burk plot ($1/v$ vs $1/S$) for the three concentrations of compound 3 tested, typical of a mixed-type inhibition with the intersecting point below the $1/S$ axis. In addition, parameters from Equation 1, $K_o = 4.23 \times 10^3\ M^{-1}$, $K'_o = 1.39 \times 10^6\ M^{-1}$, as calculated from

$$\text{y int} = \frac{1 + [I]K'_o}{V_{max}} \text{ and slope} = \frac{K_m(1 + [I]K_o)}{V_{max}}$$

(where $[I] = 4.5 \times 10^{-5}$ M, $V_{max} = 1.18 \times 10^{-3}$ M/min, $K_m = 2.7 \times 10^{-3}$ M, y int $= 5.4 \times 10^4$ min/M and slope $=$ 2.29 min) again indicate a "mixed inhibitor" [22]. This suggests that the inhibitor is able to combine with either the enzyme itself or the enzyme–substrate complex according to Equation 1.

$$\begin{array}{ccc} E & \rightleftharpoons & ES \rightarrow E + P \\ K_o \downarrow\uparrow & & \downarrow\uparrow K'_o \\ EI & \rightleftharpoons & EIS \end{array} \quad (1)$$

Since Na^+,K^+-ATPase is an allosteric enzyme [23], binding to critical SH groups is thought to prevent the conformational change required for enzyme activity. The blocking of ion translocation will affect cell volume control and result in cell death. Inhibition of Na^+,K^+-ATPase by compounds 1–4 appears to result from the interaction with enzyme thiols and may be the precursor to cell death. This study and others [7, 9] suggest that the cell membrane may be an important target for those antitumor agents which complex with cellular thiols.

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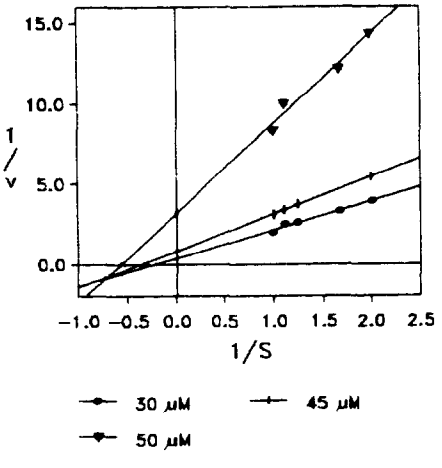

Fig. 2. Double-reciprocal plot obtained in the presence of three different concentrations of the inhibitor of Na^+,K^+-ATPase, compound 3.

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Effects of octimibate, an inhibitor of acyl coenzyme A: cholesterol acyltransferase, on cholesterol metabolism in the hamster and rat

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Inhibition of esterification of cholesterol in cells has been considered by many to be a desirable goal. In the intestine this would lead to reduced absorption of cholesterol and in arteries would result in reduced deposition of cholesterol and hence a reduction of growing atherosclerotic plaques [1, 2]. A number of compounds have been reported that inhibit the intracellular enzyme that catalyses cholesterol esterification, acyl-CoA:cholesterol acyltransferase (ACAT), but to date none has been successful in the clinic [1–4]. Octimibate (Nattermann) is one of a number of compounds of this type in development and it appears to have a number of advantages over previous compounds through its solubility and absorbability [5]. We have used inhibitors of ACAT to characterize the role of hepatic pools of cholesterol in primary cultures of rat hepatocytes and in bovine adrenal cortical cells [6, 7]. Since little information is available on octimibate we carried out a short study to characterize its effects on cholesterol metabolism in the hamster, an animal that offers a number of advantages in studies of cholesterol metabolism over the rat. We compared these results with parallel studies in the rat.

Materials and methods

Male Syrian hamsters were obtained from Shamrock Farm or Belgrave Trading Ltd and had a body weight of 104–136 g at the time of the experiments. Male Wistar strain rats were obtained from Charles River and had a body weight of 319–366 g at the time of the experiments. A week prior to dosing, the animals were housed individually in wire-bottomed cages. The rats were allowed to feed on powdered PRD chow *ad lib.* from rat food hoppers and hamsters were allowed to feed on powered PRD chow *ad lib.* from pots clipped to the grid floor. During this acclimatization period food consumption was measured. The animals were weighed on alternate days and the expected mean body weight of the animals at the time of the experiment calculated.

The appropriate amount of octimibate, calculated from the food consumption and expected body weight of the animals, was dissolved in a large volume of 99% ethanol, typically 1.2 L for a 4 kg batch of diet. This solution was then thoroughly mixed into the powdered PRD chow to give a homogeneous mixture. The wet diet was then transferred to a large photographic developing tray and placed in a fume cupboard to dry. During the experiment the daily dose of octimibate received by each animal was calculated from its food consumption and body weight. After the animals had been fed on the appropriate diet for 7 days, they were anaesthetized with diethyl ether and a blood sample (2–3 mL) was taken from the superior vena cava. The liver was perfused with cold saline and then weighed. A portion of liver (~ 1 g) was removed, weighed and homogenized in chloroform/methanol (2:1 v/v) with a Polytron homogenizer. [^{14}C]Cholesterol was added as a recovery marker to the resulting homogenate, which was then filtered through Whatman number 1 filter paper and blown down to dryness under nitrogen. The residue was taken up in 1 mL of propan-2-ol and centrifuged at 2900 *g* for 10 min in a microcentrifuge. The supernatant was assayed for free and total cholesterol using the AMES enzymatic colorimetric method. Octimibate was synthesized by the Department of Medicinal Chemistry at Smith Kline and French Laboratories in Welwyn.