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Effect of membrane active 1-alkylpiperidine *N*-oxides on glycolysis, respiration and ATP level in tumor cells

Milan Miko and Ferdinand Devinsky

Department of Microbiology, Biochemistry and Biology, Slovak Technical University, Radlinskeho 9, 812 37 Bratislava, Slovakia. Tel: (+42) 7 3260 40; Fax: (+42) 7 493 198.
[1]Faculty of Pharmacy, Comenius University, Bratislava, Slovakia

The aim of this study was to study the effect of the homologous series of 1-alkylpyrrolidine *N*-oxides on ATP-producing processes in Ehrlich ascites and L1210 murine leukemia cells. 1-Decylpiperidine *N*-oxide (dePNO), one of the active compounds, significantly stimulated the course of aerobic glycolysis of Ehrlich cells. Derivatives with longer side-chains markedly inhibited endogenous respiration of both tumor cells. dePNO, immediately after addition to the suspension of Ehrlich cells in an ice bath, markedly decreased the level of ATP in Ehrlich ascites cells. The decrease in ATP level might be explained through impairment of cell membrane integrity.

Key words: 1-Alkylpiperidine *N*-oxides, ATP, Ehrlich and L1210 cells, glycolysis, respiration, thiol groups.

Introduction

Non-aromatic amine oxides represent a large group of compounds derived from tertiary amines containing a strongly polarized N → O bond.[1,2] A great number of amine oxides occurring in nature or prepared synthetically are biologically active compounds (antimetabolites and chemotherapeutics, psychotropic and cancerostatic compounds, etc.). Though some non-aromatic amine oxides have found wide industrial utilization due to their good surface active properties,[3] relatively little attention has been paid to their biological activity, in contrast to aromatic amine oxides.[4,5]

In addition to the interesting chemical and biological activities shown by these compounds,[6-9] Ferencik *et al.*[10] have recently opened new perspectives in the field of immunomodulatory compounds due to their concentration-dependent influence on the immune system. With selected compounds of this type the immunosuppressing activity was found to be as high as that cyclosporin A,[11,12] used today as one of the most powerful immunosuppressants known.

In our previous paper[13] it was shown that cytotoxic activity of 1-alkylpiperidine *N*-oxides increased with increasing alkyl chain length, reaching a maximum at C_{12}–C_{15}. The 1-alkylpiperidine *N*-oxides containing an alkyl chain shorter than C_{10} were found to be less effective.

Up to now, however, nothing has been known about the action of 1-alkylpiperidine *N*-oxides on energy-yielding processes in tumor cells. Further, as macromolecule biosynthesis is an energy-requiring process we followed the effects of 1-alkylpiperidine *N*-oxides on energy-producing processes, i.e. on aerobic glucose consumption, lactic acid formation, content of total (T-SH) and non-protein (NP-SH) thiol groups, endogenous respiration, and levels of ATP in both Ehrlich ascites carcinoma and L1210 murine leukemia cells. The chemical structures of the substances studied are shown in Figure 1. Synthesis, properties and antimicrobial activity of the compounds have been described by Devinsky.[7-9] The compounds of this type belong to the so-called 'soft' antimicrobially active compounds.[14] Linker *et al.*[15] calculated that Ehrlich cells, grown in standard medium, produced 60% of ATP via oxidative pathways and 40% via glycolysis. Beckner *et al.*[16] showed that the motility of metastatic cells in the human melanoma line A2058 depends on the presence of glucose—primarily on energy from glycolysis. These findings suggest that inhibition of glycolysis *in vivo* might reduce the ability of tumor cells to leave the primary mass and metastasize to secondary sites.

Ehrlich ascites tumor cells have been extensively used as an experimental model for biochemical investigation.[17,18] We have also used Ehrlich cells for the study of the mechanism of action of some antibiotics,[19] ethidium bromide,[20] isothiocyanates[21] and other known cancerostatics.[22-24]

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Correspondence to M Miko

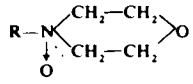
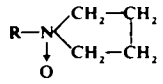
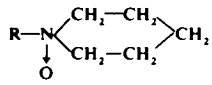
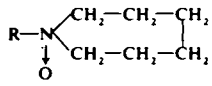
Group	Structural formula	Derivatives of	R	Number of compounds
A		morpholine	C_1 to C_{18}	18
B		pyrrolidine	$C_{12,14,16,18}$	4
C		piperidine	C_8 to C_{15}	8
D		perhydroazepine	C_9 to C_{16}	8
	Total number			38

Figure 1. Survey of *N*-oxides investigated.

Materials and methods

Cells

Ehrlich ascites carcinoma (EAC) cells were maintained and propagated in strain H Swiss albino mice (Institute of Experimental Pharmacology, Dobra Voda, Slovakia), approximately 10 weeks old and 20–25 g body weight, as described previously.[25,26] Ehrlich ascites cells were transplanted at 7 day intervals by i.p. injection of 0.2 ml ascitic fluid collected under sterile conditions. The tumor cells were obtained from the peritoneal cavity of mice and were packed by low-speed centrifugation (600 *g* for 10 min at 4°C). Mice with transplanted L1210 cells were from Dr V. Ujházy (Cancer Research Institute, Bratislava). The cells were suspended in Krebs–Ringer phosphate buffer, pH 7.4, without calcium but with ascitic serum (2.5%, v/v) and glucose (final concentration, 3.0 mmol/l). The number of cells was adjusted to 5×10^6/ml of medium.[26] All operations were performed at 0–4°C.

Drugs

Chromatographically pure amine oxides were from the Department of Inorganic and Organic Chemistry, Faculty of Pharmacy, Komensky University, Bratislava. Substances were dissolved in water immediately before use as stock solution. After appropriate dilutions (1:1) each of these solutions was then added to the cell suspensions. The chemicals and enzymes necessary for the determination of glucose consumption and lactate formation were purchased from Boehringer (Mannheim, Germany). DTNB for the determination of T-SH and NP-SH was purchased from Calbiochem (San Diego, CA). All other reagents were obtained from Sigma (St Louis, MO).

Glucose uptake and lactic acid production

The kinetics of aerobic glucose uptake and lactic acid production were determined by commercially available tests (Boehringer, Mannheim, Germany) as described earlier.[27] The concentrations of glucose and lactate were determined enzymatically in the supernatant obtained after precipitation of suspensions of EAC cells with 1 ml 0.6 mol/l glacial perchloric acid in an ice bath. The precision of these measurements is $\pm 3\%$.

Determination of T-SH and NP-SH groups

The determination was done according to the method of Ellman,[28,29] modified by Sedlak and Lindsay[30] in performance according to Drobnica *et al.*[31] The level of SH groups was determined concomitantly with glucose consumption and lactate formation. 5,5′-dithiobis(2-nitrobenzoic)-acid is reduced by SH groups giving rise to 1 mol of 2-nitro-5-mercaptobenzoic acid per 1 mol SH. This anion is of bright yellow color (E_{412} = 13 600 M^{-1} cm^{-1}).

Respiration

The effect of amine oxides on endogenous respiration of Ehrlich and L1210 cells was determined on the basis of oxygen consumption in 154 mmol/l NaCl, 6.2 mmol/l KCl, 11 mmol/l sodium phosphate buffer, pH 7.4.[32] Amine oxides were mixed with 2.0 ml of this medium and 200 μl of the cell suspension in the same buffer solution was added.

Assay of respiration

Cellular respiration was measured with a Clark-type oxygen electrode in a thermostatically controlled reaction vessel equipped with a stirring device. The reaction system and procedure were essentially as described previously.[20,21] Oxygen consumption was monitored for approximately 10 min and the linear portion of the oxygen consumption curve was used to calculate the rate of oxygen consumption. The respiratory rate was expressed as natoms of oxygen consumed per min and dry weight of the cells.

ATP determination

ATP level determination was carried out according to the method described elsewhere.[33]

Protein determination

Determination of the protein concentration in the cell suspension was done according to the method of Lowry *et al.*[34]

Results

On the basis of our previous results,[13] one of the active compounds, namely 1-decylpiperidine *N*-oxide (dePNO), was chosen for further biochemical study. The effect of amine oxide on aerobic glycolysis of EAC was investigated for dependence on both time and concentration (Figure 2). Ehrlich cells consumed glucose from the medium linearly with the time, even if its concentration fell by more than 50% of its original value. A likewise

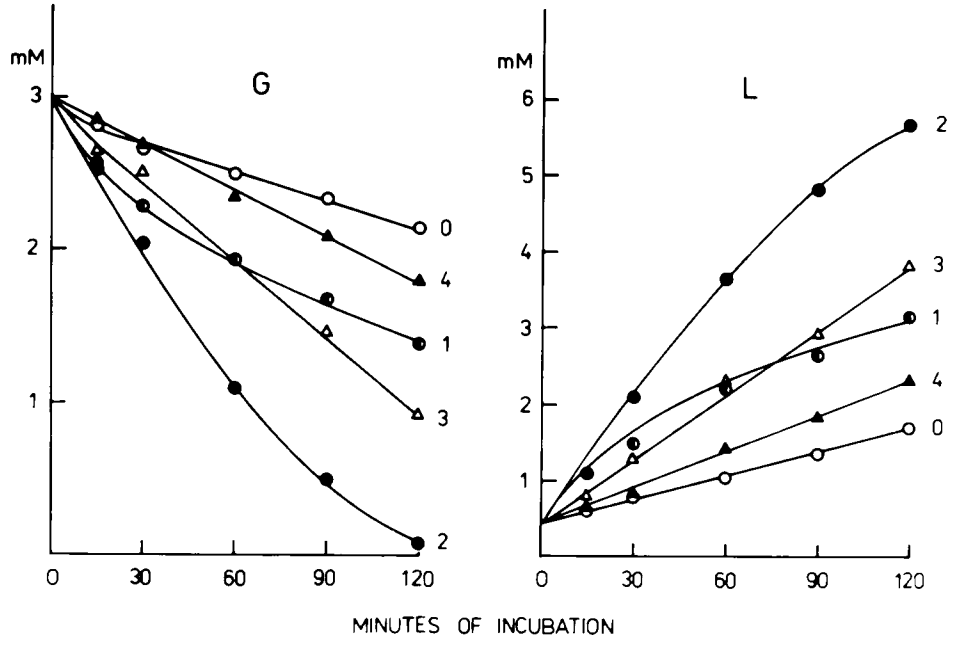


Figure 2. The effect of dePNO on the kinetics of aerobic glucose utilization (G) and lactic acid formation (L) by EAC. The cells were incubated at 37°C in the presence of different concentrations of dePNO. The initial glucose concentration was 3 mmol/l. At various times, 1 ml samples of suspension were analyzed for glucose and lactate. Compound concentrations: 0 = none (○), 1 = 600 (◐), 2 = 300 (●), 3 = 150 (△), 4 = 75 (▲) μmol/l.

proportional increase in lactate concentration in the medium was seen in control cells. As shown in Figure 2, none of the concentrations inhibited glucose consumption or lactate formation. On the contrary, the course of aerobic glycolysis was significantly stimulated, depending on concentration. The results appear to indicate that the stimulation takes place immediately on addition of amine oxide to the cancer cell suspension. In this respect, the effect of dePNO was similar to that of carbonyl cyanide phenylhydrazone,[35] which is the best known protonophoric uncoupler.[36] The conversion of glucose to lactate in control cells was approximately 76% (calculated for the first 30 min of measuring glycolysis) which is in good agreement with our previous results, where the transformation of glucose into lactate was in the range of 75.0–83.5%.[26]

It is known that the key glycolytic regulation enzymes contain cysteine SH groups which are essential for their catalytic activities.[37] However, the regulatory enzymes of glycolysis are very sensitive to SH-blocking agents.[38,39] In this regard, we investigated the level of total (T-SH, non-protein + protein SH) as well as non-protein (NP-SH) thiol groups, in Ehrlich cells (Table 1) after the action of amine oxide. dePNO decreased the level of both thiol groups in Ehrlich cells. However, the decrease in the level of NP-SH was markedly higher over the whole concentration range. The decrease in the level of T-SH groups was not large enough to affect the activity of key enzymes of glycolysis, i.e. hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12).

Amine oxide stimulated the aerobic glycolysis of EAC in the whole range of concentrations studied (Figure 2). Such a stimulation of glycolysis by amine oxide pointed to potential interference with the respiratory processes in cancer cells or in isolated mitochondria, respectively.[27] Experiments were carried out in order to verify this, the results of which are presented in Tables 2 and 3. The inhibitory effect was characterized by IC_{50} values (molar concentration of compound required for 50% reduction of oxygen uptake). As shown in Tables 2 and 3, alkylpiperidine *N*-oxides containing alkyl chains shorter than C_{10} were found to be less effective (Table 2). All other derivatives markedly inhibited endogenous respiration of both tumor cells and the extent of inhibition was dependent on the concentration of the amine oxide in the incubation medium. Maximum activities were achieved with compounds IV–VI (length of the joining chain C = 12, 13 and 14). Further lengthening of the side-chains (R) led to decrease in activity. The rate of oxygen consumed was calculated immediately after amine oxide addition.

To obtain direct evidence indicating interference of the amine oxide with energy-generating systems, we studied the effect of dePNO on the level of ATP in Ehrlich ascites cells (Table 4). Amine oxide immediately after addition to the suspension of Ehrlich ascites cells in an ice bath markedly decreased the level of ATP over the whole concentration range. After 2 h incubation at 37°C the drop in the ATP level is lower. The decrease in ATP level might be explained through the amine oxide's interaction with the processes related to oxidative phosphorylation or indirectly through impairment of cell membrane integrity.

Table 1. Effect of dePNO on T-SH and NP-SH content in Ehrlich cells after 120 min of incubation *in vitro*

μmol/l	T-SH		NP-SH	
	A^{1}_{412} nm	% of the control	A^{1}_{412} nm	% of the control
0	1.050	100	0.498	100
75	0.900	85.7	0.409	82.1
150	0.864	82.3	0.363	72.9
300	0.785	74.8	0.283	56.8
600	0.745	71.0	0.167	33.5

Discussion

The results described here show that amine oxides interfere with energy-yielding processes in tumor cells. dePNO stimulated the aerobic glycolysis of Ehrlich cells over the whole range of concentrations studied. Diamond *et al.*[40] have also shown that agents such as dinitrophenol (an uncoupler of oxidative phosphorylation) and oligomycin (an inhibitor of oxidative phosphorylation), which interfere with ATP synthesis, markedly stimulated lactic acid production by intact quiescent 3T3 cells; however, the effect of oligomycin occurs at much lower concentrations than that of dinitrophenol. Increased glycolysis, for example, may be an appropriate response to increased energy demand resulting from cellular functions (e.g. ion movement motility), but such conditions are commonly associated with decreased biosynthetic activities which may be supplied with reducing equivalents by the operation of the hexose monophosphate pathway.[41]

Table 2. Effects of 1-alkylpiperidine-*N*-oxide derivatives on endogenous oxygen uptake by Ehrlich ascites cells

No.	*R* dry weight of the cells (mg)	Formula	Molecular weight	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Inhibition (%)	IC_{50} (μmol/l)
I	octyl (14.0)	$C_{13}H_{27}NO$	213.35	0	145	0	
				454.54	145	0	
				904.97	145	0	
				1354.4	145	0	2700
				1801.98	122	16.12	
				2247.19	98	32.25	
				2690.06	75	48.38	
				3131.99	47	67.74	
II	nonyl (14.0)	$C_{14}H_{29}NO$	227.39	0	159	0	
				454.54	159	0	
				909.09	131	17.64	1320
				1363.64	70	55.88	
				1818.18	30	81.18	
				2262.44	0	100.00	
III	decyl (12.25)	$C_{15}H_{31}NO$	241.42	0	122	0	
				88.88	122	0	
				177.77	103	15.38	260
				266.6	56	53.85	
				355.5	23	80.77	
				442.48	0	100.00	
IV	dodecyl (11.47)	$C_{17}H_{35}NO$	269.46	0	136	0	
				25.72	136	0	
				45.45	75	44.83	50
				68.18	37	72.41	
				90.90	14	89.66	
				113.64	0	100.00	
V	tridecyl (12.13)	$C_{18}H_{37}NO$	283.50	0	173	0	
				22.72	140	18.9	
				45.45	56	67.56	35
				68.18	23	86.49	
				90.90	7	95.68	
VI	tetradecyl (11.47)	$C_{19}H_{39}NO$	297.52	0	150	0	
				22.72	140	6.25	
				45.45	94	37.5	55
				68.18	47	68.75	
				90.90	0	100.00	
VII	pentadecyl (11.47)	$C_{20}H_{41}NO$	311.55	0	131	0	
				22.72	131	0	
				45.45	117	10.71	77
				68.18	82	37.14	
				90.90	42	67.85	
				113.64	9	92.86	

The rate of oxygen uptake was determined immediately after the addition of inhibitors to the cells. Cell suspension (0.2 ml) was added to 2.0 ml of isotonic saline phosphate medium pH 7.4. Oxygen uptake was measured at 30°C. Amine oxides were dissolved in Krebs–Ringer phosphate medium shortly before experiments.

It has been shown that Ehrlich cells are glycolysis-dependent in support of their metabolism and growth. Therefore, they have an effective system for glucose transport. Cuppoletti *et al.*[42] showed that the density of glucose carriers in Ehrlich cells is 30 times higher than in erythrocytes. The inhibition of glucose uptake may result in the inhibition of growth in Ehrlich cells. The cytotoxic effects of methotrexate, for example, are at least partially ascribed to its ability to inhibit glucose uptake under *in vitro* conditions.[43] Recently, Medina *et al.*[44] have shown that if Ehrlich ascites cells can

Table 3. Effects of 1-alkylpiperidine-*N*-oxide derivatives on endogenous oxygen uptake by L1210 cells

No.	*R* dry weight of the cells (mg)	Formula	Molecular weight	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Inhibition (%)	IC_{50} (μmol/l)
IV	dodecyl (12.4)	$C_{17}H_{35}NO$	269.46	0	112	0	
				22.22	112	0	
				44.44	67	40	
				66.66	42	62.5	54
				88.88	23	79.17	
				111.11	9	91.67	
				133.33	5	95.83	
V	tridecyl (12.4)	$C_{18}H_{37}NO$	283.50	0	84	0	
				22.22	84	0	
				44.44	56	33.33	58
				66.66	33	61.11	
				88.88	19	77.78	
				111.11	9	88.89	
VI	tetradecyl (12.4)	$C_{19}H_{39}NO$	297.52	0	75	0	
				22.22	70	6.25	
				44.44	65	12.5	79
				66.66	51	31.25	
				88.88	28	62.5	
VII	pentadecyl (12.4)	$C_{20}H_{41}NO$	311.55	0	103	0	
				22.22	103	0	
				44.44	94	9.09	
				66.66	80	22.73	105
				88.88	75	27.27	
				111.11	42	59.09	
				133.33	33	68.18	
				155.55	19	81.82	

The rate of oxygen uptake was determined immediately after the addition of inhibitors to the cells. The experimental conditions were the same as in Table 2.

Table 4. Effect of dePNO on the level of ATP in Ehrlich ascites cells *in vitro*

Minutes of incubation	ATP level (nmol/mg) protein at inhibitor concentrations (μmol/l))				
	0	75	150	300	600
0 (0°C)	9.30	2.95	3.30	2.08	2.95
120 (37°C)	9.00	2.60	2.26	1.91	1.91

choose among different energy substrates (as it is the case in physiological conditions), they choose glucose preferentially.

The studies of several authors indicated that anticancer chemotherapy based on specific inhibitors of NADH-linked respiration may be worth investigating.[22,45] Our results indicate that a substantial percentage of anticancer agents are active respiratory inhibitors.[22] The effects of anticancer drugs on respiration can, in some cases, provide information relevant to the mechanism of action, mechanisms of toxicity and biochemical side-effects of the compounds.[45] Amine oxide inhibited endogenous respiration of both tumor cells (Tables 2 and 3). The inhibition of respiration can be due to the escape (release) of endogenous substrates from the intracellular space into the extracellular environment as a result of amine oxides interference with the integrity of biological membranes of these cells. In a previous paper[46] we observed that amine oxides show considerable cytolytic activity, particularly at higher concentrations. Membranous effects were demonstrated by several methods. It is well known that loss of ATP by diminished respiration is balanced by an approximately equal gain of ATP by an increased rate of lactate production.[47] However, due to an impairment of the integrity of biological membranes, such a compensation does not take place in a measure sufficient to maintain ATP at the required level (Table 4).

In most cell types, even in tumor cells, ATP is predominantly formed by oxidative phosphorylation. The energy-requiring processes cannot distinguish between the ATP formed by mitochondrial

oxidative phosphorylation and that produced by cytosolic glycolysis. In Ehrlich ascites cells it was found that approximately 30% of total ATP produced was consumed by protein synthesis, 5–10% by ATP- and ubiquinone-dependent proteolysis, approximately 20% by Na^+/K^+-ATPase, approximately 10% by Ca^{2+}-ATPase and approximately 10% by the transcription processes.[48]

The capacity of amine oxide to inhibit both respiration and ATP production of tumor cells makes this drug worthy of further interest. In fact, any attempt to inhibit tumor cell growth and survival by interfering with tumor cell energy production must take into account the ability of these cells to utilize equally well both oxidative phosphorylation and glycolysis to support cell growth.[49]

Although the majority of currently used anticancer drugs are cytotoxic, either by inhibiting DNA synthesis or by damaging the DNA template by alkylation or intercalation, Hill[50] emphasizes that this is an oversimplification. Most agents have multiple effective target sites within the cell, especially in the case of thiol reagents. The work by Farber[51] and others[22,49] indicates that inability to synthesize ATP in a cell leads to multiple secondary derangements in cellular metabolism.

As found recently,[52] the antimicrobials (1-methyldodecyl)dimethylamine oxide and (1-methyldodecyl)trimethylammonium bromide affect the cytoplasmic membrane of *Escherichia coli*. The interaction results in release of intracellular material (K^+, 260 nm absorbing material), effect on dehydrogenase enzyme activity and inhibition of respiration. The final effect of both substances is the same—they differ only in their dynamics. Kopecka-Leitmanova *et al.*[53] summarized their results about the mode of action of quaternary ammonium salts and amine oxides upon bacteria determined *in vitro* in three stages. In the second stage the polar and hydrophobic interactions are involved—this stage is the destructive effect on membranes.

It is evident that the site of action of the amine oxides investigated was the biological membrane which, after the interaction with amine oxides, showed changes in molecular organization and osmotic and permeability characteristics (Miko and Devinsky, in preparation). Modification of the lipid and consequent physical properties of membranes has been shown to enhance the sensitivity of neoplastic cells to certain anticancer drugs in tissue culture and this approach should be investigated for its potential therapeutic value.[54]

Tumor cell membranes are potentially important targets for selective chemotherapeutic attack.[55] Further research is needed to elucidate the functional consequences of structural and conformational changes in cell membrane molecules, in order to permit the development of new classes of selectively toxic antitumor drugs (for a review, see Hickman[56]).

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