EFFECTS OF 4-ALKYLMORPHOLINE N-OXIDES ON ATP-PRODUCING PROCESSES IN EHRLICH ASCITES AND L1210 LEUKAEMIA CELLS

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SUMMARY

The main purpose of the present investigation was to study the effect of the homologous series of 4-alkylmorpholine N-oxides on ATP-producing processes in Ehrlich ascites and L1210 murine leukaemia cells. The effects on aerobic glucose consumption, lactic acid formation, content of total (T-SH) and non-protein thiol groups (NP-SH), endogenous and exogenous respiration and the level of ATP in tumour cells incubated *in vitro* were investigated. 4-Dodecylmorpholine N-oxide (DMNO), one of the most active compounds, decreased the level of ATP immediately after addition to the suspension of Ehrlich cells in an ice bath. After 2 h incubation at 37°C the drop in the ATP level was much lower. A possible explanation for the decrease in the ATP level might be interaction of the amine oxide with the cell membrane.

KEY WORDS

4-alkylmorpholine N-oxides, Ehrlich cells, L1210 cells, glycolysis, respiration, ATP, thiol groups

INTRODUCTION

Non-aromatic amine oxides are widely known compounds with many uses. They 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 utilisation due to their surface active properties /3/, relatively little attention has been paid to their biological activity, in contrast to the 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 shown that they have a concentration dependent influence on the immune system, thus opening new perspectives in the field of immunomodulation. The immunosuppression of certain compounds of this type was found to be as high as that of cyclosporin A /11,12/, which is today one of the most powerful immunosuppressants known.

In our previous paper /13/ it was shown that cytotoxic activity of 4-alkylmorpholine N-oxides increased with increasing alkyl chain length, reaching a maximum with C_{13} to C_{15}. The 4-alkylmorpholine N-oxides containing an alkyl chain shorter than C_{10} were found to be less effective. For quantification the bilinear approach was used which is superior to the classical Hansch's parabolical model as already shown /14-17/.

Up to now, however, so far nothing is known about the action of 4-alkylmorpholine N-oxides (Figure 1) on energy-yielding processes in tumour cells. Furthermore, as macromolecular biosynthesis is an energy-requiring process we followed the effects of 4-alkylmorpholine 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 and exogenous respiration and levels of ATP, in both Ehrlich ascites carcinoma and L1210 murine leukaemia cells. Linker *et al.* /18/ calculated that Ehrlich cells, grown in standard medium, produced 60% of ATP via oxidative pathways and 40% via glycolysis. Beckner *et al.* /19/ showed that the motility of metastatic cells in the human melanoma line A2058 depends on the presence of glucose, i.e. primarily on energy from glycolysis. These findings suggest that the inhibition of

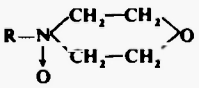
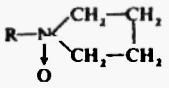
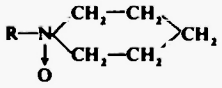
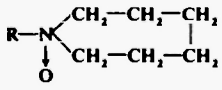
Group	Structural formula	Derivatives of	R	Number of compounds
A		morpholine	C_1 to C_{18}	18
B		pyrrolidine	$C_{12,14,16,18}$	4
C		piperidine	C_8 to C_{15}	8
D		perhydroazepine	C_8 to C_{16}	8
	Total number			38

Fig. 1: Survey of N-oxides investigated.

glycolysis *in vivo* might reduce the ability of tumour cells to leave the primary mass and metastasise to secondary sites.

Ehrlich ascites tumour cells have been extensively used as an experimental model for biochemical investigation /20,21/. We have used Ehrlich cells also for the study of the mechanism of action of some antibiotics /22/, ethidium bromide /23/, isothiocyanates /24/ and other known cancerostatics /25/.

MATERIALS AND METHODS

Ehrlich ascites carcinoma cells were maintained and propagated in strain H Swiss albino mice (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Dobra Voda, Slovakia), about 10 weeks old and 20 to 25 g body weight, as described previously /26,27/. Ehrlich ascites cells were transplanted at 7-day intervals by intraperitoneal injection of 0.2 ml of ascitic fluid collected under sterile conditions. The tumour 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. Ascitic plasma was

poured off and an incidental layer of erythrocytes was removed /28/. 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 $5x10^6$/ml of medium /27/. All operations were performed at 0-4°C.

Materials

Chromatographically pure amine oxides were from Dept. of Inorganic and Organic Chemistry, Faculty of Pharmacy, Komensky University, Bratislava. Substances were dissolved in Krebs-Ringer phosphate medium shortly before experiments. 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, California, USA). All other reagents were obtained from Sigma Chemical Co.

Glucose uptake and lactic acid production

The kinetics of aerobic glucose uptake and lactic acid production by both cell types were determined by commercially available tests (Boehringer, Mannheim, Germany) as described previously /29/. The concentration of glucose and lactate was determined enzymatically in the supernatant obtained after precipitation of suspensions of EAC and L1210 cells with 1 ml 0.6 mol/l perchloric acid in an ice bath. The precision of these measurements is ±3%. Each measurement was done in duplicate and the results are the mean values for two separate experiments, which did not vary by more than 10%.

Determination of total (T-SH) and non-protein (NP-SH) thiol groups

The determination was done according to the method of Ellman /30,31/ modified by Sedlak and Lindsay /32/ and Drobnica *et al.* /33/. 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 mole of 2-nitro-5-mercaptobenzoic acid per mole SH. This anion is of bright yellow colour (E_{412} = 13.600 $M^{-1}cm^{-1}$).

Respiration

The effect of amine oxides on endogenous respiration of Ehrlich cells was determined on the basis of oxygen consumption in an 11 mmol/l sodium phosphate buffer, pH 7.4, containing 154 mmol/l NaCl and 6.2 mmol/l KCl /28/. Amine oxides were mixed with 2.0 ml of this medium, and 200 μl of the cell suspension in the same buffer was added.

Respiration with succinate as exogenous substrate was studied in the MES medium /34/ containing 150 mmol/l NaCl, 5 mmol/l KCl, and 10 mmol/l MES 2-(N-morpholino)ethanesulphonic acid, pH 6.2. For the studies involving inhibition of cellular endogenous respiration rotenone was present at a concentration of 3 μmol/l.

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 those described earlier /23,24/. Oxygen consumption was monitored for approximately 10 min and the linear portion of the oxygen consumption curve was used to calculate 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 in detail in a previous paper /35/. The ATP level was determined by a commercially available test (Boehringer, Mannheim, Germany) in the supernatant after precipitation of Ehrlich cells with 0.6 mol/l $HClO_4$, neutralized by the addition of 5 mol/l K_2CO_3. Samples were centrifuged (4000 *g*, 10 min at 4°C) to remove potassium perchlorate salts and filtered. The supernatant was used for the quantitative determination of ATP level (0.2 ml). The decrease in $NAD+H^+$ absorbance was followed at 340 nm during 10 min. From the absorbance decrease per minute ΔA was calculated and after conversion, the ATP level was determined.

Protein determination

Protein concentration in the cell suspension was done according to the method of Lowry *et al.* /36/.

RESULTS

On the basis of our previous results /13/, one of the most active compounds, namely 4-dodecylmorpholine N-oxide (DMNO), was chosen for further biochemical study. Figure 2 demonstrates the effect of DMNO on aerobic glucose utilisation (G) and lactic acid formation (L) by Ehrlich ascites cells. Ehrlich cells consumed glucose from the medium linearly with time, even after the concentration had fallen by more than 50 per cent of its original value. A likewise proportional

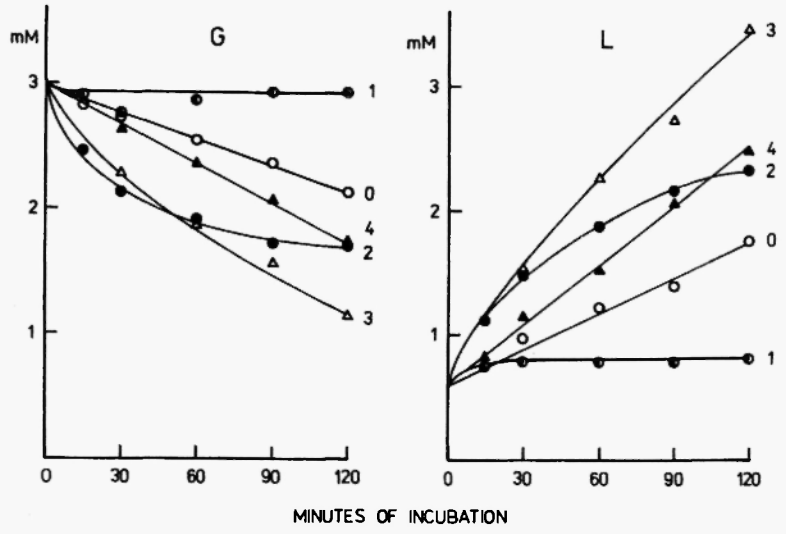


Fig. 2: The effect of 4-dodecylmorpholine N-oxide (DMNO) on the kinetics of aerobic glucose utilisation (G) and lactic acid formation (L) by Ehrlich ascites cells. The cells were incubated at 37°C in the presence of different concentrations of DMNO. The initial glucose concentration was 3 mmol/l. At various times, 1-ml samples of suspension were analysed for glucose and lactate. Compound concentrations: 0 = none (O), 1 = 600 (◑), 2 = 300 (●), 3 = 150 (Δ), 4 = 75 (▲) μmol/l.

increase in lactate concentration in the medium was seen in control Ehrlich ascites cells. The conversion of glucose to lactate in control Ehrlich ascites cells was approximately 76 per cent (calculated for the first 30 min of measuring glycolysis) which is in good agreement with our previous results /27/, in which the transformation of glucose into lactate was in the range of 75.0 - 83.5%.

As shown in Figure 2, the effect of DMNO depended on its concentration in the medium. This compound at the highest concentration tested (600 μmol/l) causes rapid and practically complete inhibition of glycolysis, as judged from the point of stopping glucose consumption or lactate formation. At an intermediate concentration (300 μmol/l), there is a progressive inhibition and after 60 min, glycolysis stops practically completely. At concentrations as low as 150 and 75 μmol/l, glycolysis is stimulated.

It is known that the key glycolytic regulation enzymes contain cysteine SH-groups which are essential for their catalytic activities /37/. On the other hand, 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 treatment with amine oxide. 4-Dodecylmorpholine N-oxide decreased both the total and non-protein content of SH groups in a concentration dependent manner. However, the decrease in the level of NP-SH was significantly higher over the whole concentration range. The decrease in the level of thiol groups might be sufficiently great (at the highest concentration) 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). The inhibition of glycolysis in the presence of amine oxide may occur as a consequence of several events. Whether this inhibition is a consequence of inactivation of key glycolytic enzymes as a result of chemical modification of catalytically active sulfhydryl groups remains to be answered by direct investigation.

Amine oxide at the lowest concentration stimulated aerobic glycolysis of Ehrlich ascites cells (Fig. 2). Such a stimulation of glycolysis at low concentrations of DMNO indicated a potential interference with respiratory processes in tumour cells, or in isolated mitochondria /29/. Experiments were carried out in order to verify this, the results of which are presented in Tables 2-4. The inhibitory effect was characterised by IC_{50} values (molar concentration of

TABLE 1

Effect of 4-dodecylmorpholine N-oxide on total SH-group content (T-SH) and non-protein SH-group (NP-SH) content in Ehrlich cells after 120 min of incubation *in vitro*.

/umol/l	T-SH		NP-SH	
	$A^1_{412\ nm}$	% of the control	$A^i_{412\ nm}$	% of the control
0	1.05	100.0	0.498	100.0
75	0.84	79.8	0.348	69.9
150	0.80	76.6	0.249	50.0
300	ND	ND	0.178	35.7
600	0.321	30.6	0.078	15.7

ND = not determined

compound required for 50% reduction of oxygen uptake). As shown in Table 2, derivatives I-X showed little effect on endogenous oxygen utilization by Ehrlich cells. Derivatives with longer side-chains markedly inhibited endogenous respiration of Ehrlich cells. The lengthening of the alkyl chain in 4-alkylmorpholine N-oxides positively affected their inhibitory activity in Ehrlich cells. Maximum activity was achieved with the compound no. XIII (IC_{50} 73 μmol/l). Further lengthening led to decrease in activity.

On the basis of the results in Table 2 we obtained a profile of the inhibitory activity of 18 derivatives of 4-alkylmorpholine N-oxides. In order to extend the present investigation, we examined the effects of selected 4-alkylmorpholine N-oxides also on endogenous oxygen uptake of L1210 murine leukaemia cells (Table 3). Amine oxides inhibited oxygen uptake in a similar way as in Ehrlich cells. The extent of inhibition was dependent on the concentration of the drug in the incubation medium.

TABLE 2

Effects of 4-alkylmorpholine N-oxide derivatives on endogenous oxygen uptake by Ehrlich ascites cells

No	R	Formula	M.W.	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Per cent of inhibition	IC_{50} (μmol/l)
	dry weight of the cells (mg)						
I-III	methyl, ethyl, propyl						> 9000
IV	butyl	$C_8H_{17}NO_2$	159.23	0	144	0	
	(16.6)			1363.6	102	29.2	1800
				2727.3	24	83.3	
				4515.4	0	100.0	
V	pentyl	$C_9H_{19}NO_2$	173.24	0	168	0	
	(15.5)			444.4	138	17.86	
				666.6	108	35.7	870
				888.8	66	60.7	
				1111.1	42	75	
				1333.3	24	85.7	
VI	hexyl	$C_{10}H_{21}NO_2$	187.28	0	168	0	
	(15.5)			666.6	168	0	
				1111.1	150	10.7	
				2222.2	144	14.3	> 4444.4
				2666.6	132	21.4	
				3333.3	132	21.4	
				4414.4	126	25.0	
VII	heptyl	$C_{11}H_{23}NO_2$	201.30	0	134	0	
	(17.7)			133.15	134	0	
				265.95	117	12.5	560
				442.47	72	45.8	
				881.05	28	79.16	

TABLE 2 (cont.)

No	R dry weight of the cells (mg)	Formula	M.W.	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Per cent of inhibition	IC_{50} (μmol/l)
VIII	oktyl (17.7)	$C_{12}H_{25}NO_2$	215.33	0	133	0	
				1108.64	134	0	
				2212.38	128	4.17	
				3311.25	117	12.5	10869.5
				4405.28	106	20.83	
				6578.9	106	20.83	
				8733.6	89	33.3	
				10869.5	67	50.0	
IX	nonyl (17.7)	$C_{13}H_{27}NO_2$	229.37	0	134	0	
				1108.64	134	0	
				2212.38	128	4.17	
				3311.25	117	16.66	6300
				4405.28	99	26.37	
				5494.5	78	41.66	
				7658.6	45	66.6	
X	decyl (17.7)	$C_{14}H_{29}NO_2$	243.40	0	134	0	
				444.25	117	16.66	
				709.85	89	33.33	1330
				1329.78	67	50.0	
				1771.48	45	66.66	
XI	undecyl (17.7)	$C_{15}H_{31}NO_2$	257.42	0	181	0	
				90.91	171	5.87	
				181.81	155	14.695	
				272.72	107	41.17	310
				372.63	69	61.76	
				452.483	37	79.4	
				545.45	27	85.29	

TABLE 2 (cont.)

No	R dry weight of the cells (mg)	Formula	M.W.	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Per cent of inhibition	IC_{50} (μmol/l)
XII	dodecyl	$C_{16}H_{33}NO_2$	271.45	0	171	0	
	(12.4)			45.45	151	11.76	
				90.91	101	41.12	110
				136.36	69	60.0	
				181.81	32	81.18	
XIII	tridecyl	$C_{17}H_{35}NO_2$	285.47	0	171	0	
	(12.4)			45.45	136	20.59	
				90.91	61	64.7	73
				136.36	35	79.4	
				181.81	15	91.18	
				227.27	10	94.12	
XIV	tetradecyl	$C_{18}H_{37}NO_2$	299.50	0	161	0	
	(12.4)			45.45	151	6.25	
				90.91	116	28.13	120
				136.36	68	57.81	
				181.81	22	86.25	
				227.27	0	100.0	
XV	pentadecyl	$C_{19}H_{39}NO_2$	313.53	0	161	0	
	(12.4)			45.45	151	6.25	
				90.91	131	18.75	
				136.36	91	43.75	150
				181.81	40	75.0	
				227.27	10	93.75	
				272.72	0	100.0	
XVI	hexadecyl	$C_{20}H_{41}NO_2$	327.56	0	152	0	
	(13.8)			113.64	126	16.7	
				226.24	71	53.36	220
				338.6	20	86.67	
				450.45	0	100.0	

TABLE 2 (cont.)

No	R dry weight of the cells (mg)	Formula	M.W.	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Per cent of inhibition	IC_{50} (μmol/l)
XVII	heptadecyl	$C_{21}H_{43}NO_2$	341.57	0	151	0	
	(13.8)			177.77	140	0	
				266.6	131	6.66	660
				355.5	117	16.66	
				442.48	112	20.0	
				530.58	94	33.3	
				618.37	74	46.6	
XVIII	oktadecyl	$C_{22}H_{45}NO_2$	355.61	0	168	0	
	(13.8)			93.5	141	0	
				186.05	141	0	
				279.07	131	7.18	> 558
				372.09	121	14.32	
				465.12	111	21.47	
				558.14	96	32.29	

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 Kiebs-Ringer phosphate medium shortly before experiments

TABLE 3

Effects of 4-alkylmorpholine N-oxide derivatives on endogenous oxygen uptake by L1210 cells

No	R	Formula	M.W.	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Per cent of inhibition	IC_{50} (μmol/l)
	dry weight of the cells (mg)						
X	decyl	$C_{14}H_{29}NO_2$	243.40	0	94	0	
	(12.75)			222.2	84	10	
				442.48	75	20	
				662.25	56	40	780
				881.06	39	58	
				1098.9	28	70	
				1315.79	23	75	
XI	undecyl	$C_{15}H_{31}NO_2$	257.42	0	98	0	
	(12.75)			88.88	98	0	
				177.77	75	23.81	
				266.6	56	42.86	300
				355.5	37	61.91	
				442.48	23	76.19	
				530.5	14	85.72	
XII	dodecyl	$C_{16}H_{33}NO_2$	271.45	0	98	0	
	(12.75)			44.44	84	14.29	
				88.88	65	33.34	120
				133.3	37	61.91	
				177.77	23	76.19	
				222.2	14	85.72	
XIII	tridecyl	$C_{17}H_{35}NO_2$	285.47	0	89	0	
	(12.75)			44.44	80	10.53	
				88.88	51	42.11	100
				133.3	30	66.32	
				177.77	17	81.05	
				222.2	0	100	

TABLE 3 (cont.)

No	R dry weight of the cells (mg)	Formula	M.W.	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Per cent of inhibition	IC_{50} (μmol/l)
XIV	tetradecyl (8.57)	$C_{18}H_{37}NO_2$	299.50	0	126	0	
				44.44	108	14.81	
				88.88	70	44.44	105
				133.3	37	70.37	
				177.7	19	85.19	
				222.2	0	100	
XV	pentadecyl (8.57)	$C_{19}H_{39}NO_2$	313.53	0	126	0	
				44.44	126	0	
				88.88	112	11.11	
				133.3	67	46.66	150
				177.7	37	70.37	
				222.2	23	81.48	
				265.6	11	88.88	

The rate of oxygen uptake was determined immediately after the addition of inhibitors to the cells. The experimental conditions are the same as in Table 1.

TABLE 4

Effects of 4-alkylmorpholine N-oxide derivatives on the oxidation of succinate by Ehrlich ascites cells

No	R dry weight of the cells (mg)	Formula	M.W.	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Per cent of inhibition or stimulation (+)
X	decyl (14.7)	$C_{14}H_{29}NO_2$	243.40	0	33	0
				319.99	26	20
				639.11	21	37.13
				957.45	14	57.13
				1274.97	9	71.42
XI	undecyl (14.7)	$C_{15}H_{31}NO_2$	257.42	0	33	0
				85.31	28	14.27
				170.47	23	28.56
				255.48	19	42.85
				340.35	19	42.85
				425.08	14	57.14
				509.66	11	65.71
XII	dodecyl (14.7)	$C_{16}H_{33}NO_2$	271.45	0	37	0
				85.31	37	0
				170.47	28	25
				255.48	23	37.5
				340.35	21	45
				425.08	23	37.5
				509.66	45	+20
				594.1	56	+50
XIII	tridecyl (14.7)	$C_{17}H_{35}NO_2$	285.47	0	33	0
				85.31	33	0
				170.47	33	0
				255.48	33	0
				340.35	61	+85
				425.08	94	+183.7
				509.66	89	+171.43

TABLE 4 (cont.)

No	dry weight of the cells (mg)	Formula	M.W.	Concentration of the inhibitor (μmol/l)	Oxygen uptake (natoms/min)	Per cent of inhibition or stimulation (+)
XIV	tetradecyl (14.7)	$C_{18}H_{37}NO_2$	299.50	0	38	0
				85.31	33	12.5
				170.47	33	12.5
				255.48	37	0
				340.35	98	+162.5
				425.05	117	+212.5
XV	pentadecyl (15.7)	$C_{19}H_{39}NO_2$	313.53	0	33	0
				85.31	28	14.27
				170.47	26	19.99
				255.48	26	19.99
				340.35	80	+142.89
				425.05	98	+142.89
XVI	hexadecyl (15.7)	$C_{20}H_{41}NO_2$	327.56	0	28	0
				85.31	28	0
				127.88	28	0
				170.47	19	33.33
				212.77	14	50
				254.02	12	56.67
				296.11	11	60.6
XVII	heptadecyl (15.7)	$C_{21}H_{43}NO_2$	341.57	0	23	0
				128.01	20	16
				170.6	14	40
				213.17	9	60
				255.69	7	68

The rate of oxygen uptake was determined immediately after the addition of amine oxides to the cells. Cell suspension (0.2 ml) was added to 2.0 ml of MES medium pH 6.2. Succinate was present at a concentration of 10 mmol/l and rotenone 3 μmol/l. Oxygen uptake was measured at 30°C.

Inhibitory activity, expressed as IC_{50} values, increased with increasing alkyl chain, reaching a maximum with C_{12} to C_{14} (Fig. 3). The 4-alkylmorpholine N-oxides containing an alkyl chain shorter than C_{10} were found to be less effective. Ehrlich cells were more "sensitive" than L1210 murine leukaemia cells (Fig. 3).

Table 4 demonstrates the effects of amine oxides on the exogenous respiration of Ehrlich ascites cells. As demonstrated by Spencer /34/, succinate may be transported across the cell membrane by the organic anion carrier system. The uptake of succinate is maximal at pH 6.2. Therefore this pH value was maintained in these experiments. The rate of exogenous respiration is considerably lower than that of endogenous respiration. The results in Table 4 show a different

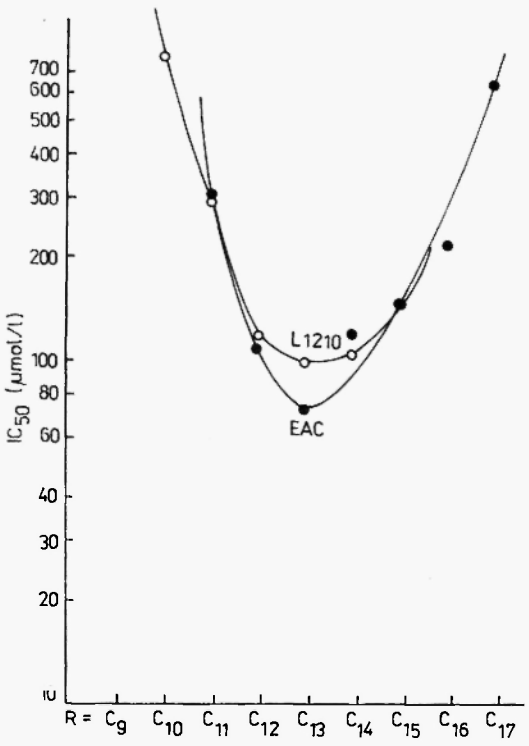


Fig. 3: Relationships between IC_{50} values (endogenous respiration) and the length of side-chain in 4-alkylmorpholine N-oxides.

picture from that in Tables 2 and 3. Some derivatives of amine oxides (decyl-, undecyl-, hexadecyl- and heptadecyl-) exert an almost identical inhibitory effect on exogenous respiration of Ehrlich ascites cells. On the other hand, derivatives dodecyl-, tridecyl-, tetradecyl- and pentadecyl-, at low concentrations, inhibited oxygen uptake of Ehrlich cells and at the highest concentrations stimulated exogenous respiration of these cells.

To obtain direct evidence indicating interference of the amine oxide with energy-generating systems, we studied the effect of 4-dodecylmorpholine N-oxide on the ATP level in Ehrlich ascites cells (Table 5). Immediately after addition of the amine oxide to the suspension of Ehrlich ascites cells in an ice bath, there was a large decrease in the ATP level. After 2 h incubation at 37°C the drop in the ATP level was much lower. The decrease in ATP level might be explained by the amine oxide's interaction with processes related to oxidative phosphorylation, or indirectly through impairment of cell membrane integrity.

TABLE 5

Effect of 4-dodecylmorpholine N-oxide on the level of ATP in Ehrlich ascites cells *in vitro*

Minutes of incubation	/umol/l				
	0	75	150	300	600
	nmoles of ATP/mg protein				
0 (0°C)	10.62	4.30	4.11	2.78	0.34
120 (37°C)	10.00	3.82	3.13	1.04	0.17

DISCUSSION

The results described here show that amine oxides interfere with energy-yielding processes in tumour cells. DMNO stimulated the aerobic glycolysis of Ehrlich cells at the lowest concentrations. Diamond *et al.* /40/ have shown that agents such as dinitrophenol (an uncoupler of oxidative phosphorylation) and oligomycin (an inhibitor of oxidative phosphorylation), that 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 may be an appropriate response to an 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/.

It has been shown that metabolism and growth in Ehrlich cells are glycolysis-dependent. Therefore, there is 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 choose among different energy substrates (as is the case under physiological conditions), they choose glucose preferentially.

The studies of several authors have indicated that anti-cancer chemotherapy based on specific inhibitors of NADH-linked respiration may be worth investigating /25,45/. The effects of anti-cancer drugs on respiration can, in some cases, provide information relevant to the mechanisms of action, mechanisms of toxicity and biochemical side effects of the compounds /25/. Amine oxides inhibited endogenous respiration of both types of tumour cell (Tables 2 and 3). The reduction of respiratory rate might be due to the effects of the amine oxides on the cell membrane. In a previous paper /46/ we observed that amine oxides show considerable cytolytic activity, particularly at higher concentrations.

In most cell types, including tumour cells, ATP is formed predominantly 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 about 30% of total ATP produced was consumed in protein synthesis, 5-10% in ATP- and ubiquinone-dependent proteolysis, about 20% by the Na^+/K^+-ATPase, about 10% by the Ca^{2+}-ATPase and about 10% by transcription processes /47/.

The capacity of amine oxide to inhibit both respiration and ATP production of tumour cells makes this drug worthy of further study. In fact, any attempt to inhibit tumour cell growth and survival by interfering with tumour cell energy production must take into account the ability of these cells to utilise both oxidative phosphorylation and glycolysis equally well to support cell growth /48/.

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 /49/ emphasises that this is an over-simplification. Most agents have multiple effective target sites within the cell, especially in the case of thiol reagents. The work by Farber /50/ and others indicates that inability to synthesise ATP in a cell leads to multiple secondary derangements in cellular metabolism.

As found recently /51,52/, the antimicrobials (1-methyldodecyl) dimethylamine oxide and (1-methyldodecyl) trimethylammonium bromide affect the cytoplasmic membrane of *E. coli*. This interaction results in release of intracellular material (K^+, 260 nm-absorbing material), an 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/ summarised their results about the mode of action of quaternary ammonium salts and amine oxides upon bacteria determined *in vitro* in three stages.

It is evident that the site of action of the amine oxides investigated was the biological membrane which, after interaction with amine oxides, showed changes in molecular organisation and osmotic and permeability characteristics.

Many types of agents have been found to have membrane action even though they were originally designed to inhibit the synthesis or function of DNA /54/. New drugs have been synthesised which have lipophilic or membrane-selective structures and some of these are in early clinical trials /54/.

The surface membrane alterations which characterise the neoplastic transformation offer a potential for cytotoxic selectivity. 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/.

Tumour 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 antitumour drugs (for review see /56/).

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