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In vitro cytotoxic activity of 1-alkylpiperidine *N*-oxides and quantitative structure–activity relationships

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The main objective of the present investigation was to screen a series of 1-alkylpiperidine *N*-oxides for *in vitro* cytotoxicity, and to find out whether there is a quantitative structure–activity correlation (QSAR) between cytotoxic effect represented here by inhibition of incorporation of [^{14}C]adenine into nucleic acid or [^{14}C]valine into proteins in Ehrlich ascites carcinoma (EAC) cells and structure (as a structural parameter the number of carbon atoms *m* in the alkyl chain was used). On the basis of primary screening, one of the most active compounds, i.e. 1-decylpiperidine *N*-oxide, was chosen for further biochemical study. The drug inhibited the incorporation rate of ^{14}C-labeled precursors (adenine, thymidine, uridine, valine) into appropriate macromolecules of Ehrlich cells, the extent of inhibition being dependent on both time and concentration of the compound in the incubation medium. The lengthening of the alkyl chain in 1-alkylpiperidine *N*-oxides positively affected their cytotoxic activity in Ehrlich cells. For these compounds the optimal *m* value is 12–15.

Key words: 1-Alkylpiperidine *N*-oxides, cytotoxicity, Ehrlich cells, mode of action, QSAR.

Introduction

Non-aromatic amine oxides are widely known and used compounds. 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.). Although 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] recently Ferencik *et al.*[10] have 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.

In the present study cytotoxic activities and mode of action of 1-alkylpiperidine *N*-oxides have been investigated. One of the goals of this presentation is to find out if there is a quantitative structure–activity correlation (QSAR) between cytotoxic effect represented here by inhibition of incorporation of [^{14}C]adenine into nucleic acids or [^{14}C]valine into proteins in Ehrlich ascites carcinoma (EAC) cells and the structure (as a structural parameter the number of carbon atoms *m* in the alkyl chain was used). The bilinear approach was used for quantification, which is superior to the classical Hansch's parabolical model, as already shown.[13–16] The chemical structure of the substances studied is 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.[17]

Ehrlich ascites tumor cells have been extensively used as an experimental model for biochemical investigation.[18,19] We have also used Ehrlich cells for the study of the mechanism of action of some antibiotics,[20] ethidium bromide,[21] isothiocyanates[22] and other known cancerostatics.[23]

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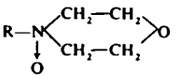
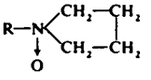
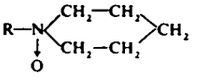
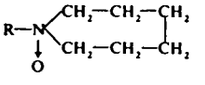
Group	Structural formula	Derivatives of	R	Number of compounds
A		morpholine	C_1 to C_{18}	18
B		pyrrolidine	$C_{12,14,16,18}$	4
C		piperidine	C_8 to C_{15}	8
D		perhydroazepine	C_9 to C_{16}	8
	Total number			38

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

Materials and methods

Cells

EAC cells were maintained and propagated in strain H Swiss albino mice (Institute of Experimental Pharmacology, Dobra Voda, Slovakia), about 10 weeks old and of 20–25 g body weight, as described previously.[24,25] EAC cells were transplanted at 7 day intervals by intraperitoneal injection of 0.2 ml ascitic fluid collected under sterile conditions. Ascitic plasma was poured off and an incidental layer of erythrocytes was removed.[26] 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.[25] All operations were performed at 0–4°C.

Materials

Chromatographically pure amine oxides were from the Department of Inorganic and Organic Chemistry, Faculty of Pharmacy, Komensky University, Bratislava. Substances were dissolved in water shortly before experiments. [8-^{14}C]Adenine sulfate (specific activity, 44 mCi/mmol), [U-^{14}C]-valine (specific activity, 175 mCi/mmol), [2-^{14}C]thymidine (specific activity, 53 mCi/mmol), and [2-^{14}C]uridine (specific activity, 53 mCi/mmol) came from the Institute for Research, Production and Application of Radioisotopes, Prague. Other chemicals were supplied by Boehringer, Mannheim, Germany. All other reagents were obtained from Sigma (St Louis, MO).

Primary biochemical screening (cytotoxicity assays)

In our laboratory, a new system has been developed and is being used routinely for mass screening of candidate compounds for antineoplastic activity.[27–29] The procedure used in evaluating the cytotoxic effect of the compounds was similar to that used when testing other metabolic inhibitors.[28,30] In short, cells were incubated for 1 h in the presence of at least four selected concentrations of the substance, under defined conditions *in vitro*, and the active synthesis of nucleic acids and proteins was followed. After 1 h of drug exposure, the test tubes were transferred into an ice bath. [8-^{14}C]Adenine was added to the first series to a final concentration of 0.187 μCi per 1.02 μg and L-[U-^{14}C]valine was added to the second series to a final concentration of 0.165 μCi per 2.64 μg. Both series were again incubated for 1 h at 37°C. In control experiments only Krebs–Ringer phosphate medium or ethanol were used. Incorporation was terminated by adding 1 ml of 5% trichloroacetic acid (TCA) to each test tube in an ice bath. The samples were filtered through synpor membrane filters, pore size 4 μm (Synthesia, Prague), the precipitate washed with 10 ml of cold 2.5% TCA and 10 ml water, and dried at 105°C. The radioactivity was measured on a methane flow counter (Frieseke und Hoepfner, Erlangen, Germany).

Kinetics of DNA, RNA and protein synthesis

To define further the mechanisms of action of selected drugs, the kinetics of DNA, RNA and protein synthesis inhibition were examined using isotope incorporation. This method has been described in detail.[30] The cells were incubated in a water bath at 37°C without shaking. At the indicated time intervals, samples of suspensions (1 ml) were analyzed for radioactivity in acid-insoluble material. Radioactivity was measured on a methane flow counter as in primary biochemical screening. In some cases, the nature of the labeled material was checked by alkaline–acid hydrolysis. In the case of adenine incorporation, 60.6% of the incorporated radioactivity corresponds to the RNA fraction and 39.4% corresponds to that of DNA. In the case of thymidine, 90% of its incorporation was found in DNA. In the case of uridine, 87.5% of the radioactivity was found in the RNA fraction.[25] All the data points are from duplicate determinations. The precision of these measurements is ±5%.

Results

Biochemical screening of cytotoxic activity

The results from primary biochemical screening of the cytotoxic activity on EAC cells are summarized in Table 1. For the chemical structures of the substances studied, see Figure 1. The numbers represent counts per minute (c.p.m.) with percentage of inhibition (or stimulation) in parentheses. The inhibitory effect was characterized by IC_{50} values (molar concentration of compound required for 50% reduction of the incorporation rate). As is seen from the results in Table 1, derivatives **I** and **II** show little effect on incorporation of both precursors. However, derivatives with longer side-chains markedly depress the incorporation of both precursors investigated (substances **III–VII**). This has been confirmed not only by percentage inhibition (given in parentheses) but also by IC_{50} values. The lengthening of the connecting (bridging) chain in 1-alkylpiperidine *N*-oxides positively

Table 1. Primary biochemical screening of 1-alkylpiperidine *N*-oxides. The measure of the cytotoxic effect was the degree of inhibition of [^{14}C]adenine (a) and [^{14}C]valine (b) incorporation into TCA-insoluble fraction of EAC cells after 2 h incubation *in vitro*. Inhibition of incorporation in c.p.m. or % (in parentheses)

No.	*R*	Formula	Molecular weight	μmol/l					IC_{50} (μmol/l)	*R*
				0	75	150	300	600		
I	octyl	$C_{13}H_{27}NO$	213.35	(a) 1887 (0) (b) 1473 (0)	1636 (13.31) 956 (35.1)	1826 (3.23) 1030 (30.08)	2280 (+20.83) 1264 (14.19)	1546 (18.07) 784 (46.78)	>600	?
II	nonyl	$C_{14}H_{29}NO$	227.39	1887 (0) 1473 (0)	1708 (9.49) 1792 (+21.06)	1682 (10.86) 1150 (21.93)	1566 (17.02) 752 (48.95)	948 (49.76) 448 (69.59)	600 325	1.84
III	decyl	$C_{15}H_{31}NO$	241.42	1887 (0) 1473 (0)	1392 (26.24) 872 (40.8)	— 440 (70.13)	376 (80.08) 188 (87.24)	368 (80.5) 208 (85.88)	175 130	1.30
IV	dodecyl	$C_{17}H_{35}NO$	269.46	2366 (0) 1766 (0)	1095 (57.3) 834 (52.8)	740 (68.7) 565 (68.0)	— 633 (64.2)	149 (93.7) 173 (90.2)	<75 <75	?
V	tridecyl	$C_{18}H_{37}NO$	283.50	1602 (0) 1720 (0)	450 (71.91) 336 (80.47)	110 (93.13) 62 (96.40)	68 (95.76) 66 (96.05)	74 (95.38) 42 (97.56)	<75 <75	?
VI	tetradecyl	$C_{19}H_{39}NO$	297.52	2400 (0) 3683 (0)	253 (89.46) 1088 (70.50)	181 (92.46) 400 (89.14)	353 (85.29) 219 (94.05)	225 (90.63) 232 (93.70)	<75 <75	?
VII	pentadecyl	$C_{20}H_{41}NO$	311.55	1887 (0) 1473 (0)	198 (89.51) 58 (96.06)	66 (96.51) 168 (88.6)	64 (96.61) 68 (95.38)	186 (90.14) 406 (72.44)	<75 <75	?
				0	12.5	25	50	100		
IV	dodecyl	$C_{17}H_{35}NO$	269.46	1450 (0) 2226 (0)	1199 (17.31) 1811 (18.64)	1043 (28.07) 1416 (36.39)	442 (69.52) 771 (65.36)	409 (71.79) 762 (65.77)	38 35	1.08
V	tridecyl	$C_{18}H_{37}NO$	283.50	1460 (0) 1867 (0)	1474 (+0.96) 1036 (44.50)	700 (52.05) 479 (74.34)	399 (72.67) 284 (84.79)	199 (86.37) 76 (95.93)	23 18	1.28
VII	pentadecyl	$C_{20}H_{41}NO$	311.55	1450 (0) 2226 (0)	731 (49.59) 1096 (50.76)	153 (89.45) 260 (88.32)	195 (86.55) 69 (96.00)	58 (96.00) 66 (97.04)	12.5 12.5	1.00

Substances were dissolved in H_2O shortly before experiments. $R = IC_{50}$ adenine:IC_{50} valine. *Stimulation over 100% against control sample.

affected their cytotoxic activity. IC_{50} values are much higher for the first three substances than for substances **IV–VII**, according to the length of the side-chain. To calculate IC_{50} values, however, much lower concentrations of amine oxides were needed and therefore we repeated the experiments as indicated in the lower portion of the Table 1. IC_{50} values for adenine as well as for valine are very similar. Maximum activity was achieved with the compounds **IV–VII** (Table 1). Further lengthening led to a decrease in activity.

Cytotoxic activity, expressed as IC_{50} values for adenine and valine, increased with increasing alkyl chain length, reaching a maximum with C_{15} (Figure 2). The 1-alkylpiperidine *N*-oxides containing an alkyl chain shorter than C_{10} were found to be less effective ($IC_{50} > 600$ μmol/l). Qualitatively, the same results were obtained for a homologous series of 1-alkylpyrolidine *N*-oxides (results not shown).

With the membrane active monoamine oxide amphiphiles investigated in this paper our interest has been focused on the study of the effect of alkyl chain length change as well as the influence of the heterocycle size upon biological activity manifested as inhibition of incorporation of radioactive labeled adenine and valine in cancer cells.

The relationships $\log(1/IC_{50}) = f(m)$ (m the number of carbon atoms in the alkyl chain $C_mH_{2m} + 1$) showed a non-linear course (Table 2) which was quantified using the bilinear approach

$$\log(1/IC_{50}) = Am + B\log(\beta 10^m + 1) + C.$$

According to the present status of knowledge on non-aromatic amine oxides biological activities in general, with these compounds one can also see that with lengthening the alkyl chain the activity increases and, after having reached a maximum, decreases again. At first analysis the heterocycle size variation (six- and seven-membered ring), in principle, has no effect upon the activity (Table 2). However, the analysis of regression coefficients (equations 1 and 2) and maximum activity [$\log(1/IC_{50})_{max}$] both for adenine and valine—in comparison with each other—leads to more precise results, i.e. the most effective are the perhydroazepine type amine oxides, then the morpholine ones and 'less' active are the piperidine *N*-oxides. It is quite difficult to explain why the piperidine *N*-oxides—although more lipophilic[15,31]—are less active than the more hydrophilic morpholine *N*-oxides. The highest activity of perhydroazepine *N*-oxides is probably due to their higher lipophilicity compared with piperidine and morpholine derivatives.

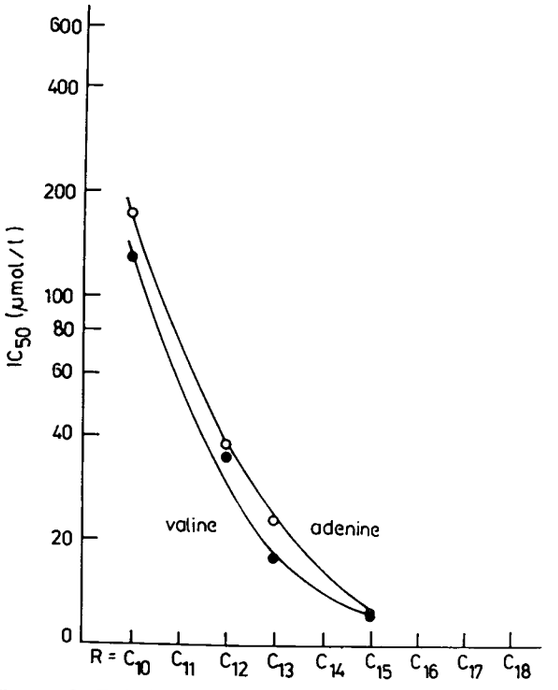


Figure 2. Relationships between IC_{50} values (adenine, valine) and the length of side-chain in 1-alkylpiperidine *N*-oxides.

Table 2. Effect of 1-alkylpiperidine *N*-oxides on [^{14}C]adenine and [^{14}C]valine incorporation into whole EAC cells [at 600 μmol/l; $\log(1/IC_{50})$]

m	[^{14}C]adenine	[^{14}C]valine
8	2.8107	3.1056
9	3.0231	3.3487
10	3.4341	3.6819
12	3.8268	3.7619
13	4.1307	4.3767
14	3.7304	3.6345
15	3.6478	3.3914

Respective regression equations calculated from above data:

$$\log(1/IC_{50})_{AD} = (0.286 \pm 0.030)m - (0.544 \pm 0.092)\log(\beta 10^m + 1) + (0.506 \pm 0.304)$$

$n = 7$, $r = 0.982$, $s_D = 0.108$, $F = 53.56$ $\log \beta = -12.807$, $m_{opt} = 12.9$, $\log(1/IC_{50})_{AD,max} = 4.0070$. (1)

where n is the number of data points used in deriving the regression equation, SD is the standard deviation from regression; r is the correlation index, F is the value of the Fischer–Snedecor test, β is the non-linear parameter in the bilinear equation and '±' values are the 95% confidence intervals.

$$\log(1/IC_{50})_{VAL} = (0.224 \pm 0.056)m - (0.743 \pm 0.209)\log(\beta 10^m + 1) + (1.337 \pm 0.581)$$

$n = 7$, $r = 0.898$, $s_D = 0.219$, $F = 8.30$, $\log \beta = -13.172$, $m_{opt} = 12.8$, $\log(1/IC_{50})_{VAL,max} = 4.0912$. (2)

It is interesting to analyze the optimal calculated values (m_{opt}, equations 1 and 2) for cytotoxic activity related to m. In the case of piperidine *N*-oxides the optimal values, i.e. the most active compounds, possess 12–15 carbon atoms in their aliphatic chain, both for adenine and valine incorporation.

On the basis of our previous results,[27–30] it is convenient to use the IC_{50} adenine:IC_{50} valine ratio (R) as a suitable parameter to indicate the possible primary mode of action of the substance investigated. All ratios, as demonstrated in Table 1, are in the range 1.00–1.84. Such ratios are also typical for other biologically active compounds which interfere with energy-generating systems of cells. Inhibition of energy metabolism may, for example, be due to direct interaction or through the disorganization of the membrane structure.

Effect on macromolecule biosynthesis

The values from biochemical screening represent the first fundamental information about cytotoxic activity of new derivatives. The data obtained in a relatively short time indicate whether the tested substance has cytotoxic activity at all, and perhaps also its possible mode of action (ratio). In a first approach to determine the mode of action of the cytotoxically active compounds, the kinetics of DNA, RNA and protein synthesis inhibition were examined using isotope incorporation. Only when the time course is known is it possible to state at what time and concentration the inhibitory effect appears.

On the basis of primary screening, one of the most active compounds, i.e. 1-decylpiperidine *N*-oxide (DPNO), has been chosen for further biochemical study. Figure 3 demonstrates the inhibitory effect of DPNO upon biosynthesis of macromolecules, indicated by incorporation of [^{14}C]adenine and [^{14}C]valine into TCA-insoluble material of EAC cells.

As can be seen from Figure 3, DPNO at the highest concentrations initially stimulated both incorporation of [^{14}C]adenine and [^{14}C]valine into appropriate macromolecules of EAC cells. However, after 60 min of action, incorporation of [^{14}C]adenine and [^{14}C]valine is markedly inhibited by the two highest concentrations of DPNO. [^{14}C]adenine incorporation was inhibited more than [^{14}C]valine incorporation. The lowest concentrations of the tested substance do not practically affect the incorporation rates of both precursors.

As [^{14}C]adenine is incorporated into both DNA

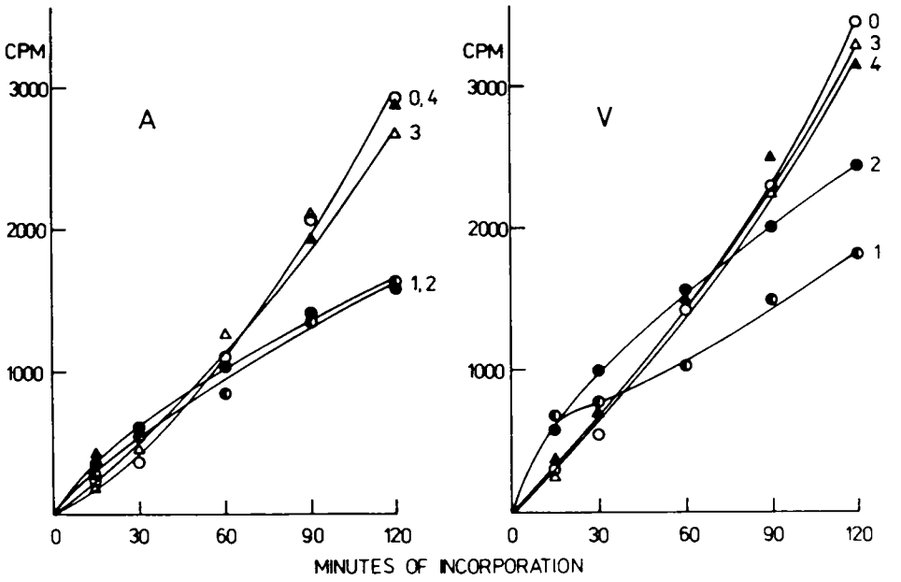


Figure 3. The effect of 1-decylpiperidine *N*-oxide on macromolecule synthesis of EAC cells. Incorporation of radioactive adenine (A) and valine (V) into acid-insoluble fractions was determined by incubating cells with appropriate [^{14}C]precursors. Radioactive precursors and amine oxide were added to the cells at the same time. The test tubes were incubated at 37°C and 1 ml samples of suspension were analyzed for radioactivity in acid-insoluble material. The results are expressed as c.p.m./5 × 10^6 cells. Concentrations: 0 = 0, 1 = 600, 2 = 300, 3 = 150, 4 = 75 μmol/l.

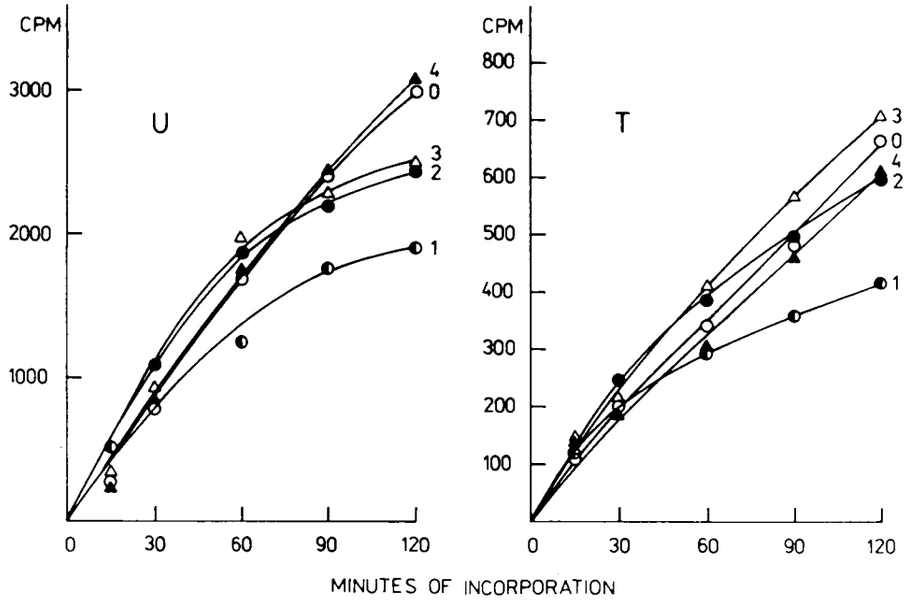


Figure 4. The effect of 1-decylpiperidine *N*-oxide on dynamics of [^{14}C]uridine (U) and [^{14}C]thymidine (T) incorporation into TCA-insoluble fractions of EAC cells. Other experimental conditions and symbols are the same as for Figure 3.

and RNA, we determined which of these nucleic acids was more sensitive by the experiments presented in Figure 4. We observed similar effects to those in Figure 3. At the highest concentration tested, a progressive inhibition of [^{14}C]uridine (RNA synthesis) and [^{14}C]thymidine (DNA synthesis) incorporation was observed. The degree of inhibition of both precursors was approximately the same. The lower concentrations of amine oxide first stimulated incorporation of [^{14}C]uridine and after 60 min of incubation inhibition was observed. The lowest concentration of DPNO caused no inhibition of [^{14}C]uridine incorporation. Incorporation of [^{14}C]thymidine was stimulated in a similar way as that of [^{14}C]uridine. The incorporation of all precursors was followed in incubation medium containing glucose as a sole energy source. These concentrations stimulated the glycolysis of EAC cells (results not shown).

Discussion

The values from biochemical screening represent the first fundamental information about cytotoxic activity of new derivatives of amine oxides. The data obtained in a relatively short time inform exactly whether the tested substance shows cytotoxic activity at all and perhaps also indicate the possible mode of action (R). We have previously reported a rapid radiometric *in vitro* technique for primary screening for anticancer substances.[27–29] This method, which measures the drug-induced inhibition of [^{14}C]adenine and [^{14}C]valine incorporation, is relatively simple, reliable and sensitive. In the EAC cells the degree of influence on metabolic activity is identified by uniformly selected concentrations of the substances, in definite conditions *in vitro*, ensuring the active synthesis of proteins and nucleic acids.

From the results presented in Table 1 it is evident that the new derivatives of amine oxides affected the incorporation of both precursors into appropriate macromolecules of EAC cells, according to concentration dependence. Maximum activity was achieved with the compoundss **IV–VII** (Table 1, lower part). On lengthening the alkyl chain, the activity increases and, after having reached a maximum, decreases again (Figure 2). Similar results were obtained by Subik *et al.*[32] and Devinsky *et al.*[33] in the study of antimicrobial activity. For reliable screening, human cells of different origin derived from cancer and from normal tissues are recommended. The ratio IC_{50} adenine:IC_{50} valine shows the difference in the cytotoxicity of the substances, and indicate primarily the similarity or diversity in the mode of action (in the initial

changes). All ratios, as demonstrated in Table 1, are in the range 1.00–1.84. Such ratios are typical for other biologically active compounds which interfere with the generation or utilization of energy in cancer cells.[29,30] Inhibition of energy metabolism may be due to direct interaction or through the disorganization of the membrane structure.

Only substances which are already present in their active form can be tested under *in vitro* conditions. Volm[34] found reasonably good correlations between a test based on the inhibition of radioactive nucleoside uptake and *in vivo* chemosensitivity of several rodent tumors.

Recently, Von Hoff *et al.*[35] developed a radiometric system for the screening of antitumor agents. The index of cytotoxic effectiveness was based on the inhibition of transformation of [^{14}C]glucose into [$^{14}CO_2$]. This radiometric system (BAC-TEC 460) was optimized with the aid of tumor cell lines of both human and animal origin. Scheithauser *et al.*[36] used this new screening system for the selection of antitumor agents for the treatment of human colorectal tumors.

Our results show that DPNO inhibited incorporation of all four precursors (Figures 3 and 4) into appropriate macromolecules of EAC cells at the highest concentrations utilized. This fact suggests that the effect of amine oxide lies at an underlying level of energy generation or transfer rather than at specific reactions in the biosynthesis of DNA and protein. The process of DNA synthesis is actually the culmination of many synthetic pathways. In the intact cell, interference with any of these pathways, as well as alterations and variation in the pool size of precursors, can alter the apparent rate of DNA synthesis and obscure specific drug effects. The rate of DNA synthesis is rapidly affected by the lowering of the level of any of the four deoxyribonucleotide triphosphates. Interference with the generation of high-energy phosphate bonds is one of the mechanisms available for induction of nucleotide deficiency. A depletion of nucleotide pools can serve as an efficient tool to inhibit cellular growth and to induce cell death under some circumstances.

Although in the case of many antineoplastic agents, attention has been focused upon their effects on DNA, RNA and protein synthesis, the data of Hill[37] emphasize that this is an oversimplification. Most agents have multiple effective target sites within the cells and the primary cytotoxic events responsible for their clinical effectiveness remain to be elucidated. The work by Farber[38] and others indicates that the inability to synthesize ATP in a cell leads to multiple secondary derangements in cellular metabolism.

The substances investigated showed a considerably inhibitory effect on the metabolic processes examined, especially at the highest concentrations utilized. We assumed, therefore, that the cytotoxic effect could be the consequence of cytolytic activity of the amine oxide investigated. As recently found,[39] 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), 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.*[40] summarized their results about the mode of action of quaternary ammonium salts and amine oxides upon bacteria determined *in vitro* in three stages. The first stage is characterized by the rate of onset of the action for which polar interactions of molecules with the bacterial membrane are responsible. In the second stage polar and hydrophobic interactions are involved—this stage means the destructive effect on membrane. The third stage is represented by hydrophobic interactions which lead to the death of cells.

The results from this QSAR study also show that there is no doubt that the mode of action of all investigated compounds for the incorporation of precursors must be the same. However, additional work is required to clarify this point, even though it has already been shown[39,41] that non-aromatic amine oxides incorporate predominantly into the outer membrane of cells and disturb their architecture and fluidity, and consequently all processes associated with the membrane.

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.[42] New drugs have been synthesized which have a lipophilic or membrane-selective structure and some of these are in early clinical trials.[42]

The surface membrane alterations which characterize neoplastic transformation offer the 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.[42]

Tumor cell membranes are potentially important targets for selective chemotherapeutic attack.[43]

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[44]).

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