1,3-Dialkyl-3-acyltriazenes, a Novel Class of Antineoplastic Alkylating Agents

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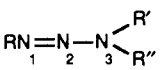
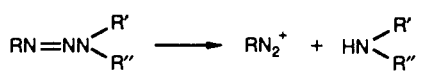
Aliphatic triazenes, such as 1,3-dimethyltriazene, are potent biological alkylating agents because they form alkyldiazonium ions. They are also subject to very rapid proteolytic decomposition, even at physiological pH. The acylated analogues 1,3-dialkyl-3-acyltriazenes are much more stable in aqueous solution, but they also give rise to alkyldiazonium ions. Four acylated 1,3-dimethyltriazenes, where the acyl groups were diethylphosphoryl (DMP), carbethoxy (DMC), acetyl (DMA), and *N*-methylcarbamoyl (DMM), were studied kinetically. Rate–pH profiles indicated that the acyl group had a profound effect on the mechanism of decomposition. The cytotoxic potential of all four compounds was studied in vitro by using the MTT-tetrazolium assay. The compounds had fair-to-good activity against some cell lines, particularly those deficient in methylation repair. In vivo assays of DMC and DMM against several tumor xenografts in nude mice showed promising activity for some cancers, particularly in the case of DMM. In vitro assays were also carried out on three 1-(2-chloroethyl)-3-methyl-3-acyltriazenes. The acyl groups were carbethoxy (CMC), acetyl (CMA), and *N*-methylcarbamoyl (CMM). The activity of these compounds largely paralleled that of bis(2-chloroethyl)-*N*-nitrosourea (BCNU), except for those cell lines which exhibited the Rem^- phenotype; triazenes were more active in those lines than BCNU. The in vivo activity of CMC, CMA, and CMM was tested in the P388 leukemia assay. All three were active but CMC and CMA proved to be rather toxic. CMM was well tolerated and was examined in several tumor xenografts in nude mice. Significant activity was found against MX-1 mammary carcinoma, against LX-1 small cell lung carcinoma, and particularly against LOX amelanotic melanoma, where complete cures were effected. The antineoplastic activity of the acyltriazenes is well-correlated with their chemical behavior.

Introduction

Triazenes, where the group attached to N1 is aromatic, have been known for many years.[1] Although some members of the series have been shown to be potent central nervous system carcinogens in rats,[2] other aromatic triazenes have been shown to possess interesting antitumor properties.[3] One aromatic triazene, 5-(3,3-dimethyltriazeno)imidazole-4-carboxamide (DTIC), is used clinically in the treatment of metastatic melanoma, soft tissue sarcoma, and Hodgkin's disease.[4] Although the mode of action of DTIC and related triazenes is not well-understood, it is known that these chemicals require metabolic activation and that they alkylate genomic DNA.[5] The alkylating agents derived from them are the corresponding alkyldiazonium ions, methyldiazonium in the case of DTIC.



Much less was known about fully aliphatic triazenes before we commenced our work. Although 1,3-dimethyltriazene (DMT) had been prepared by Dimroth in 1906,[6] it remained a chemical curiosity and few studies appeared on this or other dialkyltriazenes, until recently. Trialkyltriazenes were virtually unknown. Our interest in these compounds was sparked by the possibility that they could form alkyldiazonium ions without enzymatic activation. Synthetic methods for simple 1,3-dialkyltriazenes and 1,3,3-trialkyltriazenes were devised,[7] and the mechanisms of their proteolytic decomposition were studied in considerable detail.[8] The salient feature of these decompositions was that the intermediate product was always the corresponding alkyldiazonium ion (eq 1). Thus, the



(1)

aliphatic triazenes are excellent sources of these reactive species and do not require oxidative metabolism to accomplish the transformation. In that sense, the aliphatic triazenes are related to nitrosoureas, but the mechanism by which they form the reactive intermediate is completely different. As might be expected, aliphatic triazenes, particularly those which release the methyldiazonium ion, are potent, directly acting mutagens.[9] One member of the

Table I. Acyltriazenes Examined for Antitumor Activity

compound type	acyl group	acronym	NSC number	$t_{1/2}$,[a] min 50 °C	$t_{1/2}$,[a] min 70 °C
$CH_3N{=}NN(Ac)\text{-}CH_3$	$P(O)(OEt)_2$	DMP	378856	2.3	
	CO_2Et	DMC	378855	41.7	3.9
	$COCH_3$	DMA	378854	161	16.1
	CONHMe	DMM	378853	402	34.6
$ClCH_2CH_2\text{-}N{=}NN(Ac)CH_3$	CO_2Et	CMC	604783		47.0
	$COCH_3$	CMA	604782		264
	CONHMe	CMM	604784		470

[a] Calculated from first-order rate constants obtained at pH 7.5, with 0.1 M lysine buffer (μ = 0.25 M, $NaClO_4$).

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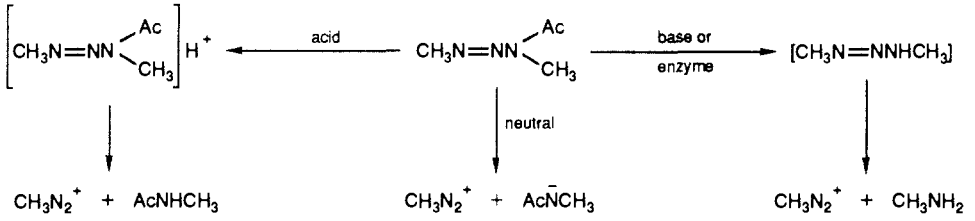
Scheme I



series, 1,3-diethyltriazene, has been shown to be a potent carcinogen in the rat.[10] Preliminary data from our laboratory revealed that simple aliphatic triazenes were strongly cytotoxic to several human tumor cell lines which were deficient in methylation repair, i.e. those cells which exhibited the Mer$^-$ phenotype.[11] Further experiments on the biological properties of aliphatic triazenes were complicated by the fact that they are extremely unstable in aqueous solutions. For example, $t_{1/2}$ for DMT at pH 7.4 was estimated to be 4.8 s. In order to design triazenes which were more stable with respect to proteolytic decomposition, we synthesized 1,3-dialkyl-3-acyltriazenes. The syntheses and the decomposition mechanism of several 1,3-dimethyl-3-acyltriazenes have been reported[12] and we have also described the synthesis of 1-(2-chloroethyl)-3-methyl-3-acyltriazenes.[13] As expected, these compounds were much more stable in aqueous media, although the mechanism of their decomposition proved to be more complex than that of the simple triazenes. Nonetheless, alkyldiazonium ions were formed during the decomposition of the acyltriazenes, and the compounds exhibited considerable biological activity. This paper describes the antitumor properties of some of these compounds in vitro and in vivo and relates the biological activity to their chemical properties. The compounds examined in this study are shown in Table I.

Results and Discussion

Chemistry. Although the details of the decomposition of 1,3-dimethyl-3-acyltriazenes in aqueous buffers have been published,[12] a brief review here will set the stage for the subsequent discussion. Acyltriazenes exhibit either biphasic or triphasic pH–rate profiles. At lower pH's, the decomposition is acid catalyzed, with the rate being inversely proportional to the pH. At intermediate pH regions, the rate is independent of hydronium ion concentration. This plateau region generally overlaps with the physiological pH. In the case of some acyl groups, such as carbethoxy or acetyl, there is a base-catalyzed domain, with the rate being directly proportional to pH. The carboxyethoxy (COOEt) group, and perhaps other acyl groups, can also be hydrolyzed by enzymatic processes, which release the highly reactive dialkyltriazene in situ.[12] These data are summarized in Scheme I.

It is interesting to note that the acid-catalyzed decomposition promotes the direct dissociation of the acyltriazene, rather than promoting the two-step process of initial hydrolysis of the acyl group followed by the acid-catalyzed decomposition of dimethyltriazene.[12] Although it is likely that the uncatalyzed reaction is most important at physiological pH, the precise location of the uncatalyzed region on the pH scale is a function of the acyl substituent. This is indicated by the rate data shown in Table II, and the pH profiles of four 1,3-dimethyl-3-acyltriazenes derived from these data and presented in Figure 1. The data suggest that, with appropriate acyl substitution, the reaction may become acid-catalyzed, even at physiological pH. For example, in the case of DMM, the transitiom from the uncatalyzed to the acid-catalyzed reaction is very close to neutral pH.

The chemistry of 1-(2-chloroethyl)-3-methyl-3-acyltriazenes is similar to the simpler dimethyl analogues. An exception exists in the enzyme-catalyzed domain and probably in the base-catalyzed reaction as well. The removal of the acyl substituent would result in the formation of 1-(2-chloroethyl)-3-methyltriazene, an unsymmetrical triazene. We have recently demonstrated[14] that the de-

Table II. First-Order Rate Constants for 1,3-Dimethyl-3-acyltriazenes Determined at 70 °C as a Function of pH[a]

pH	DMP[b]	DMC	DMA	DMM
2.10			10.8	
2.30			7.18	
2.50	4.43	25.6	5.05	
3.00		10.8	1.91	102
3.50	0.688	5.78	1.13	36.3
4.00		3.86		
4.50	0.426			3.79
5.50	0.396	3.01	0.74	1.12
6.50	0.386			
7.50	0.387 (5.16)[c]	2.96 (0.281)[c]	0.72 (0.071)[c]	0.334 (0.029)[c]
8.50	0.378			
9.50	0.396	2.93	0.81	0.286
10.50	0.372			
11.50	0.386	2.66	1.30	0.286
12.00			4.53	
12.50		4.03	13.12	0.299
13.00		4.03		

[a] Lysine buffer (0.1 M), ionic strength constant at 0.25 M maintained with $NaClO_4$. [b] The pH dependence for DMP was measured at 25 °C; the rate was too fast at 70 °C. [c] $10^3 k_{obsd}$, s^{-1}, measured at 50 °C.

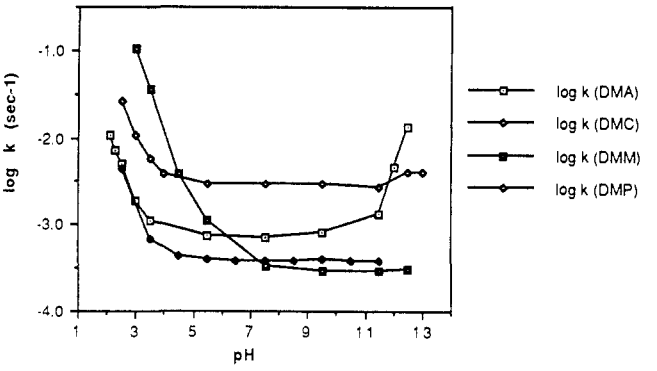


Figure 1. The kinetics measured in a 0.1 M lysine buffer (μ = 0.25 M, $NaClO_4$). The rate constants were obtained at 70 °C, except for that of DMP which was determined at 25 °C.

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Table III. IC_{50}'s[a] for Acyltriazene in Human Tumor Cell Lines Using the MTT Cytotoxicity Assay

organ	cell line	IC_{50}, μM							
		DMA	DMC	DMM	DMP	CMA	CMC	CMM	BCNU
lung	H23	42	20	22	14	20	16	18	5
	H125	53	44	50	29	42	30	45	9
	H322M	55	42	42	25	32	14	37	60
	H358M	100	80	75	50	60	50	60	98
	H460	75	65	70	42	60	50	60	80
	H522	30	18	24	18	18	10	13	20
	A427Mer$^-$[b]	11	7	9	7	12	11	18	8
	A549Rem$^-$[c]	55	45	60	30	26	14	26	90
CNS	U251	42	28	33	25	18	8	30	15
	TE-671	7	48	46	28	50	34	55	25
	A172Mer$^-$	23	20	24	14	20	18	27	18
breast	MCF-7	37	27	30	20	15	16	14	4.5
	MCF-7ADR	40	34	35	23	24	17	17	20
	WiDR	40	45	30	20	15	13	14	
	DOD-1	60	44	38	30	26	20	21	37
melanoma	LOX	30	22	19	20	8	2.4	10	7
	SK-MEL-5	37	20	20	18	15	10	13	27
	RPMI7951	60	35	37	26	17	13	18	
	MALME-3M	25	22	22	15	25	15	28	20
renal	A498Rem$^-$	48	35	37	22	18	16	20	110
	A704Rem$^-$	55	55	55	37	26	18	35	125
ovarian	OVCAR-3	55	60	40	27	25	26	20	15
	OVCAR-4	45	40	35	20	18	26	20	18
sarcoma	TE85Mer$^+$	40	37	35	20	18	26	26	
	A673Mer$^+$	65	75	50	25	35	20	35	
fibroblasts[d]	CCD-19Lu	77	62	70	37	60	45	60	300
	MAR BEL	80	75	65	40	44	46	47	

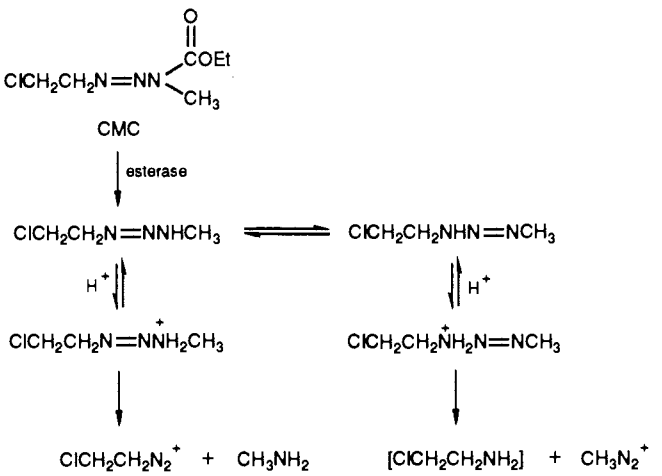
[a] IC_{50} is defined as the concentration (μM) required to cause 50% growth inhibition of each cell lines. [b] The Mer$^-$ (or Mer$^-$ Rem$^-$) phenotype is alkylation-repair deficient and is characterized by a lack of O^6-methylguanine-DNA methyltransferase (O^6MT) activity as well as other repair-related functions.[11] [c] The Rem$^-$ (or Mer$^+$ Rem$^-$) phenotype is less susceptible to alkylation-induced cytotoxicity than the Mer$^-$ phenotype but is also deficient in O^6MT. This phenotype has an intermediate sensitivity to alkylation killing, between that of Mer$^+$ Rem$^+$ and Mer$^-$ Rem$^-$ phenotypes.[16] [d] These are normal cells which provide control values for cytotoxicity in nontumor cells.

composition of unsymmetrical 1,3-dialkyltriazenes is a function of the tautomeric equilibrium, the basicities of the two tautomers and the specific rates of decomposition of the protonated tautomers. The tautomeric equilibrium makes it possible to generate two different alkyldiazonium ions. This situation is depicted in Scheme II for the esterase-catalyzed decomposition of 1-(2-chloroethyl)-3-methyl-3-carbethoxytriazene (CMC). The ratio of the two diazonium ions generated from CMC cannot be calculated a priori with precision, because it is difficult to ascertain the position of the tautomeric equilibrium in an unknown triazene, and we have not been able to prepare 1-(2-chloroethyl)-3-methyltriazene by any direct route. A rough estimate suggests that the reaction should greatly favor the formation of the methyldiazonium ion, perhaps by as much as 20:1. Therefore, we would predict that CMC should be primarily a methylating agent when activated by an esterase. Preliminary in vitro data support this conclusion (M. B. Kroeger-Koepke, data to be published). The basis for the prediction was the finding that electron-attracting groups on one end of the triazene favored the formation of the diazonium ion from the other end.[14] This is so because electron-attracting groups not directly conjugated to the cationic site tend to destabilize alkyldiazonium ions, for the same reason that they destabilize carbocations.

Biological Assays

The compounds in Table I, are listed together with their half-lives in aqueous buffer at pH 7.5, at the temperature stated. It is clear that they differ widely in stability. These compounds were studied in several types of assays, with

Scheme II



the most extensive testing being carried out in vitro, by using the MTT cytotoxicity assay[15] in a number of human cell lines. Since the data set with this assay was large, a summary in the form of a list of IC_{50}'s is presented in Table III for the seven triazenes tested, together with data on bis-(2-chloroethyl)-*N*-nitrosourea (BCNU), which was also examined in most of the lines. The data in Table III show that the triazenes, particularly CMA, CMC, and CMM, which are expected to be chloroethylating agents, behave similarly to BCNU. Interestingly, the chloroethylating

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Table IV. Summary of Activity of 1,3-Dimethyl-3-acyltriazenes against Tumor Xenografts in Nude Mice[a]

compound	tumor	dose, mg/kg	% T/C
DMC,[b] acyl = CO_2Et	M5 sarcoma	60	168
		30	158
		15	140
		7.5	131
	L1210 leukemia	30	142
		15	133
	amelanotic melanoma	120	191
		60	147
		30	132
DMM, acyl = $CONHCH_3$	M5 sarcoma	50	177
		25	158
		12.5	136
	L1210 leukemia	60	186
		36	146
		21.6	132
	MX-1 mammary	300	-13
		150	15
	LX-1 lung (small cell)	100	-55
		50	-34
		25	6
	A549 lung (nonsmall cell)	200	-27
		100	8
	LOX amelanotic melanoma	100	230
		50	188
		25	154
		12.2	131

[a] Chemicals were administered ip according to the following schedules: three injections administered at 4-day intervals for MX-1 mammary carcinoma, LX-1 lung carcinoma, A549 lung carcinoma, and LOX melanoma; five injection on 5 successive days for L1210 leukemia, four injections administered at 4-day intervals for M5 sarcoma. [b] DMC was inactive against LX-1 lung carcinoma and MX-1 mammary carcinoma.

triazenes appear to be more cytotoxic than BCNU to those cell lines characterized by the Rem^- phenotype (A549, A4988, and A704). While the reason for this is not clear, it is tempting to speculate that the aforementioned ability of these triazenes to form both methylating and 2-chloroethylating agents may be connected with the enhanced cytotoxicity in the Rem^- lines. One of the characteristics of the Rem^- phenotype is that it is unable to repair O^6-methylguanine damage produced by *N*-methyl-*N*-nitroso-*N*-nitroguanidine (MNNG) after initial pretreatment with a lower dose of MNNG. If CMA, CMC, or CMM decompose initially (at least in part) to 1-(2-chloroethyl)-3-methyltriazene, then the genomic DNA of those cells will suffer both methylation and chloroethylation damage, by vitrue of the reactions in Scheme II. Consequently, the low constitutive levels of O^6-guanine DNA alkyltransferase in the Rem^- cells will be partly scavenged by reaction with O^6-methylguanine, allowing more of the chloroethyl damage to continue on to form lethal G–C cross-links. If this is correct, then the triazenes of the CMA, CMC, and CMM type have a built-in alkyltransferase-scavenging capacity. The cytotoxicity of all of the triazenes toward the normal human fibroblast is less encouraging, being greater than that of BCNU by a factor of 4–5. We suspect that the methylation damage is again the major problem in this regard. While the chloroethylating triazenes were generally more active in vitro than their methyl analogues, the differences were not large. Interestingly, DMP was the most active of the methylating agents in vitro; it was completely inactive in vivo. We assume that this lack of antitumor activity is connected to its very high chemical reactivity.

Table V. Summary of in Vivo Tests on 1-(2-Chloroethyl)-3-acyl-3-methyltriazenes

compound	tumor	dose, mg/kg	% T/C
CMA, acyl = $COCH_3$	P388 murine leukemia	52.5	257 (2/6 cures)
		26.25	217
		13.12	157
CMC, acyl = $CO_2CH_2CH_3$	P388 murine leukemia	30.0	175
CMM, acyl = $CONHCH_3$	P388 murine leukemia	200	297 (4/6 cures)
		100	196
		50	175
	MX-1 mammary carcinoma	400	-76
		200	1
		100	15
	LX-1 Lung small cell carcinoma	200	-66
		100	-19
	LOX melanoma, amelanotic	800	309 (6/6 cures)
		400	309 (6/6 cures)
		200	309 (3/6 cures)
		100	201 (2/6 cures)

[a] Dosing schedule is given in footnote *a* of Table III. The schedule for P388 leukemia was ip injection on each of 5 consecutive days.

Several of the triazenes were examined for antineoplastic activity against various tumor xenografts in nude mice. These assays were performed under the auspices of the Developmental Therapeutics Program, DCT, National Cancer Institute. Table IV summarizes the data obtained for two methylating triazenes, DMC and DMM, which were selected for these assays. It is clear from the table that both compounds exhibit reasonably good activity in the tumor set studied. Interestingly, DMM showed some in vivo activity against the nonsmall cell lung cancer A549. This tumor was not particularly affected by the chemical in vitro (Table III). This suggests that DMM may have to be metabolized before its cytotoxic potential can be realized. Its proteolytic half-life is long, over 20 h at physiological temperature (based on $t_{1/2}$ in Table I). It follows from that that the chloroethyl analogue CMM, which is even more stable, should also require metabolic activation.

Table V presents the data for the in vivo test on the chloroethylating triazenes CMA, CMC, and CMM. CMA and CMC were studied by using only the P388 murine leukemia test system. CMM, in analogy with DMM, was selected for additional testing against several human tumor xenografts.

The data demonstrate that CMM was only slightly more active than its methyl analogue DMM, on a molar basis. It was, however, much less toxic and consequently much higher doses could be administered. In contrast, the carbethoxy derivative CMC was very toxic. For example, administration of a 60 mg/kg injection to P388 leukemia bearing animals resulted in death of all six animals before the antitumor efficacy could be evaluated.

The lack of acute toxicity of CMM, even at an extremely high dose of 800 mg/kg, and the generally good cytotoxic activity of its methyl analogue DMM suggest that the *N*-methylcarbamoyl group enhances the therapeutic index of these compounds. The presence of the methyl group in position 3 may be beneficial in terms of the aforementioned possibility of scavenging the alkyl transferase, but in general, methylation is likely to produce more undesirable effects, such as general toxicity and carcinogenicity. The new generation of triazenes being developed in our

laboratory is directed toward enhancing the delivery of the (chloroethyl)diazonium ion to the tumor tissue, without the undesirable side reactions. The methyl group in the 3-position will be replaced by groups which favor the dissociation to the (2-chloroethyl)diazonium and which do not produce toxic intermediates. The benzyl group appears to have these qualities. Likewise, the experience with the apparent activation of CMM by metabolism of the *N*-methylcarbamoyl moiety suggests that an appropriately designed acyl group, which can be cleaved by a tumor-specific enzyme, may impart much greater selectivity and greatly decreased toxicity.

Experimental Section

Synthesis. The compounds in this study were prepared by previously published methods. Thus, DMA, DMP, and DMC were prepared by the acylation of the anion of 1,3-dimethyltriazene (DMA, CMP, DMC) or, in the case of DMM, by the direct reaction of 1,3-dimethyltriazene with methyl isocyanate.[12] The compounds were isolated and characterized as described previously and were >99% pure. The (2-chloroethyl)triazenes CMA, CMC, and CMM were prepared by a multistep synthesis, also described previously.[13] These compounds were also analytically pure.

Kinetics. Rates of triazene decomposition were determined spectrophotometrically, as described previously,[12] on a Hewlett-Packard Model 8450A diode-array spectrophotometer. The thermostated (±0.1 °C) 1-cm cuvettes were charged with 1.341 mL of 0.1 M lysine buffer at the appropriate pH. The reaction was initiated by addition of 9 μL of a 3×10^{-3} M solution of the triazene in acetonitrile. The reference cuvette contained the same buffer and 9 μL of acetonitrile. The reactions were followed for at least 3.5 half-lives and at least 100 points were used to evaluate each rate constant. The calculations employed the Guggenheim approximation to determine the infinity absorbance and the rate constants were evaluated by a least-squares method. The calculations were carried out by utilizing a program written in our laboratory. Each kinetic run was carried out in duplicate and, when deviations were >3%, three or more runs were used to obtain a more accurate value.

MTT-Microculture Tetrazolium Assay. Cellular growth in the presence or absence of experimental agents was determined by using the previously described MTT assay.[17] Briefly, cells were harvested and inoculated into 96-well microtiter plates at 1000 cells/well. After 24 h, drugs were applied and cultures were incubated an additional 6 days at 37 °C. MTT was added, the formazan product was solubilized, and the absorbancy was measured at 540 nm with a Bio-Tek Model EL 312 microplate reader.

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Neural Networks Applied to Quantitative Structure–Activity Relationship Analysis[1]

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An application of the neural network to quantitative structure–activity relationship (QSAR) analysis has been studied. The new method was compared with the linear multiregression analysis in various ways. It was found that the neural network can be a potential tool in the routine work of QSAR analysis. The mathematical relationship of operation between the neural network and the multiregression analysis was described. It was shown that the neural network can exceed the level of the linear multiregression analysis.

Introduction

The first quantitative structure–activity relationship (QSAR) method is the model proposed by Hansch and co-workers.[2-4] It was the seminal contribution to this field. The success of this method has prompted many workers to reexamine the derivation of the Hansch equation by using the principles of theoretical pharmacology[5,6] or pharmacokinetics.[7-10] This model, the free energy model,[11] and its elaborations[12] have been by far the most widely used. This may be due to its direct conceptual linkage to established physical organic chemical principles. However, the method is totally dependent on the multiregression analysis. This causes the problems of orthogonality of the variables as well as the size of population.

QSAR is also regarded as the problem of pattern recognition. From this view point, techniques of pattern recognition have been applied to QSAR study, examining structural features and/or chemical properties underlying

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