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Original article

Evaluation of the local anaesthetic activity of 3-aminobenzo[*d*]isothiazole derivatives using the rat sciatic nerve model

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Abstract

On the basis of computer prediction of biological activity by PASS and toxicity by DEREK, the most promising 32-alkylaminoacyl derivatives of 3-aminobenzo[*d*]isothiazole were selected for possible local anaesthetic action. This action was evaluated using an *in vitro* preparation of the isolated sciatic nerve of the rat and compared with lidocaine which was used as a reference compound. QSAR studies showed that the polarizability, polarity and molecular shape of molecules have a positive influence on their local anaesthetic activity, while contributions of aromatic CH and singly bonded nitrogen are negative. Since the estimated PASS probabilities to find local anaesthetic activity in the most active compounds are less than 50%, these compounds may be considered to be possible NCEs.
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1. Introduction

Local anaesthetics are compounds that block nerve fibre conduction when applied locally to nerve tissue in appropriate concentrations. They block the voltage-sensitive sodium channels of every type of nerve fibre, thereby eliminating their action potential [1]. The great practical advantage of the local anaesthetics is that their action can be reversed when they are removed from the nerve tissue. In this case there is a complete recovery of the nerve function and of the action potential, with no evidence of structural damage to nerve fibres or cells [1]. Following the classic scheme of Lofgren [2], structural requirements for a local anaesthetic are: a lipophilic (aromatic) head, a hydrophilic end bearing a tertiary amine, and an intermediate substituted alkyl chain. Compounds having an amino group linked to a heterocyclic nucleus, such as the lipophilic moiety, display greater activity and less toxicity than benzene analogues [3,4]. Some derivatives of 2-aminobenzothiazole [5–9] and 2-aminothiazole [10–13] as well as 3-aminobenzo-[*d*]-isothiazole [14–17] are reported to possess local anaesthetic activity. Keeping this in mind, together with the fact that a number of local anaesthetic drugs carry alkyl-acetamido or alkyl-propionamide moieties, we synthesized different thiazole and isothiazole derivatives [18–20]. Several compounds among these heterocyclic derivatives were found to possess local anaesthetic properties. Among these compounds, a number of 3-(alkylaminoacyl)aminobenzo[*d*]isothiazoles carrying different anaesthesia-promoting basic moieties, lidocaine-like in structure, proved to be active in infiltration and trunkular anaesthesia [18].

The local anaesthetic action of the compounds was evaluated using the isolated sciatic nerve model [21–23]. The same

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model has been used previously in the assessment of the neurotoxicity of pesticides [24–26] and in the evaluation of the local anaesthetic activity of a variety of compounds [27]. In our earlier preliminary paper [27] we evaluated the local anaesthetic action of previously synthesized 18 3-(alkylaminoacyl)aminobenzo[*d*]isothiazoles [18] using the isolated sciatic nerve of the frog (*Rana ridibunda*). However, generally in this kind of preclinical *in vitro* test anyone tries to use species as close as possible to humans, like mammals. Although the sciatic nerve of the frog is an excellent preparation for fast- and low-cost *in vitro* studies, it is used to refine, replace and reduce mammalian tests in neurotoxicological studies, it has physiological properties far different from those of mammals. The frog is an ectothermic animal with a significant tolerance to temperature variation and can survive with body temperatures below 0 °C [22,23], while mammals are endothermic with minimal tolerance to body temperature changes.

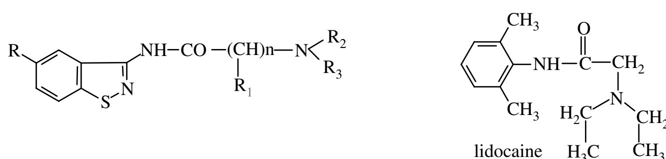
The purpose of this work is to assess and compare the action of the same 3-(alkylaminoacyl)aminobenzo[*d*]isothiazoles as well as 14 new compounds of general formula **1** (see Fig. 1) on the evoked compound action potential (CAP) of the sciatic nerve of the rat as representative of the mammalian species. To increase the chance of selecting the most promising structures (probably anaesthetic activity, high structural novelty, small likelihood of toxicity), predictions of biological activity and toxicity have been carried out with the computer programs PASS [28] and DEREK [29], respectively.

2. Results and discussion

2.1. Chemistry

The hydrochlorides of compounds **1–32** (see Table 1) were obtained by dissolving the free bases, shown in Fig. 1, in absolute ethanol treated with a few drops of concentrated hydrochloric acid. All compounds were obtained in good yield and crystallized from EtOH/Et_2O. The free bases were obtained by reaction at high temperature in anhydrous benzene, between a number of selected amines (molar excess) and 3-(haloacyl)aminobenzo[*d*]isothiazoles, prepared in turn from the appropriate 3-aminobenzo[*d*]isothiazole and haloacylhalogenides [18] (see Fig. 2).

Most of the benzisothiazolylalkylaminoacyl derivatives studied have been reported previously and the resynthetized compounds showed physico-chemical properties and spectral data which were in agreement with the reported literature values [18,27,30]. Compounds **14** and **15**, not previously described, were characterized and analysed by elemental analysis, and their analytical IR and ^{1}H NMR spectral data were consistent with the assigned structures.



lidocaine

Fig. 1. General formula of the compounds tested.

2.2. Biological evaluation

The isolated sciatic nerve preparation is suitable for the assessment of local anaesthetics. The evoked compound action potential is graded in amplitude reflecting the summation of the external currents generated by the action potential of each fibre activated in the nerve (Fig. 3A). In this case, the electrical stimulation of the nerve activates the voltage-gated sodium channels (VGNaCs) of each individual nerve fibre to generate the recorded action potential. The summation of the action potential of single nerve fibres creates the evoked CAP (Fig. 3A). The VGNaCs are the main target of local anaesthetics [31]. The isolated sciatic nerve of the rat in the three-chamber recording bath is a reliable preparation allowing quantitative and qualitative assessments of the action of local anaesthetics (see, for example [19,20,32]).

2.2.1. The action of the synthesized compounds on the CAP

Comparison has shown that all tested compounds can be divided into four groups according to the speed of action – IT_{50} values (the time which required to block action potential to 50%) (Table 1) for abbreviation see Section 4.

Group A includes compounds where the time required to decrease the CAP to 50% of its control value is shorter than the time required for lidocaine, resulting in $IT_{50} < IT_{50Lid}$ (3.14 min). Compounds **30** and **32** belong to this group. The IT_{100} values of these compounds are 1.25 and 1.45 times higher than that of lidocaine, while the RT_{50} are 1.8 and 1.2 times greater than that of lidocaine, indicating a stronger binding of these compound with the VGNaCs. The interesting point is their vitality time VT_{50}, which classifies compound **30** as neurotoxic ($VT_{50} < 660$ min), while compound **32** appears to be less neurotoxic than lidocaine, with $VT_{50} > 660$ min. The possible neurotoxicity of lidocaine has already been mentioned above; furthermore, increasing laboratory evidence suggests that local anaesthetics are potentially neurotoxic and that neurological impairment following regional anaesthesia may result from the direct neurotoxic effect of local anaesthetics [33–35]. The conclusion is that compound **32**, a morpholine derivative, can be considered for further studies as a potential new local anaesthetic.

Group B consists of compounds where $IT_{50Lid} < IT_{50} < 2 \times IT_{50Lid}$ ($3.14 < IT_{50} < 6.28$). This group contains compounds **3**, **6**, **10**, **16**, **18**, **19**, **23**, **24**, **27**. From these compounds, those with $VT_{50} < 660$ min, such as compounds **18**, **19** and **24**, were considered as neurotoxic. Moreover, there is a sub-group of compounds with $VT_{50} > 660$ min, the VT_{50} of lidocaine; namely compounds **16**, **23** and **27**. Compound **16** seems to have a very similar IT_{50}, 4.2 min, and IT_{100}, 12.9 min, to those of lidocaine (3.14 min and 14 min respectively), but there is a difference in the RT_{50}, which is 100 min for compound **16** and 48 min for lidocaine. This indicates a stronger binding of compound **16** with the VGNaCs of the nerve fibres than that of lidocaine.

Table 1
Local anaesthetic activity, experimental and predicted data

Compound (1%)	R	R_1	Z	*n*	N	IT_{50} (min)	IT_{100} (min)	RT_{50} (min)	Vitality of nerve (VT_{50}, min)	RA_{50} %	RA_{100} %	Prediction PASS
1	H	H	$N(CH_3)_2$	1	3	10.5 ± 2.5		100.0 ± 2.8	250.0 ± 2.0	29.9	–	55.3
2*	H	H	$N(C_2H_5)_2$	1	3	12.0 ± 0.5	53.9 ± 0.1	121.5 ± 5	600.0 ± 7.0	26.2	26.0	76.8
3*	H	H	Pyrrolidine	1	3	11.9 ± 2.7	28.3 ± 1.8	101.5 ± 9	705.5 ± 14.5	26.4	49.5	38.8
3	H	H	Pyrrolidine	1	4	5.0 ± 0.5	14.0	105.0	610.0 ± 1.1	62.8	100.0	38.8
4	H	H	Pyrrolidine	2	3	10.0 ± 1.0	20.4 ± 2.0	136.6 ± 16.8	870.0 ± 8.2	31.4	68.6	33.0
5*	H	H	Piperidine	1	3	8.05 ± 0.7	23.4 ± 1.4	141.2 ± 7.9	555.8 ± 39	39.0	59.8	39.8
5	H	H	Piperidine	1	4	7.0 ± 0.6	16.0 ± 1.2	142.7 ± 7	500.0 ± 30	44.9	87.5	39.8
6*	H	H	Morpholine	1	3	8.1 ± 0.4	19.0 ± 1.5	40.0 ± 2	666.7 ± 33	38.8	73.7	37.6
6	H	H	Morpholine	1	4	5.5 ± 0.25	10.0 ± 1.0	88.0 ± 5	511.0 ± 28	57.1	140.0	37.6
7	H	H	Morpholine	2	5	7.0 ± 0.7	17.9 ± 1.3	86.0 ± 4	Nt	44.9	78.2	31.8
8	H	H	$NHCH_3$	1	3	10.0 ± 0.93	34.0 ± 3.1	48.0 ± 2.3	786.0 ± 7.05	31.4	41.2	39.8
9	H	H	$NHCH_3$	2	3	10.75 ± 2.3	20.5 ± 1.9	136.0 ± 0	839.0 ± 21	29.2	68.3	28.2
10*	H	H	NHC_2H_5	1	3	11.5 ± 2.2	49.9 ± 6.6	135	658.0 ± 1.5	27.3	28.1	47.6
10	H	H	NHC_2H_5	1	3	5.0	24.2	76.0	600.0 ± 1.3	62.8	57.9	47.6
11	H	H	NHC_2H_5	2	3	26.1 ± 3.9	79.5 ± 8.6	154.8 ± 11	400.8 ± 1.5	12.0	17.6	35.0
12	H	H	NHC_3H_7	1	3	10.0 ± 0.95	17.5 ± 1.8	200.0 ± 1.5	250.0 ± 2.2	31.4	80.0	42.1
13	H	H	$NH(i)C_3H_7$	1	3	12.13 ± 1.4	30.2 ± 2.2	150.0 ± 1.2	Nt	25.9	46.4	46.9
14	H	H	Me-piperidine	1	3	25.5 ± 2.2	37.45 ± 0.77	122.0 ± 5.6	930.0 ± 22	12.3	37.4	36.0
15	H	H	Ph-piperidine	1	4	18.5 ± 1.5	–	Nt	Nt	17.0	–	28.8
16	H	Me	NHC_2H_5	1	3	4.2 ± 0.4	12.9 ± 0.8	100.0 ± 2.0	861.0 ± 30	74.8	108.5	34.7
17	H	Me	$N(CH_3)_2$	1	3	7.4 ± 1.7	14 ± 3.5	77.0 ± 0	690 ± 9	18.8	70.0	29.6
18	H	Me	$N(C_2H_5)_2$	1	3	3.85 ± 0.77	14.1 ± 1.4	57.7 ± 1.3	404.2 ± 8.4	49.8	84.8	52.0
19	H	Me	Pyrrolidine	1	3	7.7 ± 0.75	11.6 ± 1.1	135.0 ± 2.2	600.0 ± 9.5	40.8	120.7	63.0
20	H	Me	Piperidine	1	4	8.9 ± 1	20.9 ± 1.8	58.0 ± 5.8	500.0 ± 0	21.7	73.7	63.5
21	H	Me	Morpholine	1	4	11.8 ± 1.2	23.0 ± 2.0	126.0 ± 2.3	350.0 ± 30	26.6	60.9	60.4
22	H	Me	$NHCH_3$	1	3	12.8 ± 1.2	26.7 ± 2.1	68.0 ± 6.0	450.0 ± 32	24.5	121.7	23.9
23	H	Me	$NH-C_3H_7$	1	3	5.3 ± 1.47	24.1 ± 7.9	88.9 ± 10.2	750.0 ± 39	31.4	70.0	35.5
24	H	Me	$NH-C_6H_{11}$	1	4	5.8 ± 0.35	25.3 ± 0.9	52.3 ± 0.5	NO	44.9	63.6	21.9
25	Me	Me	$NHCH_3$	1	3	6.6 ± 0.6	20.0 ± 1.8	60.0 ± 5.5	780.0 ± 35	47.6	70.0	23.7
26	Me	Me	$NH(CH_3)_2$	1	3	11.6 ± 1.4	23.75 ± 1.5	162.0 ± 2.83	1012.0 ± 70.8	25.7	75.7	28.9
27	Me	Me	NHC_2H_5	1	3	4.2 ± 0.5	12.9 ± 1.2	100.0 ± 2.0	702.0 ± 22.0	74.8	108.5	36.7
28	Me	Me	NHC_3H_7	1	3	7.4 ± 0	14.8 ± 0	NO	365.0 ± 13.0	23.6	65.1	33.6
29	Me	Me	$NH-C_6H_{11}$	1	3	39.0 ± 2.0	42.5 ± 2.4	NO	NO	8.1	32.9	21.9
30	Me	Me	Pyrrolidine	1	4	1.75 ± 0.1	17.5 ± 1.1	122.0 ± 0	498.0 ± 28.0	179	80.0	59.9
31	Me	Me	Piperidine	1	4	11.0 ± 1.5	37.0 ± 3.2	NO	NO	28.5	37.8	60.2
32	Me	Me	Morpholine	1	4	2.23 ± 0.1	20.0 ± 1.2	60.0 ± 0	738.0 ± 30	140.8	70.0	58.0
Lid				1	5	3.14 ± 0.2	14.0 ± 1.1	48.75 ± 1.2	660.0 ± 20	100.0	100.0	94.7

IT_{50} – the time required to decrease CAP to 50%; IT_{100} – the time required to decrease CAP to 100%; RT_{50} – the time required to the CAP to recover to 50%; VT_{50} – vitality time; RA_{50} – relative activity calculated for IT_{50}; RA_{100} – relative activity calculated for IT_{100}; Nt: non-tested; NO: there is no recovery.

The interesting point is that VT_{50}, a parameter determining the relative toxicity of the compounds in relation to lidocaine, was much longer for compound **16** than the corresponding time for lidocaine, indicating the slight neurotoxicity of lidocaine. This situation is identical for compound **27**. Finally, compound **23** has a longer vitality time than lidocaine does, and some differences in the IT_{100} and the RT_{50} values. The results indicate that compounds **16, 23** and **27** can be considered good candidates for new local anaesthetics.

Group C consists of compounds where $2 \times IT_{50Lid} < IT_{50} < 4 \times IT_{50Lid}$ (or $6.28\ min < IT_{50} < 12.56\ min$). Compounds **2**, **4**, **5**, **7–9**, **12**, **17**, **19–21**, **25**, **26** and **28** are in this group. The most interesting compounds are those with a low relative potency of $VT_{50} > 660$ min. These are compounds **8**, **9** and **17**, **25** and **26** (Table 1). As expected, their IT_{100} was also low (mostly within the range 20–23 min), while there was one compound with $IT_{100} = 14$ min (compound **17**) and another with $IT_{100} = 34$ min (compound **8**). The majority of these compounds have an RT_{50} between 121 min and 162 min (compounds **4**, **9**, **21** and **26**), although there are some compounds in this group with an RT_{50} between 48 min and 86 min (**7**, **8**, **20** and **25**). Due to their low potency compared with lidocaine,

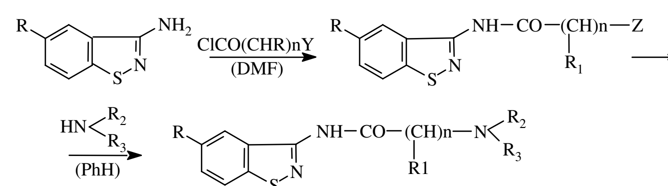


Fig. 2. Synthetic pathway for preparation of the compounds.

these compounds shall be considered for further investigations as potential local anaesthetics. There are also compounds with mild potency, like compound **20** with $VT_{50} = 500$ min, and with high potency, like compound **21** ($VT_{50} = 350$ min) and compound **28** ($VT_{50} = 365$ min) within group C.

Group D consists of compounds **11**, **14**, **15**, **22** and **29** where $IT_{50} > 4 \times IT_{50Lid}$ (or $IT_{50} > 12.56$ min). Comparison of compounds **11** and **14** is interesting. Both compounds have almost the same IT_{50} values, of 26.1 min and 25.5 min, but different IT_{100} values, with 79.5 min vs. 37.45 min, and different RT_{50} values, with 154.8 min vs. 122 min. However, it is unclear what makes the first compound extremely toxic, with $VT_{50} = 400$ min, and the second compound far less toxic than lidocaine, with a VT_{50} of 930 min.

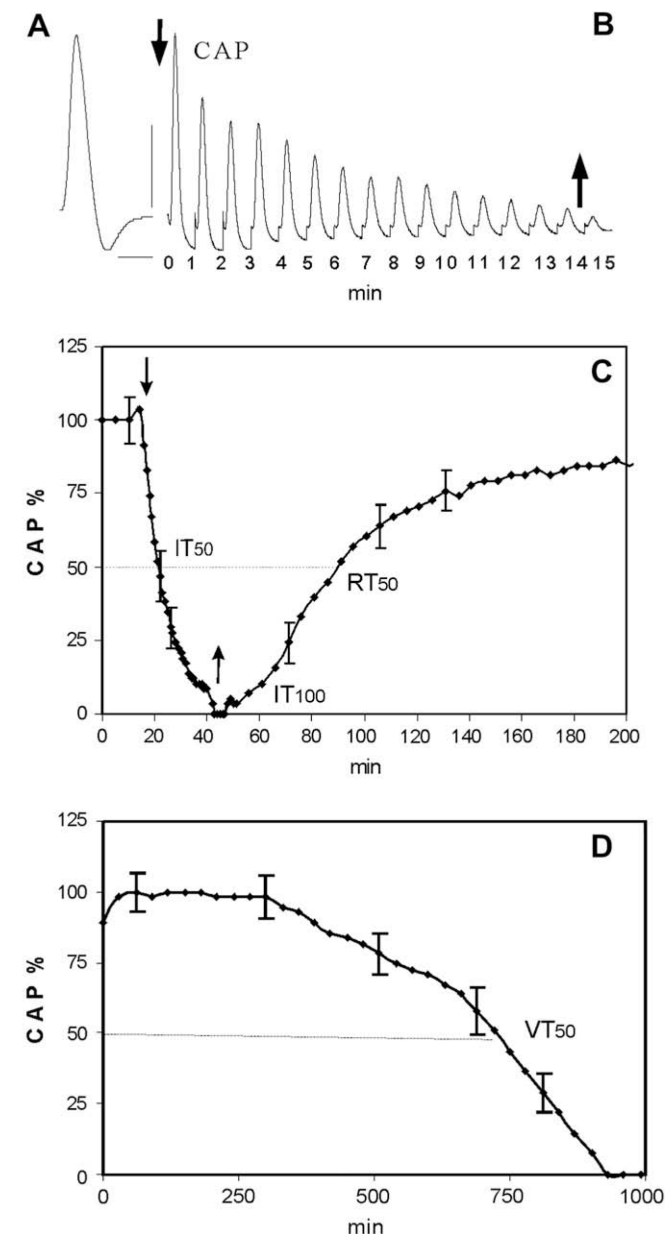


Fig. 3. Records of the evoked compound action potentials (CAPs) from the sciatic nerve of the rat isolated in the recording chamber. The nerve was bathed in saline where the compound 12 was diluted at a concentration of 1% (w/v) and the sample records were obtained every minute after application of the compound. The first arrow indicates the moment when the nerve was exposed to the compound ($t = 0$ min) and the second arrow when the nerve was washed with normal saline ($t = 15$ min). The second arrow also indicates the time required for the 100% decrease of the CAP (IT100). There was a gradual recovery of the compound action potential to 50% if its initial value (RT50) within 115 min ($t = 130$ min) after wash. The horizontal dotted line indicates the time required for the decrease of the CAP to 50% of its initial value (IT50).

The action of the compounds examined seems to be concentration dependent. In four different cases, with compounds **3**, **5**, **6** and **10**, a concentration of 0.5% was used, in addition to 1%. The results, shown in Table 1 (see **3***, **5***, **6*** and **10***), indicate a concentration-dependent response since the values of IT_{50} from 5.0 (compound **3**), 7 (**5**), 5.5 (**6**) and 5.0 (**10**) became 11.9, 8.05, 8.1 and 11.5, respectively. The other parameters, such as IT_{100}, RT_{50} and VT_{50}, were also affected. It seems that certain compounds can have equal action at the lower concentration, a fact that obviously minimises their toxic effect since the VT_{50} of 610 min (compound **3**), 511 min (**6**) and 600 min (**10**) at 1% concentration became 705 min, 666.7 min and 658 min, respectively, at 0.5% concentration.

From the structural point of view, the most potent compounds (group A) are acetamide derivatives, with a single methylene group between the lipophilic and hydrophilic moieties. Increasing the chain by one additional methylene group leads to less potent compounds. Also, the substituent in the methylene group in the intermediate chain plays an important role. It seems that the presence of a methyl group, in the methylene group of the intermediate alkyl chain, as well as piperidine as a tertiary amine, is responsible for high lipophilicity as well as for neurotoxicity (compounds **5**, **28**, **31**). Substitution of a methyl group with an ethyl group also leads to more toxic compounds (**10**).

It was observed that methyl substitution in the 5th position, as well as in the methylene group of the intermediate alkyl chain, is responsible for the high activity of pyrrolidine and morpholine derivatives (compounds **30**, **32**), while in the case of piperidine derivative (compound **31**) it leads to a decrease in activity compared with the unsubstituted compound.

The activity and lipophilicity ($C\log P$) of the tested compounds are not correlated. For example, compound **6** ($n = 1$) has almost the same structure as compound **7** ($n = 2$), with a difference only in the intermediate chain. Even though the lipophilicity of this compound is higher than that of compound **7**, it is less potent. Clearly, the lipophilicity along with other properties of the compounds plays a role in their activity.

2.3. Prediction of biological activity and toxicity

Table 1 shows the experimental activities of the compounds and local anaesthetic activity predicted by PASS. There is no correlation between the predicted values of probability Pa and the experimental data. The PASS prediction results indicate only the similarity assessment of the tested compounds with the classic local anaesthetics presented in the PASS training set. Since lidocaine is a classic local anaesthetic, it has a Pa equal to 95%.

Compounds **30** and **32** have an RA_{50} value greater than that of lidocaine, with 179.4% and 140.8%, respectively. The most active compounds, based on RA_{100} value are compounds **6** at 1% (140), **19** (121.7), **16** and **27** (108.5), **3** and **17** (100). Compounds **3**, **6**, **17** and **27** have Pa values ranging from 29.6% (**17**) to 38.8% (**3**). Hence, it appears that these compounds differ significantly from traditional local anaesthetics and may be new chemical entities (NCEs). Compounds **19**, **30** and **32** have Pa values about 60%; thus, these compounds are more similar to the anaesthetics in the PASS training set.

Toxicity predictions using DEREK indicated that carcinogenicity for all compounds (including lidocaine) is plausible. In addition, for compound **9** there is a predicted phospholipidosis risk (no rating given), and for compound **15** there is a predicted α-2-μ-globulin nephropathy risk (no rating given). Methaemoglobinaemia is also predicted as plausible for lidocaine.

It is considered that the DEREK predictions do not seem to justify the removal of any compounds; however, any compounds selected for development should be tested for carcinogenicity. If compound **10** is selected, it should also be tested for phospholipidosis, and if compound **15** is selected it should also be tested for α-2-μ-globulin nephropathy.

2.4. QSAR analysis

There have been only a few QSAR investigations of local anaesthetic activity published. Recanatini and co-workers [36] found a weak correlation ($R^2 = 0.652$) between the local anaesthetic activity of 67 lidocaine derivatives with $\log D$ (D = distribution coefficient at pH 7.4) and two indicator variables. Caliendo and co-workers [37] found a good correlation ($r^2 = 0.882$) of the local anaesthetic activity of 12 *N*-[2-(alkylamino)ethyl]benzotriazol-*x*-yl acetamides with $\log P$ (P = octanol–water partition coefficient), and Caliendo and co-workers [38] found a reasonable correlation ($r^2 = 0.750$) of the local anaesthetic activity of 12 *N*-[2-(alkylamino)ethyl]-benzotriazol-*x*-yl isobutyramides. Geronikaki and co-workers [27] obtained a reasonable correlation ($r^2 = 0.794$) of the local anaesthetic activity of 19 3-aminobenzo[*d*]isothiazole derivatives with descriptors' modelling polarity, polarizability and hydrogen bonding.

We removed six compounds (**18**, **19**, **21**, **24**, **29** and lidocaine) at random, to serve as a test set, from those listed in Table 1 before developing QSARs on the remaining compounds.

The best QSAR that we obtained for the local anaesthetic RA_{50} values was selected by GA.

$$\begin{aligned} \log RA_{50} = {} & 4.49(\pm 0.99)\text{S5CH} - 2.86(\pm 0.91)\text{SDHW6} \\ & + 0.124(\pm 0.04)\text{WTPT4} - 2.16(\pm 0.67) \\ & \text{EMIN1}\ 0.839(\pm 0.46)\text{Ca}_{max} + 0.08 \qquad (1) \\ n = 27,\ & R^2 = 0.662,\ Q^2 = 0.399,\ s = 0.184,\ F = 8.2 \end{aligned}$$

where S5CH = 5th order chain molecular connectivity, SHDW6 = standardised shadow area in *YZ* plane, WTPT4 = number of atom identities for oxygen, EMIN1 = minimum atomic *E*-state value, Ca_{max} = maximum hydrogen bond acceptor strength on an atom, n = number of chemicals in the training set, R = correlation coefficient, Q = cross-validated correlation coefficient (leave-one-out procedure), s = standard error of the estimate, and F = Fisher statistic. All p values except than for Ca_{max} were <0.01, indicating that each descriptor had less than 1% risk of being selected by chance; for Ca_{max}, $p = 0.08$, which is acceptable. There were no collinearities among the descriptors.

The best QSAR for the local anaesthetic RA_{100} values was also selected by GA.

$$\begin{aligned} \log RA_{100} = {} & -0.741(\pm 0.136)\text{FVMX} + 0.166(\pm 0.042) \\ & \text{ELOW1} - 0.000847(\pm 0.000350)\text{ECCN} + 32.1(\pm 10.7) \\ & \text{CARB-1} - 0.0285(\pm 0.0145)\text{DPM}_Z - 8.06(\pm 3.47) \\ n = 25,\ & R^2 = 0.744,\ Q^2 = 0.571,\ s = 0.125,\ F = 11.0 \qquad (2) \end{aligned}$$

where FVMX = maximum free valence value (an indicator of reactivity), ELOW1 = difference between minimum and maximum electrotopological state (*E*-state) values (polarity), ECCN = whole molecule eccentric connectivity index (molecular shape), DPM_Z = dipole moment in *Z* direction (polarity), and $\sum Q^+$ = sum of positive charges on atoms (polarity and/or hydrogen bonding), All p values were <0.02, indicating that each descriptor had less than a 2% risk of being selected by chance. There were no collinearities among the descriptors.

It is clear from Eq. (1) that a good correlation could not be obtained for RA_{50} as a measure of local anaesthetic activity. As well as a low R^2 value of 0.662 being obtained, the Q^2 value of 0.399 is below the recommended minimum of 0.5 [39,40]. Hence Eq. (1) should not be used for predictive purposes.

From Eq. (2), the positive coefficients of ELOW1, CARB1 and ΣQ^+ indicate that higher polarity and/or hydrogen bonding increase local anaesthetic activity, whilst the negative coefficient of DMP_Z suggests that molecular orientation is important in receptor-binding via polar interactions. Free valence is defined as the maximum valence of an atom minus the total electronic population on that atom; the negative coefficient of FVMX indicates that high electronic populations, leading to high polarity, are preferred. The negative coefficient of ECCN indicates that molecular eccentricity lowers activity, so more spherical molecules are preferred. As with our previous work [27], we found no indication in the present study that local anaesthetic activity is controlled by hydrophobicity, despite the findings of other workers [36–38]. It should be mentioned, however, that the factors that we have found important all contribute to hydrophobicity [41].

The correlation of Eq. (2) is shown graphically in Fig. 4. It can be seen that there is a reasonably good distribution of points, with no outliers having residuals greater than two standard deviations.

These results are in broad accordance with our previous findings [27] that molecular size/shape, polarity/polarisability and hydrogen bonding are largely responsible for local anaesthetic activity. It is difficult to say whether transport or binding is the limiting step, since molecular size and shape,

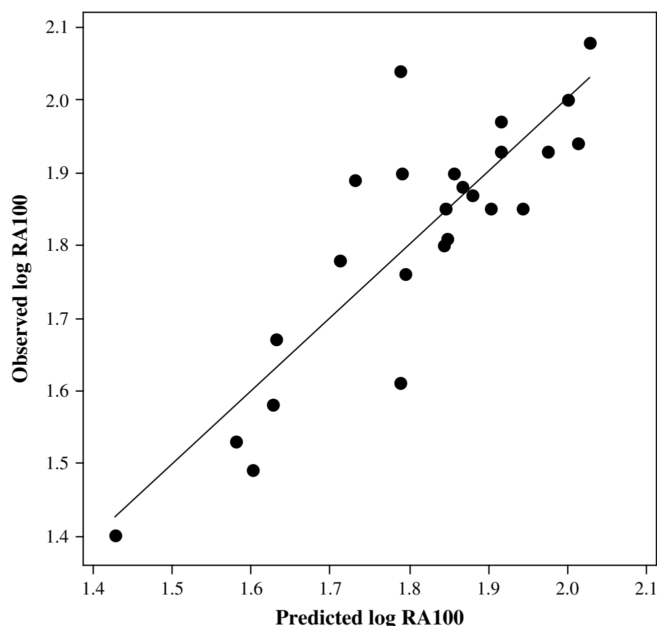


Fig. 4. Observed log RA100 values vs. those predicted from Eq. (2) for the 25 compounds in the training set.

polarity/polarisability and hydrogen bonding govern both processes. Nevertheless, Eq. (2) can be used with confidence to predict the local anaesthetic activities of compounds of the same class as those used to develop Eq. (2). This is shown by the good predictions obtained from Eq. (2) for the log RA_{100} values of the six-test set compounds, all of which are within twice the standard error of 0.125 log unit, with the exception of lidocaine. The slightly higher prediction error for lidocaine is not unexpected, because its molecular structure is somewhat different from the structures of our compounds. The predicted errors would be better presented in table (Table 2).

3. Conclusions

Based on computer predictions, the most promising 32 potential local anaesthetics were selected from the alkylaminoacyl derivatives of 3-amino-1,2-benzo[*d*]isothiazoles. The compounds were divided into four groups according to their local anaesthetic action: (a) group A – compounds which seem to have lidocaine-like action; (b) group B – compounds which seem to have better action than lidocaine; (c) group C – compounds with lower lidocaine-like action and finally (d) group D – compounds which seem to be neurotoxic with very short inhibition time but without recovery at all.

Table 2
Predicted errors

Compound	Prediction error
6	0.248
16	0.134
22	0.228
29	0.181
32	0.105
Lidocaine	0.338

From the QSAR analysis it was clear that molecular shape, polarity/polarizability, and hydrogen bonding are important for local anaesthetic action. Since the PASS probability Pa of local anaesthetic action in compounds **5**, **17**, **27**, **10** and **24** was calculated as being in the range 23–47%, the most potent compounds could potentially be new chemical entities.

Because some of the compounds studied had earlier been tested as local anaesthetics on an *in vitro* frog model, the experimental data from frog and rat models were compared. It appeared that only RT_{50} values in both models have a 40% correlation. Other values had no significant correlations. This means that the frog model may only be used in the preliminary estimation of neurotoxicity for potential local anaesthetics.

4. Materials and methods

4.1. Chemistry

Melting points (°C) were determined with a Buchi 512 apparatus and are uncorrected. New compounds were analysed on a ThermoQuest (Italia) FlashEA 1112 Elemental Analyser, for C, H, N and S. IR spectra, such as KBr pellets, were recorded on a Jasco FT-IR 300E spectrophotometer (Jasco Ltd., Tokyo, Japan). ^1H NMR spectra, in DMSO-d_6 solutions, were recorded on a Bruker AC 300 instrument at 298 K. The reactions were followed by TLC on F_{254} silica-gel precoated sheets (Merck) and the purified compounds each showed a single spot. Solvents, unless otherwise specified, were of analytical reagent grade or of the highest quality commercially available. Synthetic starting material, reagents and solvents were purchased from Aldrich Chemical Co.

4.1.1. *N-Benzo[d]isothiazol-3-yl-2-(4-methylpiperazin-1-yl)-acetamide hydrochloride (**14**)*

White crystals, yield 45%; mp 202–204 °C; Anal. calcd for $C_{14}H_{19}N_4OClS$ (326.85) C, 51.45; H, 5.86; N, 17.14; S, 9.81. Found C, 51.18; H, 5.83; N, 16.98; S, 9.60.

4.1.2. *N-Benzo[d]isothiazol-3-yl-2-(4-phenylpiperazin-1-yl)-acetamide hydrochloride (**15**)*

White crystals, yield 60%; mp 217–219 °C; Anal. calcd for $C_{19}H_{21}N_4OClS$ (388.92) C, 58.68; H, 5.44; N, 14.41; S, 8.24. Found C, 58.41; H, 5.43; N, 14.49; S, 8.07.

4.2. Biological action

Wistar rats of either sex weighing 200–250 g were used. They were killed by deep anaesthesia using sodium pentothione (100 mg/kg bodyweight, i.p.). The maintenance of the animals and the experimental procedures were conducted in accordance with the protocols outlined by Aristotle University of Thessaloniki regarding the recommended standard practices for biological investigations. The sciatic nerves were dissected from the spinal cord to the knee and mounted across a three-chambered recording bath made of Plexiglas. The recording bath has been described elsewhere and has been used in

a variety of *in vitro* neurotoxicological studies using the isolated sciatic nerve preparation [42–44]. In this instance, the propagated compound action potential was recorded using standard electrophysiological methods (CAP, Fig. 3A).

The main parameter measured from each nerve CAP, evoked by supramaximal stimuli, was the maximum amplitude, defined from the beginning of the rising phase of the CAP to its peak (see Fig. 3A). The data from the evoked CAPs (volts) were normalized to their control values, taken at time 0, the measurement time starting after 30 min of equilibration of the nerve in saline. With the sciatic nerve consists of about 7500-nerve fibres [45] the value of 100% was taken to be that of the value of the amplitude of the nerve CAP at time 0 and represents the maximum number of the stimulated sciatic nerve fibres. The values of the amplitude of the CAP were represented as a percentage of the value at time 0 and were expressed as a mean ± S.E.M. Statistical analysis was performed using the unpaired *T*-test (for significant difference, $p < 0.01$).

4.2.1. Experimental protocol

(a) For the assessment of the local anesthetic action of the compounds under investigation, lidocaine was used as a standard. The action of lidocaine was investigated first of all. For this purpose, the sciatic nerve was first immersed in normal saline for over 40 min (equilibration period), while the evoked CAP was recorded every minute. Then, the normal saline was replaced with saline with lidocaine, 1% w/v (arrow at $t = 0$, Fig. 3B). The evoked CAP was monitored continuously and as soon the CAP reached a value below 5% of its initial value (see second arrow in Fig. 3B, at $t = 15$ min), the saline with lidocaine was replaced with normal saline. The time-response curve of the lidocaine was plotted, as shown in Fig. 3C. Finally, the nerve was left in the fresh saline for at least another 15 h while recordings of the CAP were obtained automatically every 15 min (for the vitality time-response curve see Fig. 3D). From the time-response curves plotted for lidocaine, it was possible to produce the parameters used to evaluate its local anaesthetic activity. These parameters were: (a) the time required for lidocaine to decrease the CAP to 50% of its control values, the value in normal saline. This value is called inhibitory time 50 (IT_{50}) and was measured in minutes (see IT_{50} in Fig. 3C). For lidocaine, this parameter was called IT_{50Lid} and was estimated to be 3.14 ± 0.2 min ($n = 6$).

(b) The time required for a 100% reduction in CAP, called IT_{100} (see Fig. 3C), measured in minutes, and for lidocaine it was 14.0 ± 1.1 min.

(c) The time required for the CAP to recover to 50% of the control value after replacement of 1% lidocaine in the perfusion chamber with normal saline, this period is called recovery time 50 (RT_{50}, Fig. 3C) and is measured in minutes and was estimated to be 48.75 ± 1.2 min for lidocaine.

(d) The vitality time, VT_{50} (see Fig. 3D), the period of time the value of the CAP was above the RT_{50}, measured in minutes, and was 660.0 ± 20 min for lidocaine. In case when the VT_{50} for certain of the examined compounds was below 660 min, or the compounds remained without recovery at all, these compounds were considered to be neurotoxic agents [46,47].

The above values for lidocaine are summarised in Table 1 (see Lid). To assess the local anaesthetic activity of the synthesized compounds, a standard concentration of each individual compound was diluted in saline to make a final concentration of 1%. This saline was used as a vehicle to expose the part of the sciatic nerve in the perfusion chamber to the compound under investigation. The effects of a specific compound on the evoked CAP of the sciatic nerve of the rat was estimated using the four parameters described above, IT_{50}, IT_{100}, RT_{50} and VT_{50}, and were compared to the action of 1% of lidocaine.

4.3. Methods for prediction of biological activity and toxicity

4.3.1. PASS

Prediction of anaesthetic activity for the compounds under study was made using PASS software [28,48,49]. PASS (prediction of activity spectra for substances) software was developed as a tool for evaluation of general biological potential in a molecule under study on the basis of its structural formula. PASS version 1.917 (July 2005) predicts about 2000 kinds of biological activity with a mean prediction accuracy of about 87%. The list of predictable biological activities includes main and side pharmacological effects (e.g. antihypertensive, hepatoprotective, sedative, etc.), mechanisms of action, (5-hydroxytryptamine agonist, acetylcholinesterase inhibitor, adenosine uptake inhibitor, etc.) and specific toxicities (mutagenicity, carcinogenicity, teratogenicity, etc.). In the present study the accuracy of anaesthetic activity prediction calculated by leave-one-out cross-validation was 85.5%.

In PASS, biological activities are described qualitatively (active or inactive). Reflecting the result of a chemical compound's interaction with a biological object, the biological activity depends on both the compound's molecular structure and the terms and conditions of the experiment. Therefore, structure–activity relationship analysis based on qualitative presentation of biological activity describes the general "biological potential" of the molecule being studied. On the other hand, qualitative presentation allows the integration of information concerning compounds collected, from many different sources, in the PASS training set.

The 2D structural formulae of compounds are used as the basis for description of chemical structure. Structural descriptors, which are called "Multilevel Neighbourhoods of Atoms" (MNA), were designed for chemical structure representation [50].

PASS uses SDfiles as external sources of structural and activity data to prepare both the training set and the set of substances for prediction. SDfiles (*.sdf) can be exported either from ISIS/Base 2.0+ (MDL Information Systems, Inc.) or from any other molecular editor or database management system, which has the option of SDfile's export.

The PASS training set included 57,978 substances. It is a complex knowledge base, containing vocabularies of MNA descriptors and activity names, the database of the substance structures presented by MNA descriptors, their biological activity types and data on structure–activity relationships. It contains data on 463 anaesthetics.

PASS presents the biological activity spectra for each substance as a result of prediction. It is a list of biological activity types for which the calculated likelihood of being present (**Pa**) is greater than the calculated probability of not being present (**Pi**). Taking into account the fact that some substances from the training set are formally considered to be inactive, the estimated value of **Pa** is thus more reliable. However, even in this case there are some other factors that essentially influence the absolute value: the number and diversity of substances revealing such activity in the training set, recall ratio, etc. In general, the higher the **Pa** value is, the greater the probability is for a substance under investigation to be structurally similar to known biologically active substances from the training set.

The result of prediction is valuable for planning the experiment, but one should take into account some additional factors such as particular interest in some kinds of activity, desirable novelty of a substance, available facilities for experimental testing etc. Actually, each choice is always a compromise between the desirable novelty of the substance being studied and the risk of obtaining a negative result in testing.

4.3.2. DEREK

DEREK (Deductive Estimation of Risk from Existing Knowledge) is a knowledge-based system for the qualitative and semi-quantitative prediction of a number of toxicity endpoints such as carcinogenicity, mutagenicity, teratogenicity, neurotoxicity, skin sensitisation, respiratory sensitisation, irritancy, corrosivity, lachrymation, methaemoglobinaemia and anticholinesterase activity [51]. DEREK contains a number of rulebases, comprising descriptions of molecular substructures (structural alerts) that have been associated with toxicity endpoints on the basis of existing knowledge. The rules are generic in nature; that is, they are generally based on sets of related chemicals rather than on specific chemicals, and most of them are derived from mechanistic organic chemistry. For some endpoints, a semi-quantitative prediction is given of the likelihood of a compound being toxic (certain, probable, plausible, implausible, improbable, impossible); however, only the categories ‘‘certain’’ and ‘‘probable’’ are considered reason enough to halt the development of a candidate drug.

4.4. QSAR methods

The SMILES strings of all compounds were entered into the TSAR [52] software to generate and optimise the 3D structures of molecules. The optimized geometries were also transferred into ADMEWORKS Predictor [53] and HYBOT [54], and all three programs were used to calculate a large number of molecular descriptors.

In the search for the best descriptor subset by stepwise regression from a large set of descriptors, there is a major problem in relation to the mutual collinearity of descriptors, which leads to instability of the regression coefficients, overestimated standard errors and a critical loss of predictive information. In addition, although inclusion of some descriptors which have a low correlation coefficient with local anaesthetic activity in a QSAR (quantitative structure–activity relationship) model may lead to better statistical results, these descriptors do not describe the behaviour of the property well, which for purposes of external prediction may lead to serious errors. We, therefore, used MOBYDIGS software [55] to eliminate those descriptors which have a very poor correlation with local anaesthetic activity, and those with high pair-wise collinearity, leaving a total of 242 descriptors.

The MOBYDIGS genetic algorithm (GA) routine and the Minitab [56] stepwise regression (forward–backward) routine were both used for selection of the best multiple linear regression QSAR models. Cross-validation was carried out using the LOO (leave-one-out) procedure in Minitab. We did not include lidocaine in the QSAR training set, since its chemical structure is quite different from those of our compounds.

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