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ScienceDirect
European Journal of Medicinal Chemistry 44 (2009) 54–62
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://www.elsevier.com/locate/ejmech

Original article

The cytotoxic properties and preferential toxicity to tumour cells displayed by some 2,4-*bis*(benzylidene)-8-methyl-8-azabicyclo[3.2.1] octan-3-ones and 3,5-*bis*(benzylidene)-1-methyl-4-piperidones

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Received 31 October 2007; received in revised form 5 March 2008; accepted 6 March 2008
Available online 29 March 2008

Abstract

This study demonstrated that replacement of the axial protons on the C2 and C6 atoms of various 1-methyl-3,5-*bis*(benzylidene)-4-piperidones **3** by a dimethylene bridge leading to series **2** lowered cytotoxic potencies. Four compounds **2a** and **3a–c** emerged as lead molecules based on their toxicity towards different neoplasms and their selective toxicity for malignant rather than normal cells. Some possible reasons for the disparity between the IC_{50} values in the two series of compounds are presented based on molecular modeling, log *P* values and respiration in rat liver mitochondria.

Keywords: Tropinones; 4-Piperidones; Cytotoxicity; Molecular modeling; X-ray crystallography; Mitochondria

1. Introduction

The major interest in these laboratories is the development of antineoplastic agents which are structurally divergent from contemporary anticancer drugs. These novel compounds are principally conjugated unsaturated ketones which are known to react with thiols [1] but have low or nonexistent affinities for amino and hydroxy groups [2,3]. Since thiols, in contrast to amino or hydroxy groups, are not found in nucleic acids, α,β-unsaturated ketones may be bereft of the carcinogenic and mutagenic properties displayed by various anticancer drugs [4]. There are a number of critical biochemical processes which involve thiols and the importance of compounds which interact with multiple molecular targets has been emphasized recently [5,6].

The 1,5-diaryl-3-oxo-1,4-pentadienyl group has been mounted on a variety of cyclic scaffolds leading to the discovery of a number of potent cytotoxins [7,8]. This group is considered to react at a primary binding site. However, the magnitude of the bioactivity observed will be influenced by the presence of other structural units in the molecule which align at an auxiliary site. These possibilities are illustrated in Fig. 1A. In order to probe as to the nature of the groups in the vicinity of the pharmacophore which affect cytotoxic potencies, various compounds possessing the general structure **1** were prepared (Fig. 1B). Several studies revealed that

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doi:10.1016/j.ejmech.2008.03.015

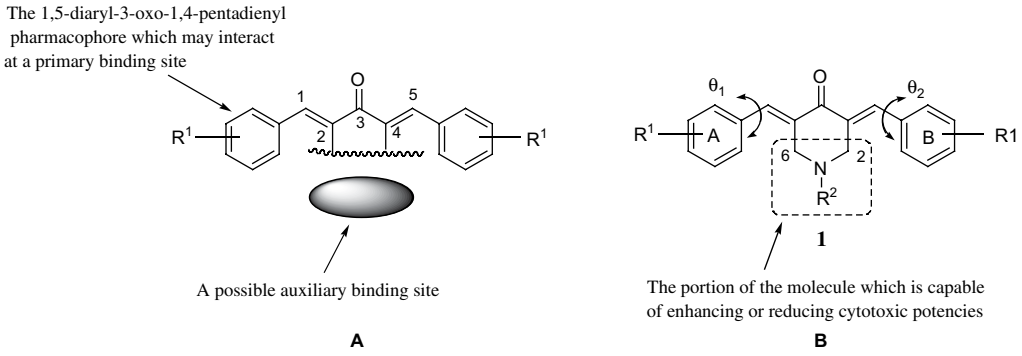


Fig. 1. A) The possible interactions of series **1**–**3** at binding sites. (B) The general structure **1**.

compounds in which R^2 is an acyl group have increased cytotoxic potencies compared to the analogs when R^2 is a hydrogen atom [9,10]. In fact a number of *N*-acyl compounds have submicromolar IC_{50} values and displayed selective toxicity to neoplasms than normal cells [11]. Thus by expanding the size of the molecules, there is the possibility of additional binding of the ligand at a receptor which results in the lowering of the IC_{50} values. The hypothesis formulated in this study is that by increasing the size of the heterocyclic scaffold, cytotoxic potencies will be elevated compared to the analogs lacking this additional structural unit. In the present case, a dimethylene bridge was placed between carbon atoms 2 and 6 of the piperidine ring to give series **2** with a view to comparing cytotoxic potencies with the analogs **3** which lack this structural feature.

Previous studies revealed that the lack of coplanarity of rings A and B with the adjacent unsaturated linkages in **1** was caused, inter alia, by nonbonded interactions between one of the *ortho* protons of each aryl ring with the equatorial hydrogen atoms at C2 and C6 [12]. The decision was made, therefore, to replace the axial and not equatorial protons on the C2 and C6 atoms by substituents. In this way, changes in the cytotoxic potencies between **1** and various analogs could be attributed to the topographical, physicochemical and chemical properties of the groups at C2 and C6 per se and the interpretation of the results would not be complicated by changes in the torsion angles θ_1 and θ_2. X-ray crystallography revealed that the displacement of the C2 and C6 axial hydrogen atoms of various piperidines by a dimethylene bridge afforded 8-azabicyclo[3.2.1]octanes [13,14]. Hence the aim of the present investigation was to prepare a small cluster of prototypic molecules related to **1** which bear C2 and C6 substituents, namely series **2**, and to compare their cytotoxic properties with the analogs having both axial protons intact on the C2 and C6 atoms viz series **3**. In particular, the information gained from this study may contribute to an understanding of those structural features which lead to marked cytotoxic properties.

2. Chemistry

The compounds in series **2** and **3** were prepared by the synthetic chemical route presented in Scheme 1. X-ray crystallography was undertaken on **2e** and an ORTEP diagram [15] of this compound is displayed in Fig. 4. Molecular models of **2a**–**e** and **3a**–**e** were built and the torsion angles θ_1 and θ_2 are recorded in Table 1.

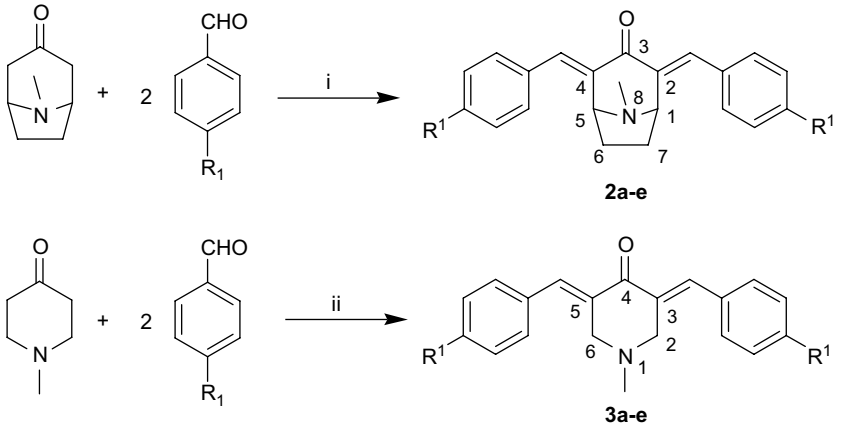
3. Bioevaluations

All of the compounds in series **2** and **3** were evaluated against human Molt 4/C8 and CEM T-lymphocytes and murine L1210 lymphoid leukemia cells. These results are portrayed in Table 1. In addition, these compounds were assayed for inhibitory effects towards human HSC-2, HSC-3 and HSC-4 oral squamous cell carcinomas and human HL-60 promyelocytic leukemia cells. Three normal human cell lines were also used, namely HGF gingival fibroblasts, HPC pulp cells and HPLF periodontal ligament fibroblasts. The data from these determinations are presented in Table 2. The effects of **2a** and **2d** on respiration and swelling of rat liver mitochondria are presented in Figs. 5 and 6, respectively. Doses of 30, 100 and 300 mg/kg of **2a**–**e** and **3a**–**e** were injected intraperitoneally into mice and the animals were observed after 0.5 and 4 h for any mortalities. In addition, using these doses and time intervals, the mice were examined for neurotoxicity by the rotorod test [16]. Two representative compounds, namely **2e** and **3c**, were administered orally to rats using a dose of 50 mg/kg and the animals were examined for mortalities and neurotoxicity after 0.25, 0.5, 1 and 2 h and in the case of **2e** after 4 h also.

4. Results and discussion

^{1}H NMR spectroscopy revealed that the compounds in series **2** and **3** are isomerically pure. X-ray crystallography of 8-methyl-2,4-*bis*(3-thienylmethylene)-8-azabicyclo[3.2.1]octan-3-one [17] as well as **2e** revealed that the olefinic double bonds adopted the *E* configuration. In addition, the same stereochemistry was noted with various 3,5-*bis*(benzylidene)-1-methyl-4-piperidones [18,19]. Hence the assumption was made that all of the compounds in series **2** and **3** are the *E*,*E* isomers.

All of the compounds in series **2** and **3** were evaluated against human Molt 4/C8 and CEM T-lymphocytes in order to ascertain the toxicity of these compounds towards human neoplastic cells. In addition, **2a**–**e** and **3a**–**e** were examined towards murine L1210 cells since a number of anticancer drugs are cytotoxic to this cell line [20] and hence it serves



Scheme 1. Synthetic routes employed in the synthesis of series **2** and **3** in which i = NaOH and ii = HCl/CH_3COOH. The R^1 substituents are as follows, namely **a**: $R^1 = H$; **b**: $R^1 = Cl$; **c**: $R^1 = NO_2$; **d**: $R^1 = CH_3$; **e**: $R^1 = OCH_3$.

as an indicator of compounds having potential clinical utility. These data are presented in Table 1.

The results indicate that among the tropinone derivatives, only **2a** displayed noteworthy cytotoxicity having an IC_{50} value of approximately 10 μM and on average possessing approximately one-quarter of the potency of melphalan. The 4-methoxy analog **2e** exhibited moderate potency towards L1210 cells but not to the T-lymphocytes while the remaining compounds in series **2** have IC_{50} values considerably in excess of 100 μM. On the other hand, in the 4-piperidone series, both **3a** and **3c** are potent cytotoxins especially towards the T-lymphocytes. Compound **3a** is 1.6 times more potent than melphalan towards Molt 4/C8 cells and is equipotent with this drug in the CEM assay. The 4-*nitro* analog **3c** is equipotent with melphalan in both the Molt 4/C8 and CEM tests. Clearly both **3a** and **3c** are useful lead molecules. Compound **3b** demonstrated modest potencies while the IC_{50} values of **3c** and **3d** are in excess of 100 μM. The unsubstituted compound in both series **2** and **3** possesses the lowest IC_{50} values which may indicate that an E_4 operating parameter is in effect [21]; i.e., the 4-substituent in **2b–e** and **3b–e** may cause an unfavourable steric impedance to the alignment of the molecules at critical binding sites. A second factor which may influence cytotoxic potencies in series **3** is the electronic contributions of the nuclear substituents. Thus in **3a–c**, the σ values of the R^1 group are 0.00–0.78 [22] while in the substantially less potent molecules **3d**,**e**, the σ constants are −0.17 to −0.27 [22]. Thus in the latter two compounds the methyl and methoxy aryl groups lower the fractional positive charge on the olefinic protons relative to **3a–c** thereby reducing electrophilicity towards cellular thiols.

The biodata presented in Table 1 reveal very clearly that the substitution of a dimethylene bridge for the axial protons attached to the C3 and C5 atoms of series **3** which generated **2a–e** leads to a reduction in cytotoxic potency. This conclusion may be drawn by noting that when the same substituents are present in the aryl rings, the compounds in series **3** are more potent than the analogs **2a–e** with the exception that **2e** is more cytotoxic than **3e** in the L1210 screen.

Table 1
Some cytotoxic and physicochemical properties of **2a–e** and **3a–e**

Compound	IC_{50} (μM)[a]				Torsion angles			
	Molt 4/C8	CEM	L1210	Average[b]	θ_1[c]	θ_2[c]	log P[d]	TPSA[d]
2a	8.51 ± 0.60	8.99 ± 0.54	11.8 ± 2.0	9.77	47.4	−47.4	4.33	20.3
2b	>500	>500	>500	>500	121.7	−45.3	5.68	20.3
2c	>500	247 ± 112	336 ± 3	>361	120.2	−120.5	4.25	112.0
2d	>500	>500	>500	>500	47.1	−47.1	5.22	20.3
2e	>500	–[e]	40.0 ± 18.4	–	46.2	−46.3	4.44	38.8
3a	1.98 ± 0.27	3.32 ± 2.30	8.77 ± 0.28	4.69	45.6	−46.0	3.95	20.3
3b	36.9 ± 8.0	33.9 ± 12.9	96.8 ± 3.5	55.9	45.5	−45.9	5.31	20.3
3c	2.42 ± 0.38	5.21 ± 3.06	14.0 ± 1.8	7.21	46.7	−47.3	3.87	112.0
3d	277 ± 6	233 ± 27	305 ± 10	171	45.5	−46.0	4.85	20.3
3e	230 ± 1	172 ± 6	281 ± 15	228	43.2	−43.7	4.06	38.8
Melphalan[f]	3.24 ± 0.56	2.47 ± 0.21	2.13 ± 0.02	2.61	–	–	–	–

[a] The IC_{50} values represent the concentrations of compounds required to inhibit the growth of the cells by 50%.
[b] These figures indicate the average of the IC_{50} figures towards the three cell lines.
[c] The θ values refer to the torsion angles between the aryl rings and the adjacent olefinic linkage.
[d] The letters log P and TPSA indicate the calculated log P and total polar surface area values, respectively, of the molecules.
[e] The percentage inhibition of CEM cells by **2e** was inconsistent viz 61 ± 7, 46 ± 3, 64 ± 4 and 12 ± 4 at concentrations of 500, 100, 20 and 4 μM, respectively.
[f] The data for melphalan was taken from Pharmazie 52 (1997) 182–186 with the permission of the copyright owner.

Table 2
Examination of **2a–e**, **3a–e** and melphalan against some human malignant and normal cells

Compound	Human tumour cells CC_{50} (μM)[a]					Human normal cells CC_{50} (μM)[a]			
	HSC-2	HSC-3	HSC-4	HL-60	Average[b]	HGF	HPC	HPLF	SI[c]
2a	21	44	23	8.3	24	298	>400	>400	>15
2b	>400	>400	>400	>400	>400	>400	>400	>400	~1.0
2c	308	>400	>400	>400	>377	>400	>400	>400	~1.1
2d	>400	>400	>400	>400	>400	>400	>400	>400	~1.0
2e	300	380	250	364	324	162	>400	366	~1.0
3a	4.2	7.9	7.4	2.0	5.4	45	64	40	9.2
3b	7.4	16	39	14	19	323	369	>400	>19
3c	16	47	22	20	26	170	>400	326	>11
3d	276	>400	338	>400	>354	>400	>400	>400	~1.1
3e	344	>400	>400	>400	>386	>400	>400	>400	~1.0
Melphalan[d]	35	115	81	6	59	>200	>200	>200	>3.4

[a] The CC_{50} values are the concentrations of the compounds required to kill 50% of the cells. Determinations were carried out in duplicate and the variation between experiments was less than 5%.

[b] The average values reflect the mean of the CC_{50} figures for the compounds generated using HSC-2, HSC-3, HSC-4 and HL-60 cells.

[c] The letters SI refer to the selectivity index (SI) values which are the quotients of the average CC_{50} figures of the compounds towards normal cells divided by the average CC_{50} data for the malignant cell lines.

[d] Solubility considerations precluded the use of concentrations higher than 200 μM. The data for melphalan are taken from Bioorganic and Medicinal Chemistry 2007; 15:3373–3380 and is reproduced with the permission of Elsevier.

Attempts were made to determine the reasons for the lowering of potencies when substituents were placed on the C2 and C6 atoms in series **3**. The information generated may assist in gleaning further knowledge of those structural features which influence cytotoxicity. First, molecular models of **2a–e** and **3a–e** were made and the torsion angles θ_1 and θ_2 are listed in Table 1. Apart from the anomolous behaviour of **2b** and **2c**, these angles are all in the range of 43–47°. Thus, in general, substitution of the C2 and C6 axial protons in both series of compounds does not affect the magnitude of the deviation from coplanarity between rings A and B and the adjacent olefinic linkages. The changes in bioactivity between series **2** and **3** are, therefore, likely due to factors in the loci of the carbon atoms attached to the nitrogen atom. The biodata in Table 1 reveal that in all three assays, **3a** possessed greater cytotoxicity potencies than **2a**. Models of both compounds are presented in Fig. 2 and the greater steric bulk in the vicinity of the basic centre and the adjacent carbon atoms in **2a** are apparent. Furthermore in order to provide some quantitative information pertaining to the steric bulk in the vicinity of the C1/C5 and C2/C6 atoms in **2a** and **3a**, respectively, the interatomic distances d_1 and d_2 as indicated in Fig. 3 were measured. The d_1 values of **2a** and **3a** are 2.21 and 1.11 Å, respectively, while the d_2 figures are 2.25 and 2.38 Å, respectively. Thus while both the C1/C5 and C2/C6 atoms in **2a** and **3a**, respectively, could align at the same portion of a binding site, the dimethylene bridge likely exerts a significant steric repulsion. The areas occupied by the axial substituents in **2a** and **3a**, i.e., $d_1 \times d_2$, are 4.97 and 2.64 sq Å, respectively. Thus, in future, the design of compounds occupying an area in between these two values may afford further information as to the effect of substituents on the C2 and C6 atoms in series **3**, e.g., a methyl or trifluoromethyl group could be placed on one of the C2 or C6 atoms in series **3**.

X-ray crystallography of a representative compound in series **2**, namely **2e**, was undertaken to confirm the *E* stereochemistry of the olefinic double bonds and to compare the θ_1 and θ_2 values with the published X-ray crystallographic data for **3e**. In the case of **2e** there are two molecules, designated **2e1** and **2e2**, in the asymmetric unit of the centrosymmetric space group $P2_1/c$. These molecules are very similar in shape and an ORTEP-3 diagram [15] of **2e1** is presented in Fig. 4. Both **2e1** and **2e2** are the *E*,*E* isomers. The C4-C9-C10-C11 (θ_1) and C2-C16-C17-C18 (θ_2) values for **2e1** are 22.1° and −14.0°, respectively, while the comparable θ_1 and θ_2 figures for **2e2** are 13.9° and −15.4°, respectively. The θ_1 and θ_2 values for **3e** are 18.4° and −26.8°, respectively [18]. In general, therefore, the X-ray crystallographic data support the concept that replacement of the axial protons in series **3** by a dimethylene bridge does not change the torsion angles θ_1 and θ_2 to an appreciable extent.

Since the hydrophobicity of molecules may influence the extent of bioactivity significantly [23], the log *P* values of **2a–e** and **3a–e** were computed. These data are presented in Table 1. The lower log *P* values of the compounds in series **3** than **2** when comparing pairs of compounds having identical aryl substituents may have contributed to the generally greater cytotoxic potencies of the 1-methyl-4-piperidones **3**. Compounds in series **2** and **3** which have the same R^1 substituents have identical total polar surface area (TPSA) values as indicated in Table 1; consequently TPSA values do not contribute to potency differences between the two series of compounds. In order to seek possible correlations between cytotoxic potencies in series **3** and both the log *P* and TPSA values, linear, semilogarithmic and logarithmic plots were made between these physicochemical parameters and the IC_{50} values of **3a–e** in each of the three assays. However, no correlations were observed ($p > 0.1$).

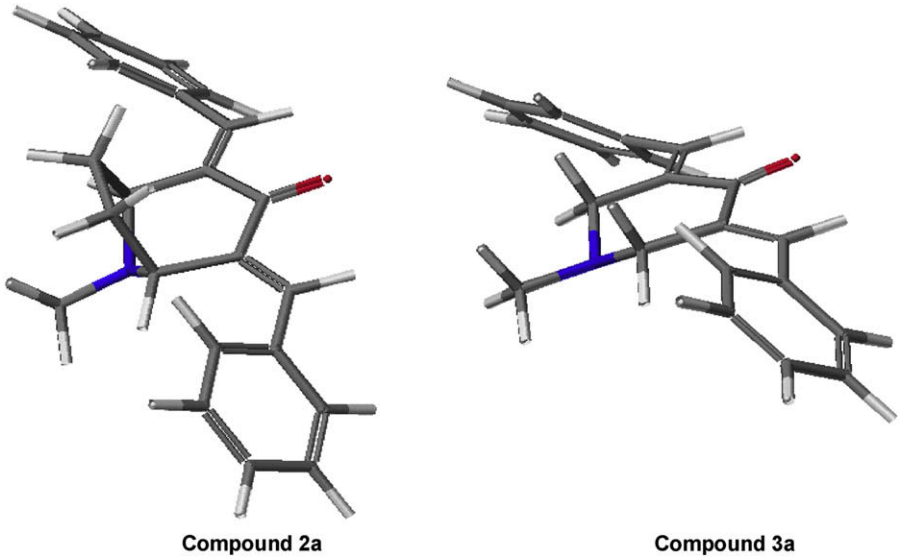


Fig. 2. Molecular models of **2a** and **3a**.

Various acyclic 3-aminoketones inhibit or stimulate respiration in mitochondria isolated from rat and mouse liver cells [24,25]. Since the compounds prepared in this study are cyclic 3-aminoketones, the question arises whether different effects on mitochondria may explain the variation in cytotoxic potencies. Accordingly two compounds displaying markedly divergent cytotoxic properties, namely **2a** and **2d**, were examined. Fig. 5 shows that after a delay both compounds exert a strong stimulating effect on mitochondrial respiration, with compound **2a** giving a significantly shorter latent period (1.96 ± 0.05 min versus 4.45 ± 0.13 min). Measurements shown in Fig. 6 reveal that **2a** produces rapid mitochondrial swelling, while **2d** does so only more slowly. Mitochondrial swelling involves opening of the mitochondrial permeability transition pore and collapse of the mitochondrial membrane potential [26]. This collapse of the membrane potential decreases the resistance to electron flow in the respiratory chain and increases mitochondrial respiration [27], which accounts for the increase in respiration observed in Fig. 5. The mitochondrial permeability transition is a critical trigger for apoptosis [28] and has been identified as a target for cancer therapy [29–31]. The greater ability of **2a** to induce mitochondrial swelling, therefore, may have contributed to its higher cytotoxic potency in the cancer cell lines.

A difference in electrophilicity may explain the difference in the ability of **2a** and **2d** to cause mitochondrial swelling. The opening of the mitochondrial permeability transition pore involves alkylation or cross-linking of a critical thiol on a protein of the permeability transition pore complex [32,33], and as noted earlier these conjugated unsaturated ketones are known to react with thiols [1]. In the less potent **2d**, the R^1 methoxy substituents are less electronegative (σ value $= -0.17$) than the R^1 protons (σ value $= 0.00$) of **2a** and would thereby decrease the electrophilicity of **2d** towards thiols compared to **2a**.

All of the compounds in series **2** and **3** were evaluated further using human HSC-2, HSC-3, HSC-4 and HL-60 neoplasms. These data are presented in Table 2. The results are similar to the biodata generated using Molt 4/C8, CEM and L1210 cells, namely in series **2** only **2a** displays noteworthy cytotoxicity while **3a–c** are substantially more potent than **3d**,**e**. Taking into consideration the average CC_{50} values towards these four cell lines, **2a** and **3a–c** possess 2.5, 10.9, 3.1 and 2.3 times the potency of melphalan and are clearly lead molecules. The CC_{50} values of **2a** and **3a–c** towards human HGF, HPC and HPLF normal cells reveal their excellent selectivity (SI) figures demonstrating the preferential toxicity of these compounds for neoplastic cells, which further confirms their importance as templates for further development.

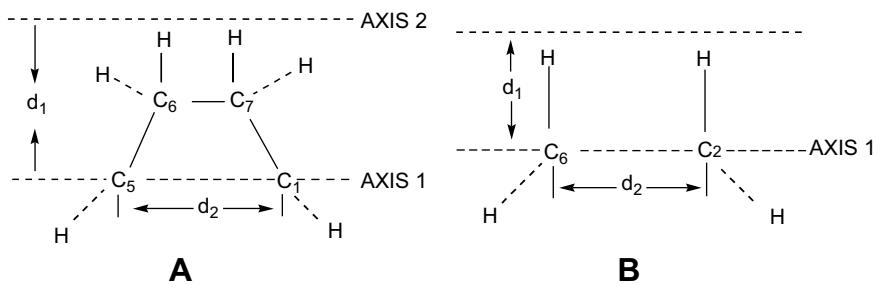


Fig. 3. A comparison of the steric bulk of portions of the structures of **2a** (A) and **3a** (B).

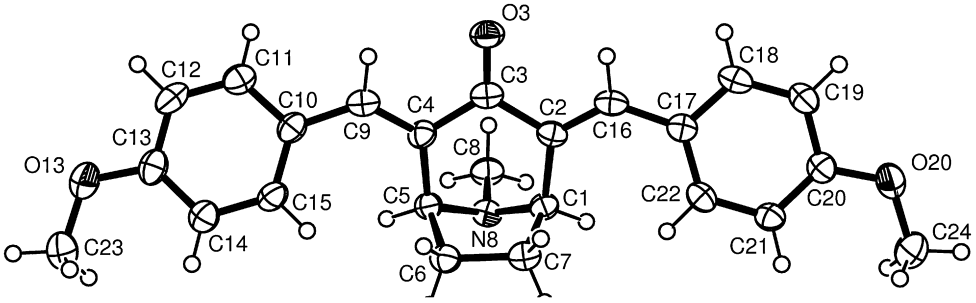


Fig. 4. An ORTEP-3 diagram of **2e1** determined by X-ray crystallography.

A number of acylic 3-aminoketones or Mannich bases are lethal to mice at low doses, e.g., 30 mg/kg, and also display neurotoxicity [34]. Since 4-piperidones may be considered cyclic 3-aminoketones, the evaluation of the compounds in series **2** and **3** with regard to mortality and neurological deficit was undertaken. Doses up to and including 300 mg/kg of **2a**–**e** and **3a**–**e** were administered intraperitoneally to mice and after 4 h, no deaths of the animals were noted. Minimal neurotoxicity was observed with **2e**, **3a**,**c**,**e** after 0.5 h and with **3a** after 4 h. A dose of 300 mg/kg of **2e** caused neurotoxicity in all of the animals. No neurological disturbances were caused by the other compounds. A dose of 50 mg/kg of two representative compounds **2e** and **3c** was administered orally to rats and the animals were examined at different time intervals up to 4 (**2e**) and 2 (**3c**) h. No mortalities or neurological deficit was noted. The conclusion drawn from this short-term toxicity evaluation is that the compounds in series **2** and **3** are well tolerated in rodents thereby enhancing their potential for future development.

5. Conclusions

This study has revealed clearly that in general replacement of the 2a and 6a protons in series **3** by a dimethylene bridge leading to **2a**–**e** is accompanied by a reduction in cytotoxic potencies. Thus development of the cytotoxic 3,5-*bis*(benzylidene)-4-piperidones in which two protons are present on the carbon atoms attached to the basic centre appears to be a prudent decision. However, limited molecular modifications whereby groups of varying sizes are placed on the 2 and 6 carbon atoms of series **3** may establish the generality or otherwise that such structural changes are disadvantageous in terms of cytotoxic potencies. The reasons for the disparity in cytotoxic potencies between series **2** and **3** may have been due to the dimethylene bridge in **2a**–**e** exerting a steric impedance to alignment at one or more binding sites as well as variation in hydrophobic properties and possibly differential effects on mitochondrial respiration.

In terms of cytotoxic potencies, the data in Table 1 reveal that **2a**, **3a** and **3c** are lead molecules while moderate potency was displayed by **3b**. The assessment of these four compounds against human tumour cell lines as revealed in Table 2 confirmed their cytotoxic properties which in these assays are on average more potent than a reference drug melphalan. The importance of these four compounds as templates for future development was enhanced by two additional observations. First, **2a** and **3a**–**c** display preferential cytotoxicity for malignant rather than normal cells as revealed by the SI

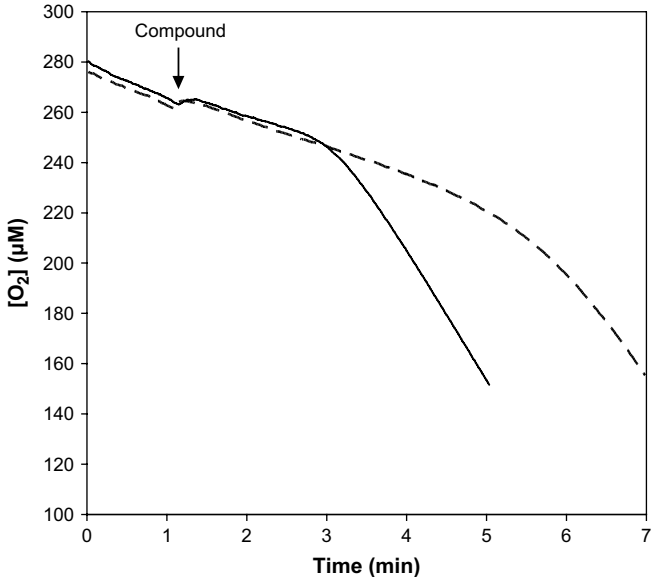


Fig. 5. Effects of **2a** and **2d** on respiration of rat liver mitochondria. Where indicated by the arrow, compounds were added to a respiring suspension of rat liver mitochondria to a concentration of 250 μM: — **2a**, – – – **2d**.

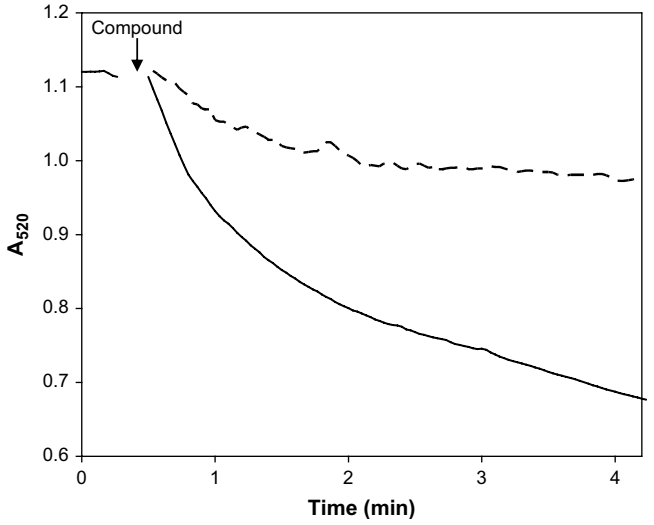


Fig. 6. Effects of **2a** and **2d** on swelling of rat liver mitochondria. Where indicated by the arrow, compounds were added to a respiring suspension of rat liver mitochondria to a concentration of 250 μM: — **2a**, – – – **2d**.

figures in Table 2. Second, these compounds and their analogs in series **2** and **3** are well tolerated in mice.

6. Experimental protocols

6.1. Chemistry

Melting points which are uncorrected were determined using a Gallenkamp instrument. ^{1}H NMR spectra were recorded using a Bruker AMX 500 FT machine while elemental analyses (C, H, N) were obtained using an Elementer analyzer and were within 0.4% of the calculated values. 4-Piperidones **3a**,**c**,**d** were crystallized with 0.25, 0.75 and 0.25 mol of water of crystallization, respectively. X-ray crystallography was undertaken using a Nonius instrument.

6.1.1. Synthesis of 2,4-bis(benzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-ones (*2a–e*)

Sodium hydroxide solution (5N, 1 ml) was added dropwise to a solution of 8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (0.5g, 0.0036 mol) and the appropriate aryl aldehyde (0.0072 mol) in ethanol (20 ml) at room temperature. The reaction mixture was stirred under nitrogen for 2 h at room temperature and then water (15 ml) was added. The precipitate was collected and recrystallized from ethanol.

*6.1.1.1. 2,4-Bis(benzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (**2a**).* Yield: 70%; m.p. 148 °C, (lit. [35] m.p. 150–151 °C). ^{1}H NMR ($CDCl_3$) δ: 2.06 (q, 2H), 2.33 (s, 3H), 2.61 (p, 2H), 4.43 (dd, 2H), 7.40 (m, 10H), 7.86 (s, 2H). Anal. calcd. for $C_{22}H_{21}NO$: C, 83.78; H, 6.74; N, 4.44%. Found: C, 83.57; H, 6.54; N, 4.40%.

*6.1.1.2. 2,4-Bis(4-chlorobenzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (**2b**).* Yield: 85%, m.p. 183 °C. ^{1}H NMR ($CDCl_3$) δ: 2.02 (q, 2H), 2.32 (s, 3H), 2.62 (p, 2H), 4.35 (p, 2H), 7.34 (d, 4H, $J = 8.38$ Hz), 7.43 (d, 4H, $J = 8.61$ Hz), 7.78 (s, 2H). Anal. calcd. for $C_{22}H_{19}Cl_2NO$: C, 68.76; H, 4.98; N, 3.64. Found: C, 68.71; H, 4.98; N, 3.77%.

*6.1.1.3. 8-Methyl-2,4-bis(4-nitrobenzylidene)-8-aza-bicyclo[3.2.1]octan-3-one (**2c**).* Yield: 73%, m.p. 247 °C. ^{1}H NMR ($CDCl_3$) δ: 2.06 (q, 2H), 2.34 (s, 3H), 2.68 (p, 2H), 4.34 (dd, 2H), 7.55(d, 4H, $J = 8.60$ Hz), 7.83 (s,2H), 8.33 (d, 4H, $J = 8.67$ Hz). Anal. calcd. for $C_{22}H_{19}N_3O_5$: C, 65.18; H, 4.72; N, 10.37. Found: C, 64.95; H, 4.67; N, 10.60%.

*6.1.1.4. 8-Methyl-2,4-bis(4-methylbenzylidene)-8-aza-bicyclo[3.2.1]octan-3-one(**2d**).* Yield: 80%, m.p. 172 °C. ^{1}H NMR ($CDCl_3$) δ: 2.05 (q, 2H), 2.33 (s, 3H), 2.42 (s, 6H), 2.63 (p, 2H), 4.43 (dd, 2H), 7.26 (d, 4H, $J = 7.92$ Hz), 7.33 (d, 4H, $J = 7.99$ Hz), 7.84 (s, 2H). Anal. calcd. for $C_{24}H_{25}NO$: C, 83.93; H, 7.34; N, 4.08. Found: C, 84.13; H, 7.02; N 4.20%.

*6.1.1.5. 2,4-Bis(4-methoxybenzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (**2e**).* Yield: 82%, m.p. 160 °C (lit. [35] m.p. 162–163 °C). ^{1}H NMR ($CDCl_3$) δ: 2.03 (q, 2H), 2.34 (s, 3H), 2.62 (p, 2H), 3.87 (s, 6H), 4.41 (dd, 2H), 6.98 (d, 4H, $J = 8.64$ Hz), 7.40 (d, 4H, $J = 8.68$ Hz), 7.82 (s, 2H). Anal. calcd. for $C_{24}H_{25}NO_3$: C, 76.77; H, 6.71; N, 3.73. Found: C, 76.89; H, 6.54; N, 3.77%.

6.1.2. Synthesis of 3,5-bis(benzylidene)-1-methyl-4-piperidones (*3a–e*)

Dry hydrogen chloride was passed into a solution of 1-methyl-4-piperidone (0.05 mol) and the appropriate aryl aldehyde (0.10 mol) in acetic acid (25 ml) at room temperature. The mixture was stirred at room temperature for 6–8 h and the precipitate was collected, washed with acetone (20 ml) and added to a solution of aqueous potassium carbonate solution (5% w/v). The free base was collected, dried under vacuum at 45–50 °C and crystallized from ethanol (**3a**), chloroform–methanol (**3b**,**d**,**e**) or chloroform–ethanol (**3c**).

*6.1.2.1. 3,5-Bis(benzylidene)-1-methyl-4-piperidone (**3a**).* Yield: 71%, m.p. 110 °C (lit. [36] m.p. 115–117 °C). ^{1}H NMR ($CDCl_3$) δ: 2.49 (s, 3H), 3.80 (s, 4H), 7.42 (m, 10H), 7.85 (s, 2H). Anal. calcd. for $C_{20}H_{19}NO.0.25H_2O$: C, 81.66; H, 6.46; N, 4.76%. Found: C, 81.90; H, 6.36; N, 4.69%.

*6.1.2.2. 3,5-Bis(4-chlorobenzylidene)-1-methyl-4-piperidone (**3b**).* Yield: 88%, m.p. 183 °C (lit. [36] m.p. 174–176 °C). ^{1}H NMR ($CDCl_3$) δ: 2.49 (s, 3H), 3.75 (s, 4H), 7.35 (d, 4H, $J = 8.44$ Hz), 7.43 (d, 4H, $J = 8.46$ Hz), 7.77 (s, 2H). Anal. calcd. for $C_{20}H_{17}Cl_2NO$: C, 67.05; H, 4.78; N, 3.91. Found: C, 66.89; H, 4.67; N, 3.81%.

*6.1.2.3. 3.5-Bis(4-nitrobenzylidene)-1-methyl-4-piperidone (**3c**).* Yield: 81%, m.p. 223 °C (lit. [36] m.p. 229–231 °C). ^{1}H NMR ($CDCl_3$) δ: 2.89 (s, 3H), 3.56 (br s, 4H), 7.52 (d, 4H, $J = 8.62$ Hz), 7.76 (s, 2H), 8.23 (d, 4H, $J = 8.61$ Hz). Anal. calcd. for $C_{20}H_{17}N_3O_5$ 0.75 H_2O: C, 61.08; H, 4.32; N, 10.69. Found: C, 61.05; H, 4.42; N, 11.07%.

*6.1.2.4. 3,5-Bis(4-methylbenzylidene)-1-methyl-4-piperidone (**3d**).* Yield: 76%, m.p. 201 °C (lit. [36] m.p. 192–195 °C). ^{1}H NMR ($CDCl_3$) δ: 2.42 (s, 6H), 2.49 (s, 3H), 3.79 (s, 4H), 7.26 (d, 4H, $J = 7.95$ Hz), 7.33 (d, 4H, $J = 8.0$ Hz), 7.82 (s, 2H). Anal. calcd. for $C_{22}H_{23}NO$ 0.25 H_2O: C, 82.00; H, 7.14; N, 4.35. Found: C, 81.92; H, 7.19; N, 4.25%.

*6.1.2.5. 3,5-Bis(4-methoxybenzylidene)-1-methyl-4-piperidone (**3e**).* Yield: 83%, m.p. 186 °C (lit. [36] m.p. 199–201 °C). ^{1}H NMR ($CDCl_3$) δ: 2.51 (s, 3H), 3.79 (s, 4H), 3.88 (s, 6H), 6.98 (d, 4H, $J = 8.71$ Hz), 7.40 (d, 4H, $J = 8.67$ Hz), 7.80 (s, 2H). Anal. calcd. for $C_{22}H_{23}NO_3$: C, 75.62; H, 6.63; N, 4.01. Found: C, 75.72; H, 6.71; N, 4.22%.

6.1.3. X-ray crystallography of 2,4-bis(4-methoxybenzylidene)-8-methyl-8-aza-bicyclo[3.2.1]octan-3-one (*2e*)

With the exception of the structure factors, data pertaining to the X-ray crystallographic determination of **2e** have been deposited with the Cambridge Crystallographic Data Centre

as supplementary publication no. CCDC 656960. This information may be obtained without cost by applying to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44 (0) 1223 336033 or e-mail deposit@ccdc.cam.ac.uk).

6.1.4. Molecular modeling

The molecular models of **2a–e** and **3a–e** were built using the BioMedCache program [37]. MOPAC optimized geometry calculations using AM1 parameters were employed in order to produce the lowest energy conformations.

6.1.5. Determination of the calculated log P and total polar surface area values of *2a–e* and *3a–e*

The log *P* and TPSA data were generated using the JME molecular editor [38].

6.1.6. Statistical calculations

The linear, semilogarithmic and logarithmic plots between the IC_{50} values of **3a–e** in different bioassays and the *c* log *P* and TPSA data were made using a software package [39].

6.2. Bioassays

6.2.1. Cytotoxicity evaluations

The assays using human Molt 4/C8 and CEM T-lymphocytes and murine L1210 cells have been described previously [40]. In brief, the cells are incubated with different concentrations of compounds in RPMI 1640 medium at 37 °C for 72 h (Molt 4/C8 and CEM cells) and 48 h (L1210 cells).

A literature procedure was utilized for the bioevaluation of **2a–e** and **3a–e** towards HSC-2, HSC-3, HSC-4, HL-60, HGF, HPC and HPLF cells [41]. In brief, with the exception of assays using HL-60 cells, different concentrations of compounds were incubated with the cell lines in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum. Cell viability was determined by the MTT method after 24 h incubation at 37 °C. A similar procedure was followed for the HL-60 cells except RPMI 1640 media containing 10% fetal bovine serum was used and cytotoxicity was assessed by the trypan blue exclusion procedure.

6.2.2. Evaluation of *2a* and *2d* on respiration in rat liver mitochondria

Rats were euthanized by isoflurane anesthesia and decapitation. The mitochondria from the liver were isolated by differential centrifugation using a literature procedure [42]. The effect of **2a** and **2d** on the consumption of oxygen in mitochondria was measured by polarography by a previously reported methodology [43]. In these measurements, freshly isolated mitochondria were incubated at 30 °C in a respiratory buffer of 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 5 mM potassium phosphate, 1 mM $MgCl_2$, pH 7.2 containing 5 mM succinate as respiratory substrate. Under the same conditions, mitochondrial swelling was measured spectrophotometrically by the loss in light scattering at 520 nm as described previously [42].

6.2.3. Toxicity and neurotoxicity evaluations in rodents

The toxicity and neurotoxicity evaluations were undertaken by the National Institute of Neurological Disorders and Stroke, USA according to their protocols [44]. Doses of 30, 100 and 300 mg/kg of **2a–e** and **3a–e** were injected intraperitoneally into mice and observed for both mortalities and neurotoxicity at the end of 0.5 and 4 h. No deaths were observed. Neurotoxicity was observed in the following cases (dose in mg/kg, time of observation in hours, number of animals displaying neurological deficit/total number of animals in the test) viz **2d** (300, 4, 2/2), **2e** (100, 0.5, 1/8), **3a** (100, 0.5, 2/8; 100, 4, 1/4), **3c** (100, 0.5, 1/8) and **3e** (300, 0.5, 1/4). In addition, doses of 50 mg/kg of **2e** and **3c** were administered orally to rats. At the end of 0.25, 0.5, 1 and 2 h (also 4 h in the case of **2e**), no deaths or neurotoxicity was observed.

Acknowledgments

The authors thank the following agencies and individuals who enabled this study to be undertaken. The Canadian Institutes of Health Research provided operating grants to J.R. Dimmock and B. Bandy. The Molt 4/C8, CEM and L1210 assays were undertaken by Mrs. Lizette van Berckelaer and funded by the Flemish Fonds voor Wetenschappelijk Onderzoek (FWO). A Grant-in-Aid was provided by the Ministry of Education, Science, Sports and Culture of Japan to H. Sakagami (No. 19592156). The Canadian Foundation for Innovation and the Government of Saskatchewan provided funding for the X-ray crystallography laboratory. The National Institute of Neurological Disorders and Stroke undertook the rodent toxicity studies while Ms. B. McCullough typed various drafts of the manuscript.

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