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Cytotoxic and Topographical Properties of 6-Arylidene-2-dimethylaminomethylcyclohexanone Hydrochlorides and Related Compounds

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A number of 2-arylidenecyclohexanones (1a–h) were converted into the corresponding Mannich bases (2a–h) and (3a,f). Evaluation against murine L1210 cells as well as human Molt 4/C8 and CEM T-lymphocytes revealed the marked cytotoxicity of the Mannich bases and also the fact that almost invariably these compounds were more potent than the precursor enones (1a–h). Further evaluation of most of the Mannich bases towards a panel of nearly 60 human tumour cell lines confirmed their utility as potent cytotoxins. In this assay, the compounds showed growth-inhibiting properties greater than the anticancer alkylator melphalan. QSAR studies revealed that in some cell lines compounds possessing small electron-attracting aryl substituents showed the greatest potencies. Molecular modeling and X-ray crystallography demonstrated that various interatomic distances and torsion angles correlated with cytotoxicity. A representative compound (2a) demonstrated weak inhibiting properties towards human N-myristoyltransferase and stimulated a tyrosine protein kinase. A single dose of 100 mg/kg of most of the compounds did not prove to be lethal in mice.

Keywords: Mannich bases; Cytotoxicity; Molecular modeling; X-ray crystallography; QSAR; Human N-myristoyltransferase; Tyrosine kinase

INTRODUCTION

A number of years ago a preliminary communication from our laboratory revealed the cytotoxicity displayed by the Mannich bases (**2**) and (**3**) towards murine P388 cells and various tumour cell lines (Fig. 1).[1] Independently the cytotoxic and anti-inflammatory properties of several of these compounds were described,[2] and so were various cyclopentyl analogues.[3] The purpose of the present study was to undertake further investigations with series (**2**) and (**3**) and related compounds in order to explore their potential as cytotoxic and anticancer agents. This report outlines the rationale for preparing these Mannich bases, the results of additional bioevaluations, some attempts to relate cytotoxicity to the shapes of molecules determined by molecular modeling and X-ray crystallography and an investigation as to a possible site of action of these compounds.

The reasons for preparing the compounds in series (**1**–**4**) included the following considerations. The incorporation of the conjugated arylideneketo group into series (**1**–**3**) was based on the affinity of α,β-unsaturated ketones for thiol groups.[4] Since thiols are absent in nucleic acids, these compounds may be devoid of the genotoxic properties associated with a number of anticancer drugs. In particular, various 2-arylidenecyclohexanones displayed cytotoxicity towards human epidermoid carcinoma of the nasopharynx,[5] and recently the growth-inhibiting properties of (**1a**,**c**,**d**,**f**,**g**) towards murine P388 and L1210 cells as well as human Molt 4/C8 and CEM T-lymphocytes was described.[6] The synthesis

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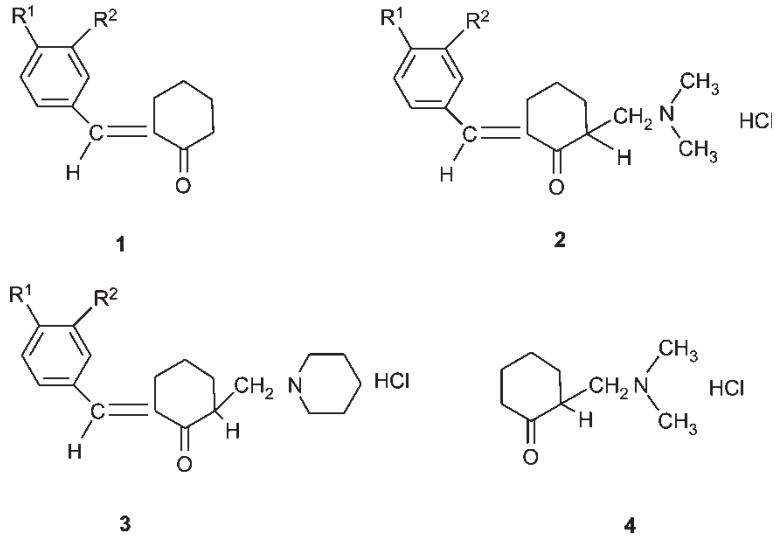

FIGURE 1 The structures of the compounds in series (**1**–**4**). The aryl substituents in series (**1**–**3**) were as follows: **a**: $R^1 = R^2 = H$; **b**: $R^1 = F$, $R^2 = H$; **c**: $R^1 = Cl$, $R^2 = H$; **d**: $R^1 = R^2 = Cl$; **e**: $R^1 = Br$, $R^2 = H$; **f**: $R^1 = CH_3$, $R^2 = H$; **g**: $R^1 = OCH_3$, $R^2 = H$; **h**: $R^1 = N(CH_3)_2$, $R^2 = H$; **i**: $R^1 = NO_2$, $R^2 = H$.

of (**1i**) was suggested based on the recent observations of the significant cytotoxic potencies of various conjugated arylidene enones containing a 4-nitrophenyl group.[7,8] The decision to convert the 2-arylidenecyclohexanones (**1a**–**h**) into the corresponding Mannich bases (**2a**–**h**) was based on two considerations. First, the rates of reaction with thiols of the Mannich bases of a number of acyclic α,β-unsaturated ketones was approximately 240 times greater than that of the precursor enones.[9] Hence the avidity for thiols in series (**2**) should be substantially greater than (**1**), which may be associated with greater cytotoxicity. Second, the release of a cytotoxic agent after initial chemical attack may be more detrimental to malignant cells than the corresponding normal cells.[10,11] Thus in considering series (**2**), thiolation could occur initially at the methine carbon atom and subsequently at a site liberated by deamination; this possibility is indicated in Fig. 2.

Replacement of the dimethylamino group of (**2a**,**f**) by a 1-piperidino function leading to (**3a**,**f**) was suggested for the following reasons. The pK_a values of dimethylamine and piperidine are 10.73 and 11.12,

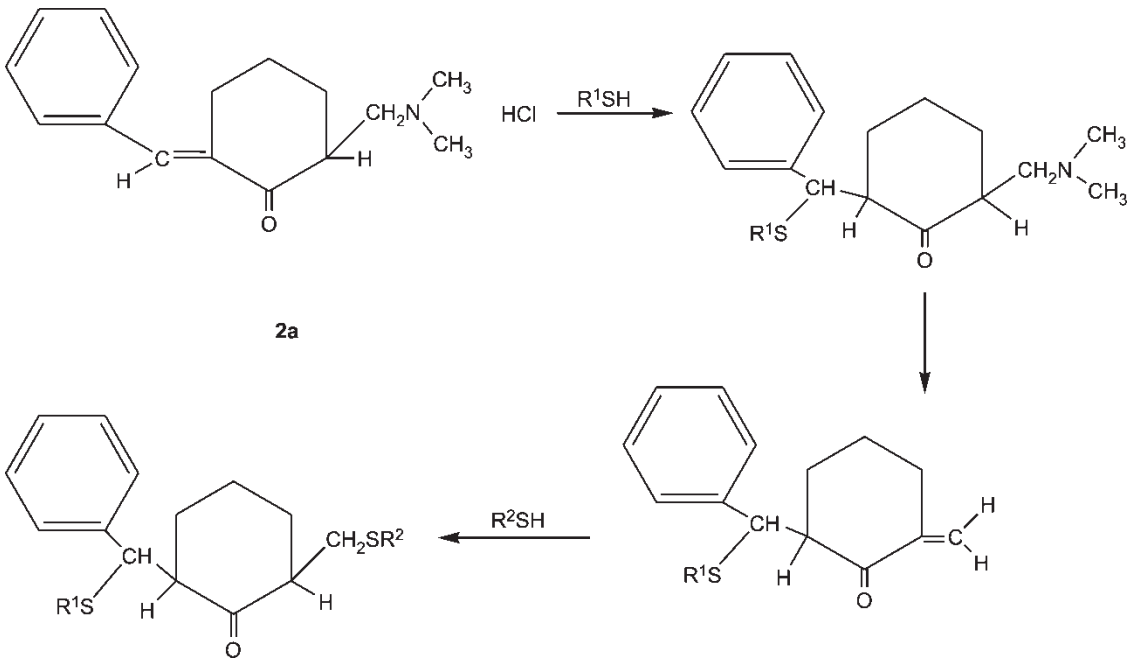

FIGURE 2 The proposed mechanism by which a representative Mannich base **2a** reacts sequentially with cellular thiols (designated as R^1SH and R^2SH).

respectively.[12] Rates of deamination are inversely proportional to pK_a values. This phenomenon may be attributed to the bond between the carbon atom of the methylene group attached to the alicyclic ring and nitrogen atom being weaker in Mannich bases derived from amines having low pK_a values and hence more susceptible to deamination than in analogues having carbon–nitrogen bonds derived from amines with higher pKa values. Hence the extent of the release of 6-arylidene-2-methylene-cyclohexanone should be greater with (**2a**,**f**) compared to (**3a**,**f**), respectively, which may lead to variations in cytotoxicity. In addition, a comparison of the potencies of (**2a**) with (**3a**) as well as (**2f**) with (**3f**) may indicate whether cytotoxicity is dependent on the nature of the basic centre. The bioevaluation of (**4**) should permit an estimate of its contribution to the cytotoxicty of the compounds in series (**2**).

A molecular target of interest in these laboratories is N-myristoyltransferase (NMT). The reasons for targeting this enzyme, which have been reviewed recently,[13] include the fact that a number of tumours express greater quantities of this enzyme than the corresponding normal cells. Hence deprivation of NMT may be more detrimental to neoplastic than normal tissues. A recent study revealed that various Mannich bases of conjugated styryl ketones inhibit this enzyme,[14] and thus the evaluation of a representative compound (**2a**) towards this enzyme may indicate, at least in part, the reason for any cytotoxicity observed. In addition, overexpression of various type 1 receptor tyrosine kinases has been observed in the development of different tumours.[15] A number of compounds which inhibited some of these kinases displayed greater inhibition towards some tumour cell lines than normal fibroblasts.[16] The evaluation of (**2a**) towards a tyrosine kinase was therefore planned.

Finally, should significant cytotoxicity be displayed by various members of series (**1**–**4**), an estimation of their murine toxicity would be warranted in order to evaluate the potential of these compounds for further development.

In summary, this study sought to prepare a limited number of prototypic molecules with a view to obtaining lead compounds. In addition, analyses of the biodata were proposed in order to gain insights as to how the project could be amplified on a rational basis.

MATERIALS AND METHODS

Chemistry

Melting points °C are uncorrected and yields are expressed as percentages. Elemental analyses were undertaken by Mr K. Thoms, Department of Chemistry, University of Saskatchewan on **1b**,**e** (C, H) and **1i** (C, H, N) as well as the intermediate aldols isolated in preparing these compounds and were within 0.4% of the calculated values. ^{1}H NMR spectra were determined routinely using Varian T-60, Bruker AM-300 and Bruker AM 500 FT NMR spectrometers.

Synthesis of 1–4

The preparation of (**1b**,**e**,**i**) was accomplished as follows. The intermediate 2-(arylhydroxymethyl) cyclohexanones required in the syntheses of (**1b**,**e**) were prepared essentially by a literature procedure,[17] while 2-(4-nitrophenylhydroxymethyl) cyclohexanone used in the preparation of (**1i**) was synthesized by minor modifications of a reported method.[18] The reaction products were recrystallized from isopropanol to give 2-(4-fluorophenylhydroxymethyl)cyclohexanone, m.p. 126–130°, 2-(4-bromophenylhydroxymethyl)cyclohexanone, m.p. 110–118° and 2-(4-nitrophenylhydroxymethyl)cyclohexanone, m.p. 165–170° in yields of 27, 46 and 70%, respectively. Dehydration of the aldols was accomplished by slight changes in a literature procedure[17] to give the following compounds (m.p., yield and recrystallization solvent in parentheses), namely **1b** (73–74, 66, n-hexane), **1e** (90, lit.[18] 77–80, 42, methanol) and **1i** (117–118, lit.[18] 118–120, 60, ethanol). The ^{1}H NMR spectrum (500 MHz) of a representative compound (**1i**) was as follows: δ ($CDCl_3$): 1.80–1.84 (m, 2H, 4-CH_2), 1.95–2.00 (m, 2H, 5-CH_2), 2.57–2.60 (t, 2H, 6-CH_2), 2.81–2.84 (m, 2H, 3-CH_2), 7.46 (s, 1H, =CH), 7.52–7.54 (d, 2H, aryl H), 8.24 (d, 2H, aryl H).

The preparation of the following compounds has been reported previously: namely, (**1h**),[19] (**2a**–**h**),[1] (**3a**,**f**)[1] and (**4**).[20]

Molecular Modeling

Models of (**1a**–**h**), (**2a**–**h**), (**3a**,**f**) and (**4**) were built using the MacroModel 8.0 programme[21] followed by a Monte Carlo search for the lowest energy conformations using an Amber force field of 1000 initial conformations. The compounds in series (2–4) were modeled as the free bases and the protonated species. Overlapping of different molecules was undertaken using the carbon atoms 3, 4 and 5 of the cyclohexane ring. Specific values of the RMS figures, torsion angles and lengths of the intramolecular hydrogen bonds in series (**2**–**4**) are available from the authors on request.

X-ray Crystallography

Suitable crystals of (**2b**) and (**2c**) for X-ray crystallography were obtained from diethyl ether-methanol

by the vapour diffusion method. Data were generated using an Enraf-Nonius CAD-4 diffractometer with an ω-2θ scan. The structures were solved using NRCVAX[22] and ORTEPII.[23] Atomic scattering factors and anomolous dispersion corrections were obtained from the literature.[24] Non-hydrogen atoms were found in E-maps and were refined anisotropically while hydrogen atoms were placed on atoms by geometry and assigned temperature factors from the attached atoms. Specific details of the X-ray crystallographic data of (**2b**) and (**2c**) may be obtained from the authors on request.

Statistical Analyses

A comparison of the potencies of the compounds in series (**1**), (**2**) and (**4**) used the Wilcoxon Signed Ranks Tests.[25] The following results were obtained (p values in the L1210, Molt 4/C8 and CEM tests, respectively, in parentheses), namely **2** > **1** (<0.01, <0.05, <0.01), **1** vs **4** (>0.05, >0.05, >0.05) and **2** > **4** (<0.05, <0.01, <0.01). The σ, π and MR values of the R^1 and R^2 groups in series (**2**) were taken from the literature[26] and combined. Linear and semilogarithmic plots were constructed using a commercial software package.[27] The following correlations using the data for (**2a**–**h**) in Table I were noted (assay, physicochemical constant, linear (l) or semilogarithmic (sl) plots, Pearson's correlation coefficient and p value in parentheses): namely, CEM, σ, l, −0.785, 0.021; CEM, σ, sl, −0.792, 0.019; CEM, MR, l, 0.697, 0.055 and CEM, MR, sl, 0.693, 0.057. The relationships established using the results presented in Table III were as follows: MG MID (mean graph midpoint vide infra), σ, l, −0.759, 0.080; MG MID, σ, sl, −0.743, 0.091; colon cancer cells, MR, l, 0.878, 0.022 and colon cancer cells, MR, sl, 0.870, 0.024.

TABLE I Cytotoxic activity of the compounds in series (**1**–**4**) and melphalan towards murine L1210 cells and human Molt 4/C8 and CEM T-lymphocytes

	IC_{50} (μM)		
Compound	L1210 cells	Molt 4/C8 cells	CEM cells
1b	69.0 ± 3.9	17.0 ± 0.3	16.6 ± 0.1
1e	45.6 ± 8.7	12.3 ± 0.2	14.3 ± 0.5
1h	91.6 ± 1.3	109.0 ± 3.5	81.1 ± 1.7
1i	54.5 ± 1.7	14.9 ± 0.5	14.5 ± 0.4
2a	2.29 ± 0.11	2.40 ± 0.18	2.18 ± 0.04
2b	2.02 ± 0.10	2.18 ± 0.13	2.18 ± 0.003
2c	1.37 ± 0.16	1.91 ± 0.13	2.16 ± 0.10
2d	2.01 ± 0.09	1.98 ± 0.12	2.32 ± 0.14
2e	1.03 ± 0.11	1.87 ± 0.03	1.87 ± 0.08
2f	2.08 ± 0.07	2.01 ± 0.17	2.18 ± 0.10
2g	1.71 ± 0.19	1.94 ± 0.03	2.52 ± 0.36
2h	2.26 ± 0.12	2.11 ± 0.16	9.11 ± 0.25
3a	1.94 ± 0.09	2.10 ± 0.06	2.19 ± 0.00
3f	1.95 ± 0.03	1.92 ± 0.06	2.01 ± 0.12
4	11.8 ± 3.92	34.0 ± 23.0	33.4 ± 22.0
Melphalan*	2.13 ± 0.03	3.24 ± 0.79	2.47 ± 0.30

*Data reproduced from reference 6.

Bioevaluations

Cytotoxicity Evaluations

The ketones described in this study as well as melphalan were evaluated against L1210, Molt 4/C8 and CEM cells using a literature methodology.[28] In brief, at least three different concentrations of compounds were incubated with the neoplastic cells at 37°C, After 48 h, the percentage inhibition of growth was recorded. The assays were conducted in triplicate at each concentration. The evaluation of selected compounds towards the panel of human tumours was undertaken by a previously reported methodology.[29] In brief, the compounds were evaluated with the cancer cell lines for 48 h using a minimum of five different concentrations at serial tenfold dilutions. The highest concentration of compounds used was 10^{-4} M, except for tamoxifen where $10^{-3.6}$ M was employed. A sulphorhodamine B protein assay was used to determine cell viability and growth. In the present investigation, 58 ± 4 cell lines were used. In the case of (**1i**) and (**2a**,**c**,**d**,**f**,**g**), melphalan, 5-fluorouracil and tamoxifen the MG MID figures are IC_{50} values, while the number of cell lines having IC_{50} figures of greater than 10^{-4} M were 1/54, 3/59, 1/56, and 1/59 for (**1e**), (**2h**) and (**3a**,**f**), respectively.

N-Myristoyltransferase and Tyrosine Kinase Assays

Evaluation of the effect of (**2a**) on NMT was undertaken by a literature methodology.[14] In brief, *Escherichia coli* DH5α with recombinant pT-7.hNMT was grown in LB medium to stationery phase at 37°C to yield NMT which was purified by a reported method.[30] The assay was carried out using cAMP-dependent protein kinase derived peptide[30] which was obtained from Research Genetics, Huntsville, AL, U.S.A. The IC_{50} figure for (**2a**) in this assay was 500 ± 46 μM.

The expression and purification of recombinant fyn kinase (pGEX-KG-fyn) was undertaken by a literature procedure.[31] In brief, *E. coli* BL21 with the fyn kinase was grown to stationery phase at 37°C in LB medium. After purification, the protein was mixed with 20% glycerol and stored at −80°C until used. The kinase assay followed a previously described methodology.[32] In brief, the reaction mixture, which included the fyn kinase and a synthetic peptide corresponding in sequence to residues 6–20 of cdc 2 (KVEKIGEGTYGVVKK), was incubated at 37°C for 0.5 h. The reaction was terminated by spotting on to Whatman P81 phosphocellulose filter paper and washed as described in the literature.[33] The radioactivity was quantified using a Beckman Ready Safe Liquid Scintillation mixture in a Beckman Liquid Scintillation Counter. The fyn kinase expressed plasmid used in these experiments was a gift from

Dr C.J. Pallen, University of British Columbia, Canada and the peptide was synthesized by the Alberta Peptide Institute. The concentrations of (**2a**) causing 50% stimulation of the activity of fyn kinase was 18.22 ± 14.85 μM. At concentrations of 100, 250 and 500 μM of (**2a**), the percentage increases in stimulation were 274, 273 and 275, respectively.

Toxicity and Neurotoxicity Evaluations

Various compounds described in this study were examined for overt toxicity using reported procedures.[34] In brief, doses of 30, 100 and 300 mg/kg of (**1b**,**e**,**i**), (**2a**–**h**), (**3a**,**f**) and (**4**) were injected intraperitoneally into mice and the animals were observed after 0.5 and 4 h. Neurotoxicity was determined by the rotorod method.[35] A dose of 50 mg/kg of (**2a**), (**3a**) and (**3f**) was administered orally to rats. No toxicity was detected at the end of 0.25, 0.5, 1, 2 and 4 h. All laboratory animals were housed, fed and handled in accord with the protocols in the National Research Council Publication "Guide for the Care and Use of Laboratory Animals". Euthanasia of the mice and rats was undertaken following the guidelines of the Institute of Laboratory Resources.

RESULTS

The compounds in series (**1**) were synthesized by condensing various aryl aldehydes with cyclohexanone. Reactions between the appropriate Mannich reagent[36] and (**1a**–**h**) or (**1a**,**f**) led to the formation of the compounds in series (**2**) and (**3**), respectively. Compound (**4**) was prepared from dimethylamine hydrochloride, paraformaldehyde and cyclohexanone. The shapes of (**2b**) and (**2c**) were determined by X-ray crystallography, while the comparative topography of the compounds in series (**1**–**4**) were examined by molecular modeling.

The compounds in series (**1**–**4**) were evaluated against murine L1210 cells as well as human Molt 4/C8 and CEM T-lymphocytes; these data are presented in Table I. Two-thirds of the compounds were examined against a wide range of human tumour cell lines and the results are summarized in Table III. A representative compound (**2a**) possessed an IC_{50} value of 500 μM towards human NMT. A concentration of 18.2 μM of this compound stimulated the activity of a tyrosine kinase by 50%. With the exception of (**3h**), different doses of all of the compounds were examined for lethal effects in mice.

DISCUSSION

The compounds in series (**1**–**4**) were evaluated against murine L1210 cells which have been used extensively in evaluating cytotoxic and anticancer agents. In order to ascertain whether the compounds prepared in this study would exert antineoplastic properties towards human cell lines as well, Molt 4/C8 and CEM T-lymphocytes were also employed. Analysis of the cytotoxicity data in Table I was made initially by comparing the IC_{50} values between different groups of compounds with a view to discerning those structural features which influence potencies. Thus, comparisons of the IC_{50} figures were made between (i) **1a**–**h** and **2a**–**h**, (ii) **2a**,**f** and **3a**,**f**, and (iii) **4** with both **1a**–**h** and **2a**–**h**.

A comparison between the potencies of (**1a**–**h**) in each screen and the analogues (**2a**–**h**) which bear the same aryl substituent was undertaken. In this procedure, the IC_{50} figure of (**1a**) was compared to (**2a**), (**1b**) with (**2b**) and so forth. The data are presented in Table II. The figures in Table II reveal that in 96% of the comparisons, greater potencies were found in the Mannich bases (**2**) then the precursor enones (**1**). The average increases in potencies in the L1210, Molt 4/C8 and CEM tests were 22.3, 12.4 and 7.13, respectively, reflecting a 13.9-fold overall increase in potency. Clearly the transformation of the 2-arylidenecyclohexanones (**1**) into the corresponding β-aminoketones (**2**) is an important molecular modification which led to potent cytotoxicities. While several compounds containing the 3-(4-nitrophenyl)-1-oxo-2-propenyl group possessed IC_{50} figures in the 0.05–2 μM range towards L1210, Molt 4/C8 and CEM cell lines,[7,8] (**1i**) showed only weak inhibiting properties towards these three tumours. Comparisons of the potencies of (**2a**) vs (**3a**) and (**2f**) vs (**3f**) revealed that in three cases, the compounds in series (**3**) possessed marginally greater potencies than the analogues in series (**2**), while in the remaining three comparisons, equal potencies were demonstrated. Thus the presence of a basic group beta to the carbonyl function appears to be more important than the specific nature of the base itself.

TABLE II Comparison of the cytotoxic potencies between (**1a**–**h**) and (**2a**–**h**)*

	Screen		
Comparison	L1210	Molt 4/C8	CEM
1a/2a	33.9	15.9	16.9
1b/2b	34.2	7.79	7.62
1c/2c	7.59	2.20	1.62
1d/2d	1.45	0.535	1.45
1e/2e	44.3	6.58	7.66
1f/2f	6.44	6.67	6.61
1g/2g	9.83	7.63	6.27
1h/2h	40.5	51.7	8.90

*The figures indicate the ratios between the IC_{50} values of each of the compounds in series (**1**) and the IC_{50} figures of the analog in series (**2**). The IC_{50} data for (**1a**,**c**,**d**,**f**,**g**) were taken from reference 6.

A further issue was whether the transformation of cyclohexanone into either the arylmethyleneketones (**1**) or the Mannich base (**4**) led to the greatest increase in potency. The IC_{50} values of (**1a–h**) were each compared with that of (**4**) in the L1210, Molt 4/C8 and CEM screens. Greater potencies were demonstrated by (**1**) and (**4**) in 21 and 29% of the comparisons, respectively, while in 50% of the cases, the IC_{50} figures were not statistically divergent. One may conclude that, in general, it is not possible to state which molecular modification led to superior potencies.

A statistical approach, while not indicating quantitative differences in potencies between the series, confirmed these general conclusions. Using the Wilcoxon Signed Ranks Test[25] applied to the biodata in all three assays revealed the greater potencies of the compounds in series (**2**) than both (**1**) and (**4**) ($p < 0.01$ or $p < 0.05$), while the IC_{50} values of (**1**) and (**4**) were not statistically different ($p > 0.05$).

Comparisons were made between the potencies of the compounds described in this study and the anticancer alkylating agent, melphalan. None of the compounds in series (**1**) and (**4**) were as potent as melphalan in the L1210 and CEM screens while in the Molt 4/C8 test, (**1c**) and (**1d**) were equipotent and 3.1 times more potent, respectively, than melphalan. On the other hand, the following compounds (screen in parentheses) were more potent than melphalan: **2c**,**e**,**g**,**3a**,**f** (L1210), **2b–h**,**3a**,**f** (Molt 4/C8) and **2e**,**3f** (CEM), while **2b**,**d**,**f**,**h** (L1210), **2a** (Molt 4/C8) and **2a–d**,**f**,**g**,**3a** (CEM) were equipotent with this established drug. Thus greater or equal potencies were displayed in series (**2**) and (**3**) in 53% and 40%, respectively, of the comparisons made with melphalan. The most potent compound was (**2e**), which possessed 2.1, 1.7 and 1.3 times the potency of melphalan in the L1210, Molt 4/C8 and CEM tests, respectively. The data in Table I clearly reveal that the Mannich bases (**2**) and (**3**) are novel prototypic antineoplastic agents. Further discussion therefore concentrates mainly on these two series of compounds and particularly the Mannich bases (**2a–h**).

The next phase of the investigation was to ascertain whether cytotoxicity was correlated with one or more physicochemical properties of the aryl substituents in series (**2**). The Hammett sigma (σ), Hansch pi (π) and molar refractivity (MR) constants reflect the electronic, hydrophobic and steric properties of aryl groups. Linear and semilogarithmic plots were constructed between the IC_{50} values in each of the L1210, Molt 4/C8 and CEM screens and the σ, π and MR figures. The following correlations were noted. The IC_{50} figures were negatively correlated with the σ constants ($p < 0.05$) and positively with the MR values ($p < 0.1$) in the CEM screen. This observation indicated that the potency increased (lower IC_{50} values) as the electron-attracting properties of the aryl substituents rose and the size of the groups diminished. No other correlations were noted ($p > 0.1$). Thus future molecular modifications should place small, strongly electron-attracting substituents in the arylidene aryl ring such as the trifluoromethyl ($\sigma_p = 0.54$, MR = 5.02)[37] and cyano ($\sigma_p = 0.66$, MR = 6.33)[37] groups.

Two additional investigations were considered regarding the choices of further aryl substituents. In theory at least, analysis of the biodata for (**2a**,**c**,**d**,**f**,**g**) should permit the utilization of a potency order table.[38] However, in practice, the very narrow range of IC_{50} values for these compounds in the L1210, Molt 4/C8 and CEM assays precluded any parameter dependencies being revealed. Nevertheless, in the L1210 screen, the potencies of (**2a**,**c**,**d**) were statistically significantly different. Hence the decision tree approach[39] was employed which indicated that future molecular modifications should include the preparation of the 4-trifluoromethyl analogue.

An investigation was initiated to determine whether the variation in cytotoxicity among the compounds in series (**1–4**) was related to the shapes of the molecules. The approach adopted was to initially determine the structures of one or more representative compounds by X-ray crystallography and subsequently utilize the observations of the stereochemistry of these ketones when building molecular models of the compounds prepared in this study.

Suitable crystals for X-ray crystallography were obtained in the case of (**2b**) and (**2c**). Both of these compounds were isolated as the *E*-isomers, which is in accord with previous studies involving 2-arylmethylenecyclohexanones and related compounds.[40–43] In addition, the dimethylaminomethyl group adopted the equatorial conformation. The alicyclic ring was found in the shape of a twisted chair while the torsion angles between the aryl ring and the adjacent olefinic group of (**2b**) and (**2c**) were $-27.0°$ and $-28.6°$, respectively.

Molecular modeling was undertaken on all of the compounds in series (**1–4**). In the majority of determinations, atoms common to the molecules whose shapes were being compared were overlapped and the root mean square (RMS) figures (range of results in parentheses) obtained. A RMS value of 1.0 Å was considered a significant difference in the shapes between two or more molecules. Under the conditions of the bioassays, the compounds in series (**2–4**) existed as a mixture of the free bases and protonated forms. The average RMS values for each of the compounds (**2a–h**), (**3a**,**f**) and (**4**) modeled as both species were 0.0689 (0.0628–0.0750), 0.0674 (0.0618–0.0711) and 0.0622 Å, respectively, and hence protonation of the bases did not change

the shapes of the molecules to any appreciable extent. Hence the remaining comparisons of the topographical features of different compounds in (**2**–**4**) refer to modeling accomplished with the free bases. It was, however, of interest to note in the case of the protonated forms, that the basic side chain assumed a conformation in which a hydrogen bond of 2.62 Å was detected between the proton on the quaternary nitrogen and the oxygen atoms.

The following comparisons of the shapes of different molecules were undertaken. First, all of the compounds (**2a**–**h**) were overlapped. The average RMS figures was 0.02598 Å (0.0000–0.0530 Å) and hence the placement of different substituents in the aryl ring exerted a minimal effect on the shapes of the molecules. A similar observation was made with the compounds (**1a**–**h**) in which case the average RMS figure was 0.0152 Å (0.0000–0.0522 Å). However, in both series of compounds, differences in the torsion angles θ between the aryl ring and the adjacent olefinic group were noted. The average θ value for (**2a**–**h**) was 71.9°, varying from 66.0 in (**2e**) to 76.5 in (**2d**) while the average θ value for (**1a**–**h**) was 70.8°, ranging from 65.5 in (**1d**) to 74.9 in (**1g**). On occasions, the presence of bioactivity in various series of compounds as well as potencies were dependent on the θ values between different groups attached to unsaturated linkages.[44] Hence linear and semilogarithmic plots were constructed between the torsion angles and the IC_{50} values of (**1a**–**h**) and (**2a**–**h**) in each of the L1210, Molt 4/C8 and CEM screens. No correlations were noted ($p > 0.05$). Hence variation in the torsion angles in series (**1**) and (**2**) is unlikely to be a dominant effect in controlling the potencies of the compounds in these assays.

Second, the overlap of the atoms common to both (**2a**) with (**3a**) and also (**2f**) with (**3f**) revealed that changes in the basic centre had little effect on the relative shapes of the molecules, since the RMS figures were 0.0139 Å and 0.0161 Å, respectively.

Third, the possibility existed that the arylidenecyclohexanone portions of (**1**) and (**2**) varied in shape. In other words, the introduction of a 2-dimethylaminomethyl group into (**1**) leading to series (**2**) may have altered the conformation of the arylidene and alicyclic moieties in (**1**). Thus each of the compounds in series (**1**) was compared with the analogue in series (**2**), i.e., the atoms common to (**1a**) and (**2a**) were overlapped, then (**1b**) and (**2b**) and so forth. The average RMS figure was 0.0462 Å (0.0307–0.1203 Å) revealing that the marked differences in cytotoxic protencies between series (**1**) and (**2**) was not due to changes in the shape of the portion of the molecules where thiolation was believed to occur. In a similar fashion, comparisons of the structures of each of the compounds in series (**2**) with 2-dimethylaminomethylcyclohexanone (**4**) revealed that the introduction of an arylidene group into (**4**) leading to series (**2**) had a minimal effect on the shape of compound (**4**) [the average RMS value was 0.0702 Å (0.0679–0.0707 Å)].

The conclusions to be drawn from the molecular modeling study were as follows. In the first place, variation in the θ values in both series (**1**) and (**2**) were noted. The θ figures in (**1**) and (**2**) were in the range of 66–77° and thus future modifications of the more potent series, namely (**2**), should include the preparation of analogues with θ values both outside and inside of this range of torsion angles. Cytotoxic evaluations may reveal the importance of this physicochemical parameter in conferring cytotoxicty. For example, modeling revealed that the θ figures of (**5a**–**d**) (the structures are indicated in Fig. 3) were 86.2, −86.0, 56.2 and −73.7, respectively, compared to a value of −71.1 for (**2a**). Secondly, the marked cytotoxic potencies of series (**2**) was due to the presence of both the 2-dimethylaminomethyl and arylidene groups at the 2- and 6-positions of cyclohexanone and not due to alterations in the shapes of either (**1**) or (**4**) by an additional substituent.

The data in Table I revealed the discovery of some novel cytotoxic molecules. In order to explore their potential further, representative compounds were examined against 58 (54–59) human tumour cell lines. The concentrations of compounds used in this assay generally ranged from 10^{-8} to 10^{-4} M. If a compound did not inhibit 50% of the growth of the cell line at the highest concentration utilized, i.e. 10^{-4} M, the figure of 10^{-4} is included in calculating the average cytotoxicity towards all cell lines. Hence the term mean graph midpoint (MG MID) rather than IC_{50} is employed. The evaluation of (**1e**,**i**), (**2a**,**c**,**d**,**f**–**h**) and **3a**,**f** towards a panel of human tumour cell lines is summarized in Table III.

The MD MID values listed in Table III will be considered first. All of the enones have lower MG MID values than the reference drug melphalan. Linear and semilogarithmic plots were made between the MG MID figures and the σ, π and MR figures of the aryl substituents. A negative trend towards significance was noted between the IC_{50} values and the σ constants ($p < 0.1$). No other correlations ($p > 0.1$) were noted. The data in

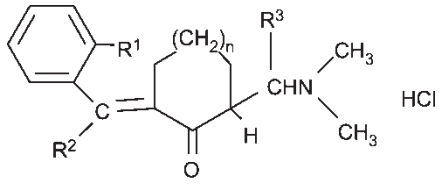

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FIGURE 3 The structures of compounds in series (**5**) namely **a** = R^1 = Cl, R^2 = R^3 = H, n = 1; **b**: R^1 = R^3 = H, R^2 = CH_3, n = 1; **c**: R^1 = R^2 = R^3 = H, n = 0; **d**: R^1 = R^2 = H, R^3 = CH_3, n = 1.

TABLE III Cytotoxic activity of (**1e**,**i**), (**2a**,**c**,**d**,**f**–**h**), (**3a**,**f**) and reference drugs towards panels of human tumours

	All cell lines		Colon cancer cells		Breast cancer cells	
Compound	MG MID (μM)*	SR†	IC_{50} (μM)	SI‡	IC_{50} (μM)	SI‡
1e	14.1	>6.61	17.7	0.80	11.8	1.20
1i	16.2	8.91	12.4	1.31	21.6	0.75
2a	3.98	38.9	2.33	1.71	2.72	1.46
2c	2.95	15.9	2.62	1.13	2.31	1.28
2d	3.09	25.1	2.88	1.07	2.21	1.40
2f	2.95	30.9	2.28	1.29	1.87	1.58
2g	3.02	27.6	2.36	1.28	1.78	1.70
2h	8.32	>8.13	3.19	2.61	5.31	1.57
3a	3.89	>16.2	2.36	1.65	1.91	2.04
3f	3.39	>6.17	2.36	1.44	2.06	1.65
Melphalan	19.1	50.1	42.9	0.45	35.5	0.54
5-Fluorouracil	24.6	26.9	7.27	3.38	–	–
Tamoxifen	5.01	5.01	–	–	4.15	1.21

* The letters MG MID refer to mean graph midpoint which is explained in the text. † The letters SR indicate the selectivity ratio and is the ratio of the IC_{50} values of the compound towards the most refractory and the most sensitive cell lines. ‡ The letters SI refer to the selectivity index figures. These values were generated by dividing the MG MID figure of the compound for all cell lines by the average IC_{50} value towards either colon cancer or breast cancer cell lines.

Table III also revealed that the potencies of the Mannich bases were superior to (**1e**) and (**1i**) and hence discussion will subsequently revolve around the compounds in series (**2**) and (**3**).

In tandem with the data obtained in the L1210, Molt 4/C8 and CEM screens, in general the compounds in series (**2**) and (**3**) had similar potencies towards the human tumour cell lines. The average MG MID figure for (**2a**,**c**,**d**,**f**–**h**), (**3a**,**b**) was 3.95 μM, which was 4.8 times higher than the value of melphalan. One of the goals of cancer chemotherapy is to obtain compounds with selective toxicity for neoplastic cells which necessitate that candidate drugs should display divergent toxicities towards different cells. A review of the mean graphs[45] was made and the following two observations were noted. First, while the MG MID figures of the Mannich bases in series (**2**) and (**3**) were similar, in general, there was considerable variation in the sensitivities of the cell lines to the compounds. This variation was revealed in the selectivity ratio (SR) figures which are the ratios of the IC_{50} values of the most resistant and most sensitive cell lines. Assuming that a tenfold disparity in potencies reflects useful lead molecules, then (**2a**,**c**,**d**,**f**,**g**) and (**3a**) met this criterion. Second, the mean graphs revealed that the colon cancer and breast cancer subpanels were the most sensitive to the compounds prepared in this study. The IC_{50} values for the cell lines in these two subpanels were computed and compared to the MG MID figures to give the selectivity index (SI) values. The SI data for 5-fluorouracil and tamoxifen are presented in Table III, since they are useful drugs in treating colon[46] and breast[47] cancers, respectively. A SI figure of 1.5 was arbitrarily chosen to reflect noteworthy subpanel specificity. This criterion was met by (**2a**,**h**) and (**3a**) in regard to colon tumours and by (**2f**,**g**,**h**) and (**3a**,**f**) towards breast cancer cell lines. The most promising compounds were (**2h**) and (**3a**), which possessed 0.8 and 0.5 times, respectively, the SI figures for 5-fluorouracil towards colon cancers and 1.3 and 1.7 times the SI values of tamoxifen when breast cancer cell lines were considered. Linear and semilogarithmic plots were made between the σ, π and MR values of the aryl substituents present in (**2a**,**c**,**d**,**f**,**h**) and the IC_{50} figures for the colon and breast cancer subpanels. A positive correlation ($p < 0.05$) between the IC_{50} values and MR constants was noted in the case of colon cancer cell lines. No other correlations were observed ($p > 0.1$). Thus subsequent development of this series of compounds should incorporate relatively small groups in the aryl ring in order to obtain specificity for colon cancer cells.

The conclusions to be drawn from the evaluation of various Mannich bases in series (**2**) and (**3**) towards a panel of human tumour cell lines are that the compounds are more potent than melphalan, display differential toxicities to cancer cell lines and, in particular, have some selectivity towards colon and breast tumours. The QSAR investigation indicated that potencies were influenced positively by small, electron-attracting aryl substituents.

A cysteine-169 mercapto group has been proposed at the binding site for substrates of NMT[48] and hence the thiol alkylators described in this study may exert their cytotoxicity by inhibition of this enzyme. The IC_{50} of one of the Mannich bases (**2a**) towards NMT was 500 μM. However, the data presented in Tables I and III indicate that inhibition of the growth of various neoplastic cells was achieved by substantially lower concentrations of this compound. Nevertheless this figure of 500 μM was significantly less than the IC_{50} values of 1.5–25 mM obtained with a series of bis-Mannich bases of conjugated enones towards NMT[14] and hence further molecular modifications of (**2a**) and analogues may lead to compounds possessing greater inhibitory properties towards NMT.

Molecules with divergent chemical structures inhibit protein tyrosine kinases including herbimycin A.[49] This latter compound contains a substituted quinone ring which may be regarded as a cyclic α,β-unsaturated ketone. In the present investigation, (**2a**) did not inhibit tyrosine kinase but stimulated the activity of this enzyme. This result could be due to (**2a**) changing the conformation of the enzyme thereby facilitating the transfer of the gamma phosphoryl group from adenosine triphosphate to the tyrosine residue of the substrate.

Finally, in considering the potential of these novel cytotoxic agents for further development, the in vivo toxicity was addressed. Doses of 30, 100 and 300 mg/kg of (**1b**,**e**,**i**), (**2a**–**h**), (**3a**,**f**) and (**4**) were administered to mice and the animals were observed after 0.5 and 4 hours. No mortalities were noted with (**1b**,**e**,**i**) and (**4**). All members of series (**2**) and (**3**) were tolerated at 30 and 100 mg/kg doses, except for mice receiving 100 mg/kg of (**2g**) and (**2h**) which died after 0.5 hour. A dose of 300 mg/kg of the remaining compounds in series (**2**) and (**3**) was lethal after 0.5 (**2a**,**f**,**3a**,**f**) or 4 (**2b**–**e**) hours. Three representative compounds, namely (**2a**), (**3a**) and (**3f**) were administered orally to rats using a dose of 50 mg/kg. During the time frame of 0.25–4 hours, no overt toxicity was detected. Two conclusions drawn from these observations are as follows. First, among the Mannich bases (**2**), the greatest murine toxicity was displayed by (**2g**) and (**2h**), which had the greatest electron-donating substituents in the aryl ring. This observation reinforces the QSAR study which revealed that future molecular modifications should concentrate on placing electron-attracting groups in the aryl ring. Second, in general, compounds prepared in this study were significantly less toxic than many current anticancer drugs such as melphalan, for which the LD_{50} in mice is 21.7 mg/kg.[50]

CONCLUSIONS

This study has demonstrated that the 6-arylidene-2-dimethylaminomethylcyclohexanone hydrochlorides (**2**) and related Mannich bases (**3**) display noteworthy cytotoxic properties towards a wide range of tumour cell lines. The compounds are structurally divergent from currently available anticancer drugs and hence are likely to be devoid of cross resistance to contemporary medications including the antineoplastic alkylating agents. The observation that certain drug-resistant tumour cell lines were not cross-resistant to a series of Mannich bases of conjugated arylideneketones[51] reinforces this possibility. Guidelines for the expansion of this series of compounds based on QSAR, molecular modeling and X-ray crystallography were obtained. In general, doses of 100 mg/kg of the compounds were tolerated in mice. A possible molecular target of a representative compound (**2a**) is NMT but other sites of action are clearly involved in the cytotoxic response, and these should be identified in the future.

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