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Cytotoxic 4′-aminochalcones and related compounds

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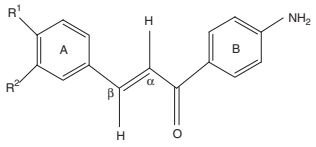
A series of 4′-aminochalcones **1** and related maleamic acids **2** and Schiff bases **3** were designed and synthesized as candidate cytotoxic agents. The atomic charges on different atoms of representative compounds were calculated. Evaluation of the enones **1**–**3** against human Molt 4/C8 and CEM T-lymphocytes as well as murine P388 and L1210 leukemic cells revealed that approximately 40% of the IC_{50} values generated were less than 10 μM. In some cases cytotoxicity was correlated with the Hammett σ values of the aryl substituents and less frequently with the aryl Hansch π values. Evidence was obtained that in general these compounds displayed selective toxicity for certain malignant cells and were well tolerated in mice. This study has revealed various directions whereby the project may be amplified in the future with a view to finding compounds with increased cytotoxicity to tumour cells.

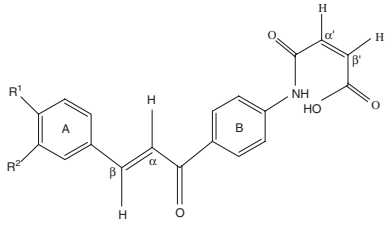
1. Introduction

The interests of this laboratory include the syntheses and bioevaluations of α,β-unsaturated ketones as candidate cytotoxic and anticancer agents. These compounds have a marked affinity for thiols in contrast to amines [1]. Since a number of clinically useful anticancer drugs have genotoxic effects [2] due to interactions with the amino groups of nucleic acids, α,β-unsaturated ketones may be devoid of this important side effect. Recently a study of the cytotoxicity of various chalcones was completed which revealed that the parent compound 1,3-diphenyl-2-propen-1-one or chalcone possessed approximately 20–25% of the potency of melphalan towards human Molt 4/C8 and CEM T-lymphocytes [3]. In the present investigation, the chalcone motif was retained while three different approaches were implemented with a view to designing prototypic molecules having the potential of preferential toxicity to malignant cells rather than the corresponding normal tissues. These studies were undertaken in the anticipation that the biodata generated for each of the compounds would provide information for subsequent amplifications of the project.

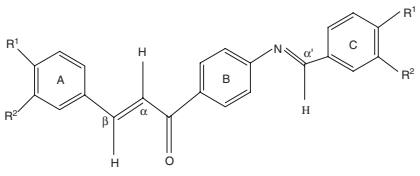
The first approach involved the placement of an amino group in ring B of chalcone for the following reason. A number of tumour tissues has a lower pH than the corresponding normal cells due to increased anaerobic glycolysis in malignant cells [4]. The percentage of the protonated ammonium species will increase as the pH falls [5]. Hence the strongly electron-attracting ammonium group having a σ_p value of 0.53 [6] will increase the fractional positive charge on the olefinic carbon atom adjacent to ring A. This, in turn, may lead to enhanced electrophilic



1 a-e



2 a-g



3 a-d,f

The aryl substituents in series **1**–**3** are as follows: **a**: $R^1 = R^2 = H$; **b**: $R^1 = Cl$; $R^2 = H$; **c**: $R^1 = R^2 = Cl$; **d**: $R^1 = CH_3$; $R^2 = H$; **e**: $R^1 = NO_2$; $R^2 = H$; **f**: $R^1 = OCH_3$; $R^2 = H$; **g**: $R^1 = R^2 = OCH_3$. **1a**–**d** were obtained as the hydrochloride salts

attack in cancerous cells. These considerations led to the decision to prepare compound **1a**. In addition, the proposal was made to synthesize **1b–e**. The substituents in ring A of these compounds had varying electronic, hydrophobic and steric properties. In particular, if cytotoxicity was influenced by the chemical reactivity of the enone group, a correlation between the IC_{50} values of **1a–e** and the Hammett σ values should emerge.

The second approach considered the synthesis and bioevaluation of series **2** based on the following hypothesis. The olefinic double bond in the N-acyl group in series **2** is attached to two electron-attracting substituents which should render the olefinic carbon atoms highly electrophilic. These molecules therefore have two locations which are capable of interacting with cellular thiols, namely at the β carbon atom and also at either the α′ or β′ carbons. Thus the reaction with thiols should proceed sequentially, namely at the most electron-deficient carbon atom followed by a second thiolation at the olefinic carbon atom bearing the next highest atomic charge. This point is of relevance insofar as sequential chemical insults have been shown to result in increased cytotoxicity against tumour cells than against the corresponding normal cells [7–9], i.e., chemosensitization of malignant cells occurred. Hence the compounds in series **2** have the potential to display preferential cytotoxicity for malignant cells.

A possible disadvantage of the compounds in series **1** and **2** is that ionization of some of the molecules may inhibit cellular penetration. Thus, the synthesis and bioevaluation of the azomethines **3** was envisaged which are potential prodrugs of aminochalcones. Azomethines have very low basicities, e.g., the pKa of 4-chlorophenylmethyleneaniline is 2.80 [10], which means that under the pH conditions of both malignant and normal cells, compounds **3** will be essentially unionized. Furthermore, azomethines are more susceptible to hydrolysis under acidic conditions than in neutral or alkaline media [11, 12]; hence preferential liberation of **1** in tumours may be achieved.

The evaluation of the murine toxicity of the compounds was also planned with a view to guiding the direction of future expansion of the project.

In summary, the objectives of the present investigation were to prepare the compounds in series **1–3**, evaluate their cytotoxicity towards several cell lines as well as their tolerability in mice and to identify those factors which would aid in developing the project.

2. Investigations and results

Condensation between 4′-aminochalcone and various aryl aldehydes under acidic conditions led to compounds **1a–d**, while a basic medium was necessary to prepare **1e**. ^{1}H NMR spectroscopy revealed that the double bond adopted the E configuration. The reaction between 4′-aminoacetophenone and maleic anhydride led to 4′-maleamoylaminoacetophenone which was condensed with different aryl aldehydes leading to the compounds in series **2**. The condensation between 4′-aminoacetophenone and different aldehydes in a 1 : 2 molar ratio gave rise to the azomethines **3**.

After minimization of the energy of **1a**, **2a** and **3a** and related compounds, the atomic charges at various unsaturated carbon atoms were calculated. These data are presented in Table 1.

All the compounds in series **1–3** were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine P388 and L1210 leukemic cells. These data are presented in Table 2. In addition, the potency of **2c** was

Table 1: Atomic charges of various compounds

Compd.	Atomic charges (esu)			
	α	β	α′	β′
Chalcone	−0.089	0.071	–	–
1a	−0.088	0.052	–	–
1a′ [a]	−0.093	0.081	–	–
2a	−0.089	0.063	−0.004	−0.061
2a′ [b]	−0.089	0.062	0.016	−0.053
3a	−0.100	0.072	0.170	–

[a] **1a′** is the protonated species of **1a**, i.e., a 4-$NH_3^{(+)}$ group is present in ring B
[b] **2a′** is a geometrical isomer of **2a** in which the olefinic group in the maleamoyl function of **2a** now has the *E* configuration

Table 2: Evaluation of 1a–e, 2a–g and 3a–d, f using human Molt 4/C8 and CEM T-lymphocytes and murine P388 and L1210 cells

Compd.	IC_{50} (μM)		SR[a]	IC_{50} (μM)		SR[a]
	Molt 4/C8	CEM		P388	L1210	
1a	8.57 ± 1.25	8.29 ± 0.45	1.03	5.25 ± 0.2	9.14 ± 0.74	1.74
1b	7.70 ± 0.32	7.44 ± 0.76	1.03	3.44 ± 0.2	8.62 ± 0.54	2.51
1c	8.96 ± 1.75	6.41 ± 0.12	1.40	3.94 ± 0.1	8.22 ± 0.20	2.09
1d	8.38 ± 1.26	8.48 ± 0.65	1.01	6.25 ± 0.9	10.4 ± 1.6	1.66
1e	7.28 ± 0.74	8.44 ± 0.59	1.16	3.70 ± 0.3	6.09 ± 3.02	1.65
2a	202 ± 8	186 ± 5	1.09	30.9 ± 1.2	160 ± 5	5.18
2b	92.2 ± 61.9	108 ± 93	1.17	16.4 ± 0.5	68.4 ± 32.9	4.17
2c	44.5 ± 1.8	43.5 ± 9.7	1.02	7.94 ± 1.6	25.0 ± 4.0	3.15
2d	89.1 ± 59.2	126 ± 109	1.41	18.1 ± 1.3	72.0 ± 36.8	3.98
2e	33.1 ± 5.8	34.2 ± 10.9	1.03	0.70 ± 0.03	26.6 ± 24.3	38.0
2f	205 ± 20	211 ± 11	1.03	57.5 ± 0.1	165 ± 20	2.87
2g	210 ± 3	239 ± 41	1.14	23.4 ± 2.7	205 ± 10	8.76
3a	8.84 ± 0.02	8.30 ± 0.61	1.07	3.40 ± 0.1	9.05 ± 0.14	2.66
3b	304 ± 105	223 ± 30	1.36	19.0 ± 0.6	175 ± 44	9.21
3c	>500	343 ± 31	>1.46	13.3 ± 0.2	320 ± 64	24.1
3d	41.4 ± 1.1	39.4 ± 6.4	1.05	15.6 ± 1.2	35.9 ± 2.7	2.30
3f	21.4 ± 1.1	21.1 ± 12.5	1.01	8.04 ± 1.1	19.6 ± 3.1	2.44
Melphalan[b]	3.24 ± 0.79	2.47 ± 0.30	1.31	0.22 ± 0.01	2.13 ± 0.03	9.68

[a] SR indicates the selectivity ratio, i.e., the ratio between the highest and lowest IC_{50} values of either the T-lymphocytes or the murine leukemic cells
[b] The data for melphalan are reproduced from the Eur. J. Med. Chem. **35**, 970 (2000)

assessed against a panel of 57 human tumour cell lines while **2a** and **2d** were evaluated for inhibitory activity towards three strains of both *Aspergillus fumigatus* and *Candida albicans*. The murine toxicity of each compound was assessed as well as possible protective effects in the maximal electroshock (MES) and subcutaneous pentylenetetrazole (scPTZ) screens.

3. Discussion

The data in Table 1 reveal that in the case of chalcone, **1a** and **1a′** (the protonated form of **1a**), the relative charges on the β carbon atoms were as predicted. In other words, the electron-releasing amino group of **1a** lowered the fractional positive charge on the β carbon atom relative to chalcone, while protonation of **1a** yielding **1a′** led to a compound with increased electrophilicity. In the case of **2a**, attack by cellular nucleophiles would be predicted to occur at the β carbon atom initially and then at the α′ position. Replacement of the two protons of **1a** by a phenylmethylene group led to **3a** which possessed a greater fractional positive charge on the β carbon atom than **1a** and also a highly electron deficient α′ atom. Thus, the compounds in both series **2** and **3** likely undergo a cascade of alkylations with cellular nucleophiles due to differences in the atomic charges at the β and α′ carbon atoms. As mentioned earlier, such a process could be more detrimental to malignant cells than to normal ones.

The bioevaluations of **1a–e**, **2a–g** and **3a–d, f** using two human T-lymphocytes and two murine leukemic cells lines is summarized in Table 2. The Molt 4/C8 and CEM screens were utilized in order to gain some idea of the potencies of the compounds prepared in this study towards human cells. The murine neoplasms were chosen on the basis of the claim of their being predictors of compounds with clinical utility as anticancer agents [13]. A review will be made first of the relative potencies of the compounds prepared in this study, second whether some selective toxicity was displayed by the enones and third, the possibility of correlations between certain physicochemical constants of the aryl substituents and cytotoxicity.

The IC_{50} values of 1,3-diphenyl-2-propen-1-one or chalcone in the Molt 4/C8, CEM, P388 and L1210 screens were 12.7, 12.5, 9.63 and 41.4 μM, respectively [3]. Hence the insertion of a 4′-amino group into chalcone leading to **1a** produced a compound with lower IC_{50} values as suggested by the hypothesis. In fact in the Molt 4/C8 and CEM tests, **1a** has 38% and 30%, respectively, of the potency of melphalan. Cytotoxicity was retained in **1b–e** when different substituents were placed in aryl ring A.

A review of the cytotoxicity of the compounds in series **2** revealed the significant potency of **2e** towards P388 cells. This compound possesses approximately one-third of the potency of the anticancer alkylating agent melphalan. In general **2e** had lower IC_{50} values than **2a–d**, **f**, **g** and is clearly a useful lead molecule. A comparison of the cytotoxicity of **1a–e** with **2a–e** in all four screens was made in the following manner. The IC_{50} values of **1a** and **2a** in each of the four screens were compared which was followed by comparisons between **1b** vs. **2b**, **1c** vs. **2c**, **1d** vs. **2d** and **1e** vs. **2e**. The standard deviations were taken into consideration. Of the 20 comparisons made, greater potency was found in the compounds in series **1** in 18 cases; the exceptions being the greater cytotoxicity of **2e** than **1e** in the P388 screen and the equiactivity of **1e** and **2e** towards L1210 cells. Thus, in general, N-acylation of the primary amino group of **1a–e** leading to **2a–e** was detrimental, at least in terms of potencies, which may possibly have been due to the presence of the polar carboxy group. Hence, esterification of the compounds in series **2** should be undertaken in the future. Furthermore, as the data in Table 1 reveal, the atomic charge at the α′ carbon atom of the E,E isomer **2a′** is substantially higher than is found in **2a** suggesting that a change in the stereochemistry of the olefinic portion of the N-acyl group could lead to increased potencies.

Replacement of the two protons of the primary amino group of **1a–d** by an arylmethylene function led to **3a–d**. In order to determine the effect on potencies of the attachment of carrier groups to **1a–d**, comparisons were made in all four screens between the IC_{50} values of each of the compounds **1a–d** with the analogues having the same substituents in ring A, i.e., **1a** was compared with **3a**, and so forth. The comparisons revealed very clear structure-activity relationships. The unsubstituted compounds **1a** and **3a** had the same potencies in the Molt 4/C8, CEM and L1210 screens, while **3a** had 1.5 times the potency of **1a** against P388 cells. However **1b–d** had lower IC_{50} values than **3b–d** in all four tests. Thus, while the phenylmethyleneimino function as a carrier group for 4′-aminochalcone did not reduce the potency of **1a**, the insertion of substituents into ring C in series **3** invariably led to a significant reduction in potency.

One may summarize the evaluation of the biodata in terms of potencies as follows. The insertion of a primary amino group into ring B of chalcone increased potency in general and the inhibitory activity of **2e** towards P388 cells is noteworthy. The phenylmethyleneimino group appears to be a good carrier function for 4′-aminochalcone. On the other hand, in general, the N-maleamoyl acids **2** and azomethines **3** were less potent than the analogues in series **1**.

However potency is not the only consideration in the quest for novel cytotoxics. An important characteristic of a prototypic cytotoxic agent is a demonstration of selectivity, i.e., there is a variation in the potencies towards different cell lines, with such molecules exerting a preferential toxicity for neoplastic cells relative to normal tissue [14]. Hence, the ratios between the IC_{50} values of the compounds towards the two human T-lymphocytes and also the two murine leukemic cells were determined. These figures, referred to as selectivity ratio (SR) values, are presented in Table 2.

The following observations were made. First, the average SR values of **1a–e**, **2a–g**, **3a, b, d, f** using the T-lymphocytes and the murine leukemic cells were 1.12 and 5.77, respectively, revealing an approximately fivefold greater differential sensitivity to these compounds when the P388 and L1210 and not the Molt 4/8 and CEM IC_{50} values were compared. A similar observation was noted with melphalan whereby the SR values of the murine leukemic cells and human T-lymphocytes 9.68 and 1.31, respectively. Second, in considering the T-lymphocytes, **1c**, **2d**, **3b** and **3c** possessed higher SR values than melphalan, while in the case of the murine cell lines, both **2e** and **3c** had substantially larger SR figures than the reference drug. Third, a comparison was made of the SR values of the compounds in series **1**, **2** and **3** when the aryl substituents were the same. Thus the SR figures of **1a**, **2a** and **3a** for the T-lymphocytes were compared and a value of 3 was ascribed to the compound with the highest SR number, 2 to the enone with the next highest SR figure and the compound with the lowest SR value was given a rat-

ing of 1. The process was repeated using **1b**, **2b**, **3b**, then **1c**, **2c** and **3c** and finally **1d**, **2d** and **3d**. The combined scores for **1a–d**, **2a–d** and **3a–d** were 5, 10 and 9, respectively. In the case of the murine leukemic cells, the combined scores of **1a–d**, **2a–d** and **3a–d** were 4, 10 and 10, respectively. These data reveal clearly that the compounds in series **2** and **3** exert a greater disparity in potency to the cell lines than the analogues in series **1** and suggest that expansion of all three series of compounds **1–3** is warranted.

A further approach aimed at discerning physicochemical factors which contributed to cytotoxicity was undertaken. In this process, linear and semilogarithmic plots were made between the Hammett sigma, Hansch pi and molar refractivity (MR) constants of aryl substituents and the IC_{50} values in each series of compounds and in each screen. The following relationships were observed. In series **1**, negative correlations were noted between the IC_{50} values and both the σ constants in the L1210 screen and the π values when CEM cells were used. For series **2**, negative correlations between the σ figures and the IC_{50} values were noted in the Molt 4/C8, CEM, P388 and L1210 tests. In series **3**, positive correlations were observed between the IC_{50} values and the σ and π constants in both the CEM and L1210 screens.

The negative correlations established between the IC_{50} values and the σ constants in series **1** and **2** are consistent with the hypothesis that potency increases as the electrophilicity of the olefinic carbon atoms rises. In other words, the electron densities on various unsaturated carbon atoms is inversely proportional to their capacity for interaction with cellular thiols, leading to increases in the cytotoxic properties of the molecules. The observation that positive, not negative correlations between potency and σ values where observed in series **3** suggests that these compounds may exert their cytotoxic effect principally by means other than interactions with cellular thiols. It is of interest to note that while a negative correlation between the π values and IC_{50} figures in the CEM screen for series **1** was observed, in the case of the compounds in series **3**, positive relationships between cytotoxicity in the CEM and L1210 screens with the π constants were noted. These observations further support the possibility that the mode of action of the Schiff bases **3** is different from that of the other two series of compounds. Since no correlations were noted between the IC_{50} values and the MR figures, the steric properties of aryl substituents apparently do not govern cytotoxicity. Thus in considering the amplification of series **1** and **2**, strongly electron-attracting and markedly hydrophobic substituents should be placed in the aryl ring A while for series **3**, electron-releasing and hydrophilic groups inserted into rings A and C may lead to more potent cytotoxic compounds.

The issue of whether the SR figures were influenced by the electronic, hydrophobic and steric properties of the aryl substituents was also addressed. A positive correlation between the MR figures of the aryl groups in series **1** and the SR values using human T-lymphocytes was noted. In the case of series **3**, positive correlations were observed between the σ values and the selectivity ratios of both human and murine cell lines and also the π values in the case of the SR data for the murine leukemic cells. No correlations were noted in the case of series **2**. Once again the statistical data suggest that the Schiff bases **3** act in a different manner to the compounds in series **1** and **2**.

A representative compound **2c** was evaluated against a number of human tumour cell lines from the following groups of tumours: leukemia, melanoma, non-small cell lung, colon, central nervous system, ovarian, renal, prostate and breast cancers. The IC_{50} figure of **2c** towards the cell lines was 19.5 μM. Observation of the mean graph [15] revealed that little differential cytotoxicity towards the neoplastic cells was noted except for the RPMI-8226 leukemic cell line. In this case the IC_{50} figure for **2c** was 0.112 μM, i.e., these cells were 174 times more sensitive to **2c** than the average cell line cytotoxicity. Melphalan possessed IC_{50} figures of 7.99 and 5.89 μM towards all cell lines and the RPMI-8226 neoplasm, respectively. Thus in comparison to melphalan, the maleamic acid **2c** had 41% of its overall potency while having 53 times greater cytotoxicity to the RPMI-8226 cell line.

In order to determine whether the compounds prepared in this study were general biocidal agents or had a preferential or possibly exclusive toxicity towards neoplastic cells, two approaches were utilized. First, two representative compounds were evaluated against pathogenic fungi. At the maximum concentrations utilized, **2a** and **2d** displayed no antifungal effect. The second method of a more substantial nature involved the intraperitoneal injection of 30, 100 and 300 mg/kg doses of **1a–d**, **2a–g** and **3a–d, f** into mice with a view to detecting toxicity in general and neurotoxicity in particular. The animals were observed 0.5 and 4 h after administration of the compounds. Under the experimental conditions employed, no mortalities were noted, i.e., the animals tolerated a dose of 300 mg/kg of all of the compounds. As well, no neurotoxicity was observed at the lowest dose, i.e., 30 mg/kg. Neurotoxicity was absent in **1e, 2b, e, f** and **3a, b, d** suggesting that in general the 4-nitro (**1e**, **2e**) and 4-chloro (**2b**, **3b**) analogues were well tolerated while the presence of substituents in both the 3 and 4 positions of aryl ring A appears to be detrimental. Using the same doses and times of observations as the neurotoxicity evaluation, the compounds were examined in the MES and scPTZ screens. Protection was noted in the MES test by **1a–e** and also by **1b, d** and **2a** in the scPTZ screen. Oral administration of **1a**, **d** and **1e** to rats did not reveal any pathological symptoms while, in general, marginal protection in the scPTZ screen was noted. The conclusion to be drawn from the antifungal and in vivo experimentation is that the compounds are not general biocidal agents and are well tolerated in mice.

In conclusion, this study has revealed that the placement of the 4′-amino, 4′-maleamoylamino and 4′-arylmethyleneamino groups in various chalcones leading to series **1–3**, respectively, gave rise to clusters of novel cytotoxic agents. Selective toxicity for different neoplastic cells was demonstrated. Various ways in which the project may be expanded with a view to increasing cytotoxicity have been indicated.

4. Experimental

4.1. Chemistry

4.1.1. General procedures for synthesis and spectroscopy

Melting points are in degrees Celsius and are uncorrected. Elemental analyses (C, H, N) were undertaken on **1a–e**, **2a–g** and **3a–d, f** by Mr. K. Thoms, Department of Chemistry, University of Saskatchewan and were within 0.4% of the calculated values. The enones **1d**, **1e**, **2a**, **3a** and **3c** were obtained with 0.25 mol of water of crystallization and **3b** with 0.5 mole of water crystallization. ^{1}H NMR spectra (500 MHz) were determined on all compounds using a Bruker AM 500 FT-NMR instrument.

4.1.2. Synthesis of series **1**

Compounds **1a–e** were prepared by condensation between 4′-aminoacetophenone and the appropriate aryl aldehyde using literature procedures for **1a–d** [16] and **1e** [3]. Compounds **1a–d** were isolated as the hydrochloride salts and **1e** as the free base. Purification of **1a–e** was accomplished by recrystallization from chloroform. The melting points and percentage yields of the compounds in series **1** were as follows: **1a**: 180–182, 67; **1b**: 200 (dec), 37; **1c**: 200 (dec), 66; **1d**: 180 (dec), 62; **1e**: 206–207 (lit. [16] m.p. 221–223 °C), 57. The ^{1}H NMR spectrum of a representative compound **1c** was as follows: δ (dmso-d_6): 6.28 (3 H, bs, NH_3^+), 6.73 (2 H, d, J = 8.5 Hz, C3′H, C5′H), 7.57 (1 H, d, J = 15.6 Hz, CH=CHCO), 7.68 (1 H, d, J = 8.3 Hz, C5H), 7.82 (1 H, dd, J = 8.4, 1.8 Hz, C6H), 7.95–8.00 (3 H, m, C2′H, C6′H, CH=CHCO), 8.23 (1 H, d, J = 1.8 Hz, C2 H).

4.1.3. Synthesis of series **2**

The required intermediate Z-4-(acetylphenylamino)-4-oxo-but-2-enoic acid **4** was prepared by a literature method [17]. It was purified by digestion in chloroform to give the desired compound in 92% yield, m.p. 196–202 (lit. [18] m.p. 198–200).

A solution of the appropriate aryl aldehyde (0.004 mol) in ethanol (3 mL) was cooled in an ice bath for 20 min. after which a solution of sodium hydroxide (0.006 mol) in water (20 mL), which had previously been cooled to ice bath temperatures, was added dropwise. A mixture of **4** (0.004 mol), sodium hydroxide (0.006 mol) and water (20 mL) was added dropwise to the solution of the aryl aldehyde over 0.25 h. The reaction mixture was stirred at room temperature for 13 h, acidified with hydrochloric acid (20% w/v) and stirred for another 2 h. The resultant precipitate was collected, washed with cold water and dried. In the case of **2e** and **2f**, gelatinous masses were obtained which were repeatedly extracted with ethyl acetate, the combined extractions dried and evaporation of the solvent led to the crude products. Purification of the compounds in series **2** was accomplished by recrystallization from methanol (**2a**, **2f** and **2g**) and in the remaining cases by digestion with methanol. The melting points and percentage yields of the compounds in series **2** were as follows: **2a**: 173–174, 63; **2b**: 208–209, 66; **2c**: 223–224, 65; **2d**: 205–206, 63; **2e**: 184–186, 56; **2f**: 182–183, 63; **2g**: 173–174, 67. The ^{1}H NMR spectrum of a representative compound **2d** was as follows: δ (dmso-d_6): 2.35 (3 H, s, CH_3), 6.34, (1 H, d, J = 12.0 Hz, Z-vinylic), 6.50 (1 H, d, J = 12.0 Hz, Z-vinylic), 7.27 (2 H, d, J = 7.9 Hz, C3H, C5H), 7.70 (1 H, d, J = 15.6 Hz, CH=CHCO), 7.76 (2 H, d, J = 7.9 Hz, C2H, C6H), 7.80 (2 H, d, J = 8.5 Hz, C2′H, C6′H), 7.88 (1 H, d, J = 15.6 Hz, CH=CHCO), 8.15 (2 H, d, J = 8.6 Hz, C3′H, C5′H), 10.65 (1 H, s, NHCO), 12.92 (1 H, bs, COOH).

4.1.4. Synthesis of series **3**

Utilization of a literature procedure [19] gave the compounds in series **3** which were purified by recrystallization from chloroform (**3a, d, f**) or digestion in either chloroform (**3b**) or methanol (**3c**). The melting points and percentage yields were as follows: **3a**: 141 (lit. [20] m.p. 143–144), 56: **3b**: 197–198, 67; **3c**: 203–204, 43; **3d**: 185–188 (lit. [20] m.p. 188), 55; **3f**: 148–149 (lit. [20] m.p. 149–150), 57. The ^{1}H NMR spectrum of a representative compound **3c** was as follows: δ (dmso-d_6): 7.40 (2 H, d, J = 7.5 Hz, C3′H, C5′H), 7.66–7.72 (2 H, m, C5H, CH=CHCO), 7.80 (1 H d, J = 8.0 Hz, C6H), 7.85 (1 H, d, J = 7.7 Hz, C5″H), 7.95 (1 H, d, J = 7.6 Hz, C6″H), 8.03 (1 H, d, J = 15.6 Hz, CH=CHCO), 8.17–8.28 (4 H, m, C2H, C2′H, C6′H, C2″H), 8.69 (1 H, s, N=CH).

4.1.5. Molecular modeling

Energy minimization of the molecules was accomplished using MOPAC while the charge density calculations were obtained by the extended Huckel method in the Chem 3D Pro® programme [21].

4.1.6. Statistical analysis

The σ, π and MR aryl constants employed in the statistical analyses were taken from the literature [22]. Linear and semilogarithmic plots were generated using a commercial software package [23]. The following correlations in series **1–3** were noted [physicochemical constant, cytotoxicity assay, linear (l) or semilogarithmic (sl) plots, "r" value, p value] namely **1**: σ, L1210, l, −0.93, <0.05; σ, L1210, sl, −0.91, <0.05; π, CEM, l, −0.87, <0.1; π, CEM, sl, −0.88, <0.1; MR, SR (Molt 4/C8-CEM), l, 0.82, <0.1; MR, SR (Molt 4/C8-CEM), sl, 0.82, <0.1; **2**: σ, Molt 4/C8, l, −0.81, <0.05; σ, Molt 4/C8, sl, −0.89, <0.01; σ, CEM, l, −0.89, <0.01; σ, CEM, sl,−0.95, <0.01; σ, P388, l, −0.79 <0.05; σ, P388, sl, −0.88 <0.01; σ, L1210, l, −0.80 <0.05; σ, L1210, sl,−0.89 <0.01; **3**: σ, CEM, l, 0.94 <0.05; σ, L1210, l, 0.95, <0.05; σ, L1210, sl, 0.82, <0.1; π, CEM, l, 0.93 <0.05; π, CEM, sl, 0.91, <0.05; π, L1210, l, 0.94, <0.05; π, L1210, sl, 0.93, <0.05; σ, SR (Molt 4/C8-CEM), l,0.93, <0.1; σ, SR (Molt 4/C8-CEM), sl, 0.93, <0.1; σ, SR (P388-L1210), l, 0.95, <0.05; σ, SR (P388-L1210), sl, 0.97, <0.01; π, SR (P388-L1210), l, 0.91, <0.05; π, SR (P388-L1210), sl, 0.89, <0.05.

4.2. Biological evaluations

4.2.1. Cytotoxicity screening

The evaluation of **1a–e**, **2a–g**, **3a–d, f** and melphalan against Molt 4/C8 and CEM T-lymphocytes as well as L1210 cells was undertaken by a literature procedure [24]. These compounds were also screened against P388 cells using a reported method [25]. The maleamic acid **2c** and melphalan were assessed against a number of human tumour cell lines using a method that has been described previously [15].

4.2.2. Antifungal evaluation of **2a**, **2d** *and reference drugs*

The maleamic acids **2a**, **2d,** amphotericin B and itraconazole were evaluated against three strains of *Aspergillus fumigatus* (ATCC 208995–208997) and three strains of *Candida albicans* (ATCC 28367, 32354, 90028) by the broth dilution method [26]. The minimum inhibitory concentrations (MIC) figures for **2a** and **2d** were in excess of 25 μg/mL. The MIC values, in μg/mL, of amphotericin B and itraconazole, respectively, towards the microorganisms were as follows: *A. fumigatus* ATCC 208995: 0.39, 0.19; ATCC 208996: 0.39, 0.19; ATCC 208997: 0.78, 0.39; *C. albicans* 28367: 0.19, 0.19; ATCC 32354: 0.097, 0.19; ATCC 90028: 0.39, 0.19.

4.2.3. Toxicity and anticonvulsant evaluation

The toxicity and anticonvulsant activity in mice and rats was undertaken by a literature procedure [27].

Compounds **1a–e**, **2a–g** and **3a–d, f** were evaluated for murine toxicity and anticonvulsant activity in the MES and scPTZ screens after intraperitoneal injections of 30, 100 and 300 mg/kg doses of the compounds. The animals were observed after 0.5 and 4 h. Neurotoxicity was measured using the rotating rod procedure [28]; in addition, mice were observed for other pathological symptoms. Neurological deficit was displayed after 0.5h by the following compounds (in parentheses are the number of animals displaying neurotoxicity/total number of animals; dose of compound) namely **1a** (3/8, 100; 1/4, 300), **1b** (4/8, 100; 3/4, 300), **1c** (2/8, 100; 1/4, 300); **1d** (3/8, 100; 3/4, 300); **2a** (1/4, 300), **2c** (2/4, 300), **2d** (2/8, 100), **2g** (1/8, 100; 2/4, 300), **3c** (2/8, 100; 3/4, 300) and **3f** (1/4, 300). Mice receiving doses of 100 and 300 mg/kg of **1b** were cyanotic. After 4h, the following compounds displayed neurotoxicity, namely **1a** (1/2, 300), **1b** (1/4, 100; 2/2, 300), **1d** (2/2, 300), **2a** (1/4, 100; 1/2, 300), **2d** (1/4, 100; 1/2, 300), **2g** (1/2, 300), **3c** (1/4, 100; 1/2, 300). Mice which received doses of 300 mg/kg of **1a** and **1d** were unable to stay on the rotorod and the 300 mg/kg dose of **1b** caused cyanosis.

Protection in the MES screen was displayed after 0.5 h in mice receiving 300 mg/kg of **1a** and **1d**. The following compounds displayed activity in the scPTZ test after 0.5 h, namely **1a** (5/5, 100; 5/5, 300), **1b** (1/1, 300), **1c** (1/5, 300), **1d** (4/5, 100; 4/5, 300) and **1e** (2/5, 300). Myoclonic jerks were noted in mice receiving 300 mg/kg of **1b** and 100 mg/kg of **1d** in this test. After 4 h, 1/1 animals were protected in the scPTZ screen which had received 300 mg/kg of **1b**, **1d** and **2a**. Myoclonic jerks were noted in mice receiving 300 mg/kg of **2a** after 4 h in the scPTZ test.

Compounds **1a, d, e** were administered orally to rats using a dose of 50 mg/kg. The animals were observed for various pathological symptoms after 0.25, 0.5, 1, 2 and 4 h and none were detected. Using the same dose and time intervals, the following enones afforded some protection in the oral scPTZ screen in rats, namely **1a** (1/4, 1 h), **1d** (1/4, 0.25 and 4 h) and **1e** (3/4, 0.25 h and 1/4, 0.5, 1 and 2 h).

The recommendations of the National Research Council Publication 'Guide for the Care and Use of Laboratory Animals' were followed in regard to the housing, feeding and handling of the mice and rats used in these experiments.

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