DOI: 10.1002/cmdc.200900288

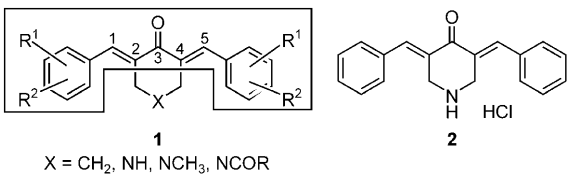
3,5-Bis(benzylidene)-4-oxo-1-phosphonopiperidines and Related Diethyl Esters: Potent Cytotoxins with Multi-Drug-Resistance Reverting Properties

Swagatika Das,[a] Umashankar Das,[a] Ponniah Selvakumar,[b] Rajendra K. Sharma,[b] Jan Balzarini,[c] Erik De Clercq,[c] Joseph Molnár,[d] Julianna Serly,[d] Zoltán Baráth,[d] Gabriele Schatte,[e] Brian Bandy,[a] Dennis K. J. Gorecki,[a] and Jonathan R. Dimmock*[a]

A series of 3,5-bis(benzylidene)-4-piperidones **3** were converted into the corresponding 3,5-bis(benzylidene)-1-phosphono-4-piperidones **5** via diethyl esters **4**. The analogues in series **4** and **5** displayed marked growth inhibitory properties toward human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemia L1210 cells. In general, the *N*-phosphono compounds **5**, which are more hydrophilic than the analogues in series **3** and **4**, were the most potent cluster of cytotoxins, and, in particular, 3,5-bis-(2-nitrobenzylidene)-1-phosphono-4-piperidone **5g** had an average IC_{50} value of 34 nM toward the two T-lymphocyte cell lines. Four of the compounds displayed potent cytotoxicity toward a panel of nearly 60 human tumor cell lines, and nanomolar IC_{50} values were observed in a number of cases. The mode of action of **5g** includes the induction of apoptosis and inhibition of cellular respiration. Most of the members of series **4** as well as several analogues in series **5** are potent multi-drug resistance (MDR) reverting compounds. Various correlations were noted between certain molecular features of series **4** and **5** and cytotoxic properties, affording some guidelines in expanding this study.

Introduction

A major interest in our research groups is the design of antineoplastic agents that contain the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. A number of reasons for the inclusion of this group into the structures of candidate cytotoxins have been collated recently.[1] Two important considerations are as follows: First, conjugated unsaturated ketones are thiol alkylators with little or no capacity to interact with amino or hydroxy groups, which are found in nucleic acids.[2] Thus these molecules should be free of the mutagenic effects elicited by certain alkylating agents used in cancer chemotherapy.[3] Second, the concept of sequential cytotoxicity states that successive alkylations of cellular constituents may be more detrimental to malignant cells than to the corresponding normal tissues.[4] This theory is based on the observation that an initial chemical insult caused by a bifunctional alkylator, for example, may be greater in neoplasms than in the corresponding normal cells.[5,6] Thus selective toxicity to tumors may result when the 1,5-diaryl-3-oxo-1,4-pentadienyl group is present in candidate cytotoxins, as illustrated in Figure 1.



1 X = CH_2, NH, NCH_3, NCOR **2**

Figure 1. Structures of series **1** (the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore is boxed) and compound **2**.

The excellent cytotoxic properties of various groups of compounds possessing the general structure **1** have been reported.[1] In particular, when X is a secondary amino group in series **1**, in a number of cases the IC_{50} values toward various transformed and malignant cells are in the low micromolar and sub-micromolar range.[7,8] For example, the free base of **2** has an IC_{50} value of 7.96 μM toward murine L1210 leukemic cells.[7] However, when assessment of **2** was made using this cell line passaged in mice, there was no increase in the life span of the animals.[8] A possible reason for this observation is the lipophilicity of 3,5-bis(benzylidene)-4-piperidones; for example, the

[a] *S. Das, Dr. U. Das,[+] Dr. B. Bandy, Prof. Dr. D. K. J. Gorecki, Prof. Dr. J. R. Dimmock*
Drug Design and Discovery Research Group
College of Pharmacy and Nutrition
University of Saskatchewan, Saskatoon, SK S7N 5C9 (Canada)
Fax: (+1) 306-966-6377
E-mail: jr.dimmock@usask.ca

[b] *Dr. P. Selvakumar,[+] Dr. R. K. Sharma*
Department of Pathology and Laboratory Medicine
University of Saskatchewan, Saskatoon, SK S7N 4H4 (Canada)

[c] *Prof. Dr. J. Balzarini, Prof. Dr. E. De Clercq*
Rega Institute for Medical Research
Katholieke Universiteit Leuven, 3000 Leuven (Belgium)

[d] *Prof. Dr. J. Molnár, J. Serly, Dr. Z. Baráth*
Institute of Medical Microbiology and Immunology
University of Szeged, Szeged 6720 (Hungary)

[e] *Dr. G. Schatte*
Saskatchewan Structural Sciences Centre
University of Saskatchewan, Saskatoon, SK S7N 5C9 (Canada)

[+] These authors contributed equally to this work.

log*P* value for the free base of **2** is 3.38.[9] Hence, the conversion of these amines into the corresponding 1-phosphono derivatives was considered, as the π value of the phosphono groups is −1.59.[10] We therefore decided to embark on a synthetic strategy leading to the compounds of series **3**–**5** as indicated in Scheme 1 in order to explore the hypothesis that cytotoxic potencies are greater in **5a**–**i** than in the precursor enones **3a**–**i**.

The choice of aryl substituents was made on the basis of the considerable differences in their electronic, hydrophobic, and steric characteristics. One or more of these properties may correlate with cytotoxic potencies. The aryl groups in series **3**–**5** are identical and, hence, if the compounds bearing the same substituents in the aryl rings have identical IC_{50} values, their potencies could be ascribed to the 1,5-diaryl-3-oxo-1,4-pentadienyl group. On the other hand, variations in potencies, for example between **3c**, **4c**, and **5c**, would point to a contribution to the magnitude of the bioactivity by the substituent on the piperidyl nitrogen atom. In addition, a comparison of the IC_{50} values of the 4-piperidones in series **4** and **5** may give some indication of whether masking of the acidic groups present in the 1-phosphono analogues is beneficial in terms of cytotoxic potencies.

A previous study revealed that while a small series of 3,5-bis(benzylidene)-4-piperidones with an average log*P* value of 3.90 had little or no capacity to reverse P-glycoprotein-associated multi-drug resistance (MDR), conversion into the corresponding amides with an average log*P* value of 5.52 led to clusters of potent MDR reverting agents.[9] Hence the decision was made to examine whether the phosphoramidates **4** and **5** possess this important biological property and whether lipophilicity affects the potencies of these compounds.

In summary, the objectives of the present study included examining the compounds in series **3**–**5** for cytotoxic properties as well as **4a**–**i** and **5a**–**i** as candidate MDR reverting agents. In addition, experiments were designed to find some of the reasons for any variation in potencies observed in the different biological evaluations.

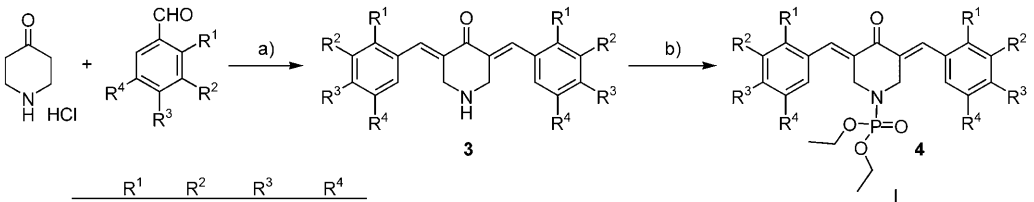
Results

The synthetic route for the preparation of the 4-piperidones **3**–**5** is presented in Scheme 1. The compounds in series **3** were prepared by acid-catalyzed condensation between a variety of aryl aldehydes and 4-piperidone. Reaction of **3a**–**i** with diethyl chlorophosphonate led to the formation of the corresponding amides **4**, which were hydrolyzed with trimethylsilyl bromide to yield the phosphonic acids **5a**–**i**. The Clog*P* values of the 4-piperidones in series **4** and **5** were computed and are listed in Table 1. The X-ray crystallographic structure of **4g** is presented in Figure 3 below.

All of the compounds in series **4** and **5** were evaluated against human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemia L1210 cells. These data are presented in Table 1. The biological data from these three assays were reported previously for **3a**,**c**,**f**,**i**[7] and also for **3b**.[11] Thus **3d**,**e**,**g**,**h** were prepared, and their growth inhibiting properties from the Molt 4/C8, CEM, and L1210 assays are listed in Table 1. The 4-piperidones **4a**,**c**,**d** and **5c** were examined by NCI against a panel of 58–59 human tumor cell lines, and these results are presented in Table 5 below. Two mode-of-action studies used human colon cancer HT29 cells: First, the effect of **5g** on these cells was examined by flow cytometry, and the results are shown in Figure 4. Second, evaluations of **5d** and **5g** on respiration in HT29 cells were undertaken, and the effects are illustrated in Figure 5. All of the compounds in series **4** and **5** were examined as candidate MDR reverting agents and the results are presented in Table 1.

Discussion

X-ray crystallography of a number of compounds having the general structure **3**[7,8,12] and related *N*-acyl derivatives[7,13,14] revealed that the olefinic double bonds adopt the *E* configuration. In addition, a representative compound prepared in this study, namely **4g**, is the *E*,*E* geometrical isomer as revealed by X-ray crystallography. Hence, the compounds in series **3**–**5** are considered to be the *E*,*E* isomers.



	R^1	R^2	R^3	R^4
a	H	H	H	H
b	H	H	CH_3	H
c	H	H	OCH_3	H
d	H	OCH_3	OCH_3	H
e	H	OCH_3	OCH_3	OCH_3
f	H	H	Cl	H
g	NO_2	H	H	H
h	H	NO_2	H	H
i	H	H	NO_2	H

Scheme 1. Synthesis of series **3**–**5**: a) HCl, CH_3COOH; b) $(C_2H_5O)_2P(O)Cl$, K_2CO_3, KI; c) $(CH_3)_3SiBr$.

All of the compounds in series **4** and **5** were evaluated against human Molt 4/C8 and CEM T-lymphocytes in order to determine whether cytotoxic properties would be exhibited toward human transformed cells. A number of anticancer drugs display growth inhibiting properties in the L1210 bioassay,[15] and this assay was also used to detect promising lead compounds. These data, along with the results of evaluating **3d**,**e**,**g**,**h**, which have not been assessed previously against these cell lines, are presented in Table 1.

The biological evaluations reveal that both series **4** and **5** demonstrate potent cytotoxicity toward human T-lymphocytes. No fewer than 94% of the IC_{50} values of **4a–i** and **5a–i** in the Molt 4/C8 and CEM screens are <10 μM, and 61% of these are in the sub-micromolar range. In particular, the high potency of **5g**, with IC_{50} values of 34 ± 2 nM toward both T-lymphocyte lines should be noted. This establishes this compound as a lead molecule. The marked potencies of these compounds toward Molt 4/C8 and CEM cells is confirmed when comparisons are made between these biological data and the results for melphalan, which is an alkylating agent used in cancer chemotherapy. In series **4**, the 4-piperidones **4a**,**d–i** are more potent than melphalan in both assays, that is, in 78% of the comparisons made. Furthermore, **5a**,**b**,**d–i** and **5b–i** have statistically significantly lower IC_{50} values than melphalan in the Molt 4/C8 and CEM screens, respectively, that is, in 89% of the data for series **5**. In particular, **5g** has 90-fold greater potency than this reference drug toward Molt 4/C8 cells, and is 76-fold more potent than melphalan in the CEM test. While the murine L1210 cells are more refractory to the 4-piperidones in series **4** and **5**, 78% of the IC_{50} values are <10 μM, and both **5d** and **5e** possess sub-micromolar IC_{50} values.

The next part of the biological data analysis involved comparison of the potencies of the compounds in series **3**–**5**. The approach involved dividing the IC_{50} value of a compound in series **3** by that of the analogue in series **4** or **5** having the same aryl substituents. This procedure gave rise to a number of $\Delta_{3/4}$ and $\Delta_{3/5}$ values in of each of the Molt 4/C8, CEM, and L1210 screens which are presented in Tables 2 and 3.

The results in Table 2 indicate that **4c**,**f**,**h**,**i** are more potent than **3c**,**f**,**h**,**i** in all three bioassays, that is, in 44% of the comparisons made. Three (11%) of the $\Delta_{3/4}$ values (indicated as footnote [b] in Table 2) denote equal potency. In the remaining cases (45%), higher potency was observed for the analogues in series **3**. Thus overall there was neither an increase nor decrease in potencies, although in some cases such as **4c** and **4h**, the IC_{50} values were considerably lower than for **3c** and **3h**, respectively. In addition, the fact that the compounds in series **4** are potent cytotoxins suggests that analogue development should be pursued vigorously, such as the preparation of a variety of related esters.

Table 1. Cytotoxicity of series **3**, **4**, and **5** compounds and Clog*P* values and fluorescence activity ratio (FAR) data for **4a–i** and **5a–i**.

Compd	IC_{50} [μM][a]			Clog *P*[b]	FAR[c]
	Molt 4/C8	CEM	L1210		
3d	0.25±0.13	0.37±0.05	1.33±0.37	–	–
3e[d]	0.31±0.02	0.30±0.02	0.53±0.34	–	–
3g[d]	0.37±0.03	0.36±0.11	1.17±0.37	–	–
3h	1.64±0.81	4.90±2.33	33.0±2.20	–	–
4a	1.36±0.20	1.52±0.19	8.46±0.18	4.01±0.43 (0.72)	51.6
4b	12.60±1.3	24.0±3.00	49.40±4.9	4.86±0.48 (0.76)	17.1
4c	2.90±1.07	5.82±0.07	38.5±3.9	3.95±0.58 (0.70)	49.6
4d	0.27±0.03	0.85±0.01	2.00±0.20	3.52±0.75 (0.69)	57.9
4e	0.45±0.02	0.51±0.05	3.00±1.01	3.38±1.09 (0.76)	52.9
4f	0.83±0.10	0.99±0.11	5.20±0.00	5.11±0.34 (0.65)	23.6
4g	1.58±0.08	1.91±0.01	7.12±0.54	3.75±0.40 (0.70)	1.86
4h	0.19±0.01	0.16±0.01	1.49±0.11	3.81±0.42 (0.68)	17.5
4i	1.02±0.06	1.30±0.06	8.63±0.04	3.76±0.32 (0.69)	11.2
5a	0.46±0.41	1.88±0.34	6.20±1.49	2.21±0.92 (−1.08)	3.66
5b	0.45±0.09	0.99±0.03	9.89±0.07	2.95±0.99 (−1.15)	13.3
5c	5.22±3.24	2.00±0.09	47.10±3.9	2.08±0.95 (−1.17)	2.53
5d	0.16±0.03	0.19±0.02	0.73±0.31	1.83±0.94 (−1.00)	60.5
5e	0.11±0.07	0.25±0.03	0.16±0.11	1.48±1.18 (−1.14)	43.7
5f	0.47±0.11	1.25±0.35	10.3±1.80	3.28±0.87 (−1.18)	1.51
5g	0.03±0.03	0.03±0.03	1.41±0.28	1.90±0.87 (−1.15)	2.86
5h	0.36±0.06	0.41±0.08	2.01±0.66	1.97±0.90 (−1.16)	1.45
5i	0.13±0.08	0.91±0.04	6.92±0.25	1.93±0.89 (−1.14)	0.99
melphalan[e]	3.24±0.79	2.47±0.30	2.13±0.03	–	–

[a] Concentration required to inhibit cell growth by 50%. [b] Values in parentheses are the differences in Clog*P* values from the respective analogue in series **3** that possesses the same aryl substituent; a negative value indicates lower hydrophobicity for the molecule than the series **3** analogue. [c] FAR values are the ratios of the fluorescence intensities of rhodamine 123 in treated versus untreated murine L-5178Y cells transfected with the human *mdr1* gene; compound concentration is 20 μM; the reference compound verapamil has a FAR value of 8.23 when 22 μM of this drug is used. [d] Evaluated as the HCl salt. [e] These data were reported previously in reference [36].

The $\Delta_{3/5}$ values are listed in Table 3. In 78% of the comparisons, **5a–i** are more potent than the analogues in series **3**, whereas in 19% of the cases equal potency was observed. The only case in which greater potency is displayed in series **3** is the IC_{50} value of **3b**, which is lower than that of **5b** in the L1210 screen. The $\Delta_{4/5}$ values were also computed and are listed in Table 3. In 70% of the comparisons, the analogues in series **5** have lower IC_{50} values than **4a–i**, whereas in 15% of the cases equal potency was noted. Thus, not only are series **4** compounds a group of promising cytotoxins, but hydrolysis of the ester groups of **4a–i** led to a highly potent cluster of cytotoxic molecules, namely series **5**.

To guide future expansion from these initial groups of compounds in series **4** and **5**, different approaches were adopted, including QSAR studies and molecular modeling. The magnitudes of the electronic, hydrophobic, and steric proper-

Table 2. Comparison of the potencies of **4a–i** with respective analogues in series **3** that have the same aryl substituents.

Compd	Ar Group	$\Delta_{3/4}$[a] Molt 4/C8	CEM	L1210
4a	H	1.2[b]	1.1[b]	0.9
4b	4-CH_3	0.1	0.1	0.2
4c	4-OCH_3	99	28	6.3
4d	3,4-$(OCH_3)_2$	0.9[b]	0.4	0.7
4e	3,4,5-$(OCH_3)_3$	0.7	0.6	0.2
4f	4-Cl	16	8.7	8.0
4g	2-NO_2	0.2	0.2	0.2
4h	3-NO_2	8.8	30	23
4i	4-NO_2	8.1	3.4	3.8

[a] The designation $\Delta_{3/4}$ refers to the quotient of the IC_{50} value of a compound in series **3** divided by that of the analogue in series **4** which bears the same aryl substituent. [b] No statistical difference in the IC_{50} values when standard deviations are taken into account.

Table 3. Comparison of the potencies of **5a–i** with compounds in series **3** and **4** that have the same substituents on the aryl rings.

Compd	Ar Group	Molt 4/C8 $\Delta_{3/5}$[a]	Molt 4/C8 $\Delta_{4/5}$[a]	CEM $\Delta_{3/5}$[a]	CEM $\Delta_{4/5}$[a]	L1210 $\Delta_{3/5}$[a]	L1210 $\Delta_{4/5}$[a]
5a	H	3.6	2.9	0.9[b]	0.8[b]	1.3	1.4
5b	4-CH_3	3.8	28	1.7	24	0.9	5.0
5c	4-OCH_3	55	0.6[b]	82	2.9	5.2	0.8
5d	3,4-$(OCH_3)_2$	1.6[b]	1.7	2.0	4.5	1.8[b]	2.7
5e	3,4,5-$(OCH_3)_3$	2.7	4.0	1.2	2.1	3.3[b]	19
5f	4-Cl	28	1.8	6.9	0.8[b]	4.0	0.5
5g	2-NO_2	10	44	11	59	0.8[b]	5.1
5h	3-NO_2	4.6	0.5	12	0.4	16	0.7[b]
5i	4-NO_2	62	7.7	4.9	1.4	4.8	1.3

[a] The $\Delta_{3/5}$ and $\Delta_{4/5}$ values are the quotients of the IC_{50} values of a compound in series **3** divided by that of either a respective series **5** ($\Delta_{3/5}$) or series **4** ($\Delta_{4/5}$) compound having the same substituents on the aryl rings. [b] No statistical difference in the IC_{50} values when standard deviations are taken into account.

ties of aryl substituents are indicated by the Hammett σ values (and Taft σ* values for *ortho* substituents), Hansch π constants and molecular refractivity (MR) values, respectively. Linear and semilogarithmic plots were made between these constants and the IC_{50} values of **4a–i** and **5a–i** in the Molt 4/C8, CEM, and L1210 screens. In addition, logarithmic plots were made between the MR values and the IC_{50} values. The following correlations ($p < 0.05$) or trends to significance ($p < 0.1$) were noted in series **5**. Negative correlation values were observed between the IC_{50} values of **5a–i** and the σ/σ* constants in the Molt 4/C8 screen ($p < 0.1$) as well as the MR values in the CEM assay ($p < 0.05$) and L1210 test ($p < 0.1$). A positive correlation with regard to the π constants in the Molt 4/C8 screen ($p < 0.1$) was also noted. No other correlations were observed in either series **4** or **5** ($p > 0.1$). Thus in developing these compounds, large electron-withdrawing groups of low hydrophobicity should be placed in the aryl rings.

The calculated log*P* values of the esters **4** and phosphonic acids **5** are listed in Table 1. The differences between the hydrophobicity of these compounds and the analogues **3a–i** are also indicated in Table 1. Thus, on average, series **4** is more hydrophobic than **3a–i** by 0.71 log*P* units, while the phosphonic acids **5** are more hydrophilic than **3** by 1.13 log*P* units. It is conceivable that a physicochemical parameter which contributes to the IC_{50} values of series **5** being lower than those of series **3** and **4** is their greater hydrophilic properties. In addition, we investigated whether the cytotoxic potencies of each of the series **3–5** compounds are influenced by the log*P* values. Thus linear, semilogarithmic, and logarithmic plots were constructed between the IC_{50} values and the Clog*P* data. Positive correlations were observed between the IC_{50} values in the L1210 screen and the Clog*P* values of series **3** ($p < 0.1$) and **5** ($p = 0.05$). This observation is in agreement with the recommendation made earlier of decreasing the magnitude of the π values of the aryl substituents. Hence, as a general rule, a variety of hydrophilic groups should be included in the future expansions of the compounds in series **3–5**.

In some cases biological potencies are influenced by the torsion angles (θ) between an aryl ring and the adjacent unsaturated group.[16] Hence the torsion angles θ_1 and θ_2, as indicated in Figure 2, were calculated by molecular modeling, and the

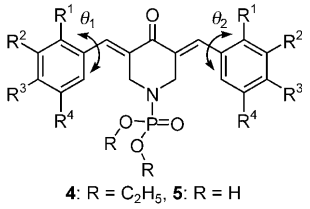


Figure 2. Designation of the torsion angles θ_1 and θ_2 in series **4** and **5**.

data for the compounds in series **3–5** are presented in Table 4. The θ_1 and θ_2 angles were calculated in a clockwise fashion and revealed that rings A and B rotate in opposite directions. In each series, the greatest torsion angles are found in the *ortho*-nitro analogues, namely **3g**, **4g**, and **5g**. Because **5g** is the most potent compound toward both T-lymphocytes among the 4-piperidones examined in this study, the placement of substituents of varying size at one or both of the *ortho* locations of rings A and B may establish whether a correlation is present between the magnitude of the torsion angles and cytotoxic potencies. In general, the torsion angles in series

Table 4. Torsion angles created between the aryl rings and the adjacent olefinic group in compound series **3–5**, **a–i**.

Compd	θ_1 [°] 3	4	5	θ_2 [°] 3	4	5
a	60.03	56.65	58.43	122.10	122.88	124.29
b	56.37	55.87	55.02	125.43	127.09	129.68
c	55.50	54.96	54.11	127.05	126.88	125.46
d	68.39	53.57	54.07	107.15	131.07	127.84
e	62.02	54.03	55.49	122.40	126.18	124.22
f	63.81	55.67	57.44	117.16	123.76	125.35
g	98.87	70.56	78.22	104.74	82.21	81.15
h	56.50	62.33	61.12	128.07	123.31	121.50
i	73.26	58.07	61.21	107.73	126.15	122.40

5 are not substantially different from those found in **3a–i** and **4a–i**, and hence θ values *per se* are unlikely to be the principal reason for the greater cytotoxic potencies of the analogues in series **5**.

Linear, semilogarithmic, and logarithmic plots were constructed between the θ_1 and θ_2 values in series **3**–**5**, and the IC_{50} values in each of the Molt 4/C8, CEM, and L1210 screens. Negative correlations were observed in the plots between the θ_1 values in series **5** and the IC_{50} values obtained in the Molt 4/C8 ($p < 0.1$) and CEM ($p < 0.05$) assays. On the other hand, positive correlations were observed from the plots between the θ_2 values in series **5** and the IC_{50} values toward both Molt 4/C8 ($p < 0.1$) and CEM ($p < 0.05$) cells. No other correlations were found ($p > 0.1$). This observation is important for the development of series **5**, whereby further analogues should ensure that θ_1 values are large and conversely θ_2 values are small. This objective can be achieved by placing large groups in the *ortho* position of ring A, and having ring B either unsubstituted or possessing small groups at the *para* position of the aryl ring.

A representative compound **4g** was examined by X-ray crystallography, and an ORTEP diagram[17] is presented in Figure 3.

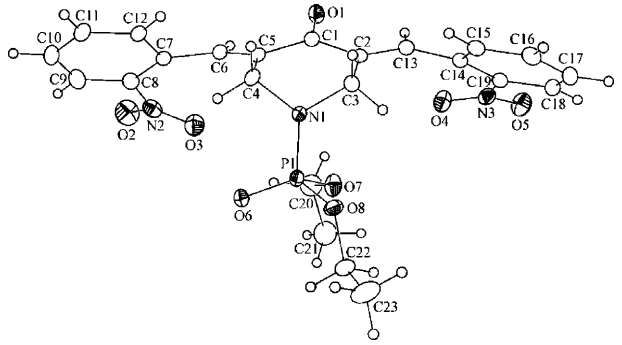


Figure 3. ORTEP diagram of **4g**.

The torsion angles C2–C13–C14–C19 (θ_1) and C5–C6–C7–C8 (θ_2) are 141.7(3) and −145.0(2), respectively. The piperidone ring adopts a half-chair conformation. The two aryl rings are orientated in an almost perpendicular fashion toward the piperidone ring. The nitro groups point away from both the heterocycle and the phosphonate moieties.

The biological data summarized in Table 1 for the potencies of various clusters of compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group toward Molt 4/C8, CEM, and L1210 cells are encouraging. Thus an important question is whether cytotoxicity toward a greater number and variety of neoplasms can be demonstrated. Hence, four compounds, namely **4a**,**c**,**d** and **5c**, were evaluated against 58–59 human tumor cell lines which originated from nine different neoplastic conditions: leukemia, melanoma, non-small-cell lung carcinoma, and colon, CNS, ovarian, renal, prostate, and breast cancers.[18] The results of these evaluations are presented in Table 5. When considering the toxicity toward all cell lines, the term GI_{50} rather than IC_{50} is used, because the average potencies listed include IC_{50} values that are greater than the maximum concentration used. The biological data reveal that **4a**,**c**,**d** and **5c** are potent cytotoxins, especially **4d**, which has a sub-micromolar average GI_{50} value, and is 21-fold more potent than melphalan. A positive feature of a candidate anti-neoplastic agent is that it displays varying toxicity toward different cell lines, which may be reflected in causing greater damage to tumors than the corresponding normal cells. Notably, the very high selectivity index (SI) values displayed by **4c** establish it as a lead molecule. Examination of the mean graphs[19] revealed that in general, colon cancers and leukemic cells are particularly sensitive to these compounds. In the case of the colon cancer cell lines, 64% of the IC_{50} values of **4a**,**c**,**d**, and **5c** are sub-micromolar, and 18% possess double-digit nanomolar values. Where specific data are available, the IC_{50} values of 5-fluorouracil (5-FU), which is a drug used in treating colon cancer, are in general substantially higher than the data obtained for **4a**,**c**,**d** and **5c** against colon cancer cells. In regard to anti-leukemic properties, the data in Table 5 reveal that **4a**,**c**,**d** and **5c** have high potencies: 63% of the IC_{50} figures are sub-micromolar. In particular, the IC_{50} values of <10 nM and 32 nM displayed by **4a** and **4c**, respectively, toward RPMI 8226 cells are impressive. The average IC_{50} values reveal that **4a**,**c**,**d** and **5c** possess 58-, 35-, 93-, and 15-fold greater potency than melphalan, which is used clinically in treating various types of leukemia. The data in Table 5 afford ample evidence to pursue series **4** and **5** as excellent leads for the future development of candidate anti-neoplastic agents.

A further issue to be addressed is the way in which the compounds prepared in this study exert their cytotoxic activity. Ex-

Table 5. Evaluation of **4a**,**c**,**d** and **5c** against a panel of 59 human tumor cell lines

Compd	All cell lines		Colon cancers, IC_{50} [μM]								Leukemic cell lines, IC_{50} [μM]					
	GI_{50} [μM][a]	SI[b]	COLO 205	HCT 116	HCT 15	HT29	KM12	SW 620	HCC 2998	Avg. IC_{50}	HL60 (TB)	K-562	RPMI 8226	SR	CCRF CEM	Avg. IC_{50}
4a	1.20	>381	1.62	0.21	1.26	0.37	0.20	0.23	2.14	0.86	1.12	2.29	<0.01	0.19	0.32	0.79
4c	1.58	>3152	1.41	0.05	0.81	0.04	0.04	0.04	0.07	0.35	4.90	–	0.03	0.06	0.16	1.29
4d	0.93	240	1.55	0.39	1.10	0.42	0.27	0.33	2.09	0.88	0.91	0.49	0.30	0.44	0.29	0.48
5c	3.47	>355	2.04	0.34	2.24	0.72	0.32	0.68	2.00	1.19	3.72	3.63	0.28	5.25	2.19	3.01
melphalan	19.1	513	32.4	39.8	36.3	70.8	57.5	26.9	52.5	45.2	0.38	195	28.2	3.24	0.39	45.4
5-FU	12.0	>10000	14.80	1.91	2.29	28.2	<0.25	22.4	<0.25	<10.0	–	35.5	1.70	2.09	14.1	13.4

[a] Average GI_{50}; GI_{50} is used instead of IC_{50}, as the average potencies listed include IC_{50} values greater than the maximum concentration used. [b] Selectivity index: quotient of the IC_{50} values from the most refractory versus most sensitive cell lines for a given compound.

periments to monitor the effects on both cell cycle and respiration using HT29 human colon cancer cells were undertaken. The IC_{50} value of **5g** after incubation with HT29 cells for 96 h is 4.25 μM. The effect of this compound at 5 μM on the cell cycle is illustrated in Figure 4, which reveals that the sub-G_1 phase

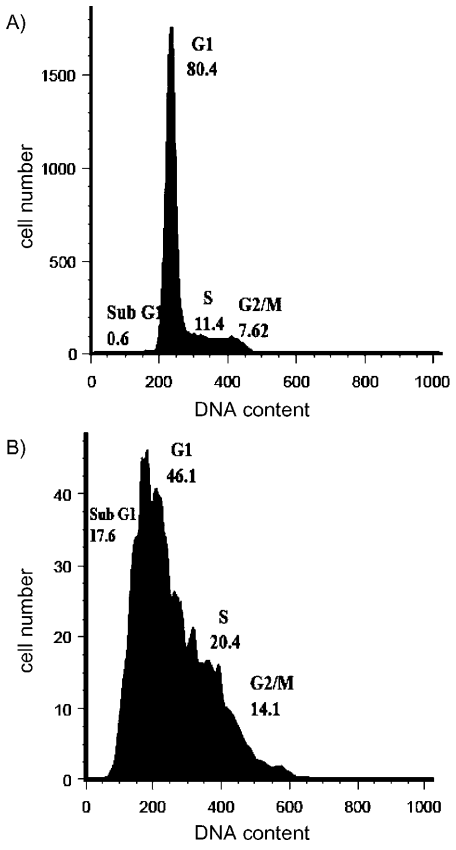


Figure 4. The effect of compound **5g** on the cell cycle of HT29 cells: A) control; B) **5g** at 5 μM.

has increased 29-fold, indicating that apoptosis has occurred. Previous work from our research groups has revealed that various compounds containing the 1,5-diaryl-3-oxo-1,4-pentadienyl group cause stimulation of respiration in rat liver mitochondria.[20–22] In the present investigation, two of the potent cytotoxins, **5d** and **5g**, as well as 5-FU were examined for their effects on respiration in HT29 cells. A concentration of 25 μM was chosen, which is close to the IC_{50} value of 5-FU toward this cell line. The results are presented in Figure 5, which reveals that only inhibition of respiration was observed. Hence, interference with mitochondrial respiration is one way in which the cytotoxicity of **5d** and **5g**, and presumably analogues of these compounds, is mediated. The significant inhibition of respiration by 5-FU suggests that this is an important mode of action for this anticancer drug.

The final question is whether the compounds in series **4** and **5** have MDR reverting properties or not. The assays for P-glycoprotein MDR reversal employed murine L-5178Y lymphoma cells transfected with the human *mdr1* gene. The concentrations of the dye rhodamine 123 in treated and untreated trans-

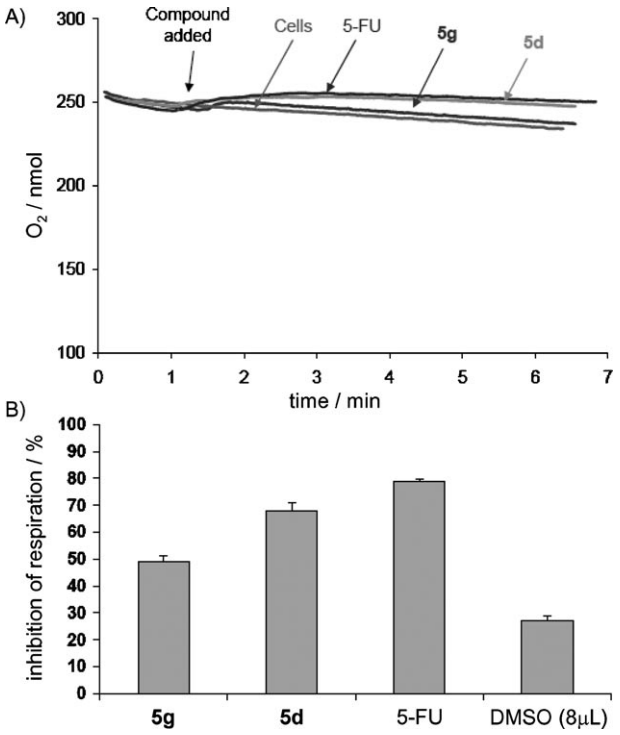


Figure 5. Effect of **5d**, **5g**, and 5-fluorouracil (25 μM each) on respiration in colon HT29 cancer cells: A) kinetics of oxygen consumption; B) percent inhibition of respiration. Error bars indicate the standard deviations from three replicates.

fected and parental cells were measured, and the relative fluorescence intensities are referred to as the fluorescence activity ratio (FAR) values. A FAR value of >1 indicates MDR reversal has occurred. These data are presented in Table 1. In general, MDR reversal is more pronounced in series **4** than in **5**, as revealed from the following observations: First, the average FAR values for series **4** and **5** are 32 and 15, respectively. Second, with the exception of **4d** and **4g**, for the same substituent in the aryl rings, the analogues in series **4** have the higher FAR values. A number of MDR reversal agents have high lipophilicity.[23,24] Because the average Clog*P* values in series **4** and **5** are 4.02 and 2.18, respectively, the greater hydrophobicity of **4a–i** than **5a–i**, in general, may contribute to the higher MDR reverting properties of the compounds in series **4**. The following 4-piperidones possess FAR values in excess of 20 and are lead molecules, namely **4a**,**c–f** and **5d**,**e**. Notably, the two compounds in each of series **4** and **5** with the highest MDR reverting properties, i.e., **4d**,**e** and **5d**,**e**, have the same aryl substituents, namely 3,4-dimethoxy and 3,4,5-trimethoxy groups. Hence the placement of a number of methoxy and related alkoxy substituents at various locations on the aryl rings may be worth pursuing in future searches for novel MDR reverting agents. Furthermore, to determine whether MDR reversal is governed by one or more of the physicochemical properties of the aryl substituents, linear and semilogarithmic plots were made between the σ/σ^*, π, and MR constants of the groups in the aryl rings and the FAR values in both series **4** and **5**. A negative correlation was observed between the FAR values of **4a–i** and the σ/σ^* constants ($p < 0.01$). In addition, a positive corre-

lation was found between the MR values of the aryl substituents of the compounds in series **5** and the FAR data ($p = 0.01$). A trend toward a negative correlation ($p < 0.1$) was observed between the FAR values of **5a–i** and the σ/σ^* constants of the aryl substituents. No other correlations were noted ($p > 0.1$). One may conclude that when developing the compounds in series **4** and **5** as candidate MDR reverting agents, strongly electron-releasing substituents should be placed in the aryl rings. In the case of series **5**, increasing the size of the aryl substituents will likely increase the magnitude of MDR reversal. An intriguing question is whether any correlation exists between the FAR values and the IC_{50} values generated for series **4** and **5** in the Molt 4/C8, CEM, and L1210 assays. Hence linear, semilogarithmic, and logarithmic plots were constructed, and a negative correlation was observed for **5a–i** in the murine L1210 screen ($p < 0.05$). No other correlations were found ($p > 0.1$). Consequently the design of analogues **5a–i** for greater cytotoxic potencies should be accompanied by increased MDR reversal.

Conclusions

A series of 3,5-bis(benzylidene)-1-phosphono-4-piperidones **5** and the related diethyl esters **4** were synthesized. These compounds display potent cytotoxicity toward human Molt 4/C8 and CEM T-lymphocytes as well as murine leukemia L1210 cells. In general, greater potencies are observed for series **5** than for the more hydrophobic analogues **4a–i**. In particular, **5g**, with an average IC_{50} value of 34 nM toward the T-lymphocyte lines, is clearly a lead molecule. Many of the compounds are more potent than the anticancer drug melphalan. Various physicochemical properties were shown to influence the magnitude of the IC_{50} values generated. Four of the 4-piperidones, namely **4a**,**c**,**d** and **5c**, are substantially more potent than melphalan and 5-fluorouracil toward nearly 60 human tumor cell lines. In this biological evaluation, approximately two-thirds of the IC_{50} values toward several colon cancer cell lines and leukemic cells are sub-micromolar, and several are in the double-digit nanomolar range. The modes of action of representative compounds include the induction of apoptosis and interference with cellular respiration. Most of the compounds in series **4** as well as **5b**,**d**,**e** have significant MDR reverting properties. Thus this study has disclosed the discovery of two novel series of cytotoxic compounds, some of which have pronounced MDR reverting properties. A number of guidelines for expanding this project have been made.

Experimental Section

Chemistry

Synthesis of 3d,e,g,h, 4a–i, and 5a–i: Melting points were determined on a Gallenkamp instrument and are uncorrected. ^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker Avance spectrometer equipped with a 5 mm BBO probe. Chemical shifts (δ) are reported in ppm. Elemental analyses were conducted with an Elementer analyzer. Mass spectra were measured using a Micromass Quattro II mass spectrometer.

Synthesis of 3,5-bis(arylidene)-4-piperidones (3a–i): The syntheses of **3a–c**,**f**,**i** were reported previously.[7,11] Compounds **3d**,**e**,**g**,**h** were prepared following the same procedure.

3,5-Bis-(3,4-dimethoxybenzylidene)-4-piperidone (3d): Yield: 67%; mp: 162 °C; ^1H NMR (DMSO): $\delta = 7.56$ (s, 2H, 2×=CH), 7.07 (d, 6H, Ar-H, $J = 14.74$ Hz), 4.03 (s, 4H, 2×NCH_2), 3.82 (s, 12H, 4×OCH_3); Anal. calcd for $C_{23}H_{25}NO_5$: C 69.86, H 6.37, N 3.54, found: C 69.76, H 6.14, N 3.32.

3,5-Bis-(3,4,5-trimethoxybenzylidene)-4-piperidone hydrochloride (3e): Yield: 68%; mp: 251 °C; ^1H NMR (DMSO): $\delta = 9.5$ (br d, 2H, $^+NH_2$), 7.84 (s, 2H, 2×=CH), 6.86 (s, 4H, Ar-H), 4.58 (s, 4H, 2×NCH_2), 3.84 (s, 12H, 4×OCH_3), 3.73 (s, 6H, 2×OCH_3); Anal. calcd for $C_{25}H_{30}ClNO_7$: C 61.04, H 6.15, N 2.85, found: C, 60.78, H 6.10, N 2.75.

3,5-Bis-(2-nitrobenzylidene)-4-piperidone hydrochloride (3g): Yield: 52%; mp: 218 °C; ^1H NMR (DMSO): $\delta = 9.15$ (br s, 2H, $^+NH_2$), 8.28 (d, 2H, Ar-H, $J = 8.15$ Hz), 8.14 (s, 2H, 2×=CH), 7.90 (t, 2H, Ar-H), 7.76 (t, 2H, Ar-H), 7.57 (d, 2H, Ar-H, $J = 7.45$ Hz), 4.19 (s, 4H, 2×NCH_2); Anal. calcd for $C_{19}H_{16}ClN_3O_5$: C 56.74, H 3.98, N 10.45, found: C, 56.45, H 3.96, N 10.25.

3,5-Bis-(3-nitrobenzylidene)-4-piperidone (3h): Yield: 68%; mp: 214 °C; ^1H NMR (DMSO): $\delta = 8.34$ (s, 2H, Ar-H), 8.27 (d, 2H, Ar-H, $J = 8.20$ Hz), 7.96 (d, 2H, Ar-H, $J = 7.71$ Hz), 7.77 (t, 2H, Ar-H), 7.72 (s, 2H, 2×=CH), 4.06 (s, 4H, 2×NCH_2); Anal. calcd for $C_{19}H_{15}N_3O_5$: C 62.46, H 4.14, N 11.50, found: C, 62.34, H 3.99, N 11.59.

Synthesis of [3,5-bis(arylidene)-4-oxo-1-yl]phosphonic acid diethyl esters (4a–i). *General procedure:* A mixture of **3a–i** (0.01 mol), diethylchlorophosphate (2.07 g, 0.012 mol), anhydrous K_2CO_3 (2.07 g, 0.015 mol), and a catalytic amount of KI (0.166 g, 0.001 mol) in acetone (30 mL) was held at reflux for 2–3 h. Reaction progress was monitored by TLC (solvent: MeOH/$CHCl_3$ 5:95 *v/v*). The solvent was evaporated under vacuum at 40–45 °C. An aqueous solution of K_2CO_3 (5% *w/v*, 50 mL) was added to the crude mass and stirred for 2 h. The solid was removed by filtration, dried, and crystallized from a suitable solvent.

[3,5-Bis(benzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4a): Yield: 61%; mp: 127 °C (acetone); ^1H NMR ($CDCl_3$): $\delta = 7.86$ (s, 2H, 2×=CH), 7.44 (m, 10H, Ar-H), 4.50 (d, 4H, 2×NCH_2, $J = 7.86$ Hz), 3.96 (m, 4H, 2×OCH_2), 1.20 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 187.02$, 136.99, 134.77, 132.59, 132.55, 130.46, 129.40, 128.97, 128.79, 62.74, 62.69, 46.28, 46.26, 16.02, 15.98; MS (ESI): *m/z* 450.01 $[M+K]^+$, 434.13 $[M+Na]^+$, 412.15 $[M+H]^+$; Anal. calcd for $C_{23}H_{26}NO_4P \cdot 0.25\,H_2O$: C 66.35, H 6.25, N 3.36, found: C, 66.37, H 6.32, N 3.20.

[3,5-Bis-(4-methylbenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4b): Yield: 66%; mp: 151 °C (*i*PrOH); ^1H NMR ($CDCl_3$): $\delta = 7.82$ (s, 2H, 2×=CH), 7.34 (d, 4H, Ar-H, $J = 8.03$ Hz), 7.26 (d, 4H, Ar-H, $J = 7.96$ Hz), 4.49 (d, 4H, 2×NCH_2, $J = 7.56$ Hz), 3.96 (m, 4H, 2×OCH_2), 2.42 (s, 6H, 2×Ar-CH_3), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 187.08$, 139.78, 136.91, 132.04, 131.88, 131.84, 130.60, 129.53, 62.68, 62.64, 46.31, 46.29, 21.48, 16.06, 16.01; MS (ESI): *m/z* 440.16 $[M+H]^+$; Anal. calcd for $C_{25}H_{30}NO_4P \cdot 0.25\,H_2O$: C 67.57, H 6.75, N 3.15, found: C, 67.77, H 6.85, N 3.06.

[3,5-Bis-(4-methoxybenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4c): Yield: 64%; mp: 144 °C (MeOH); ^1H NMR ($CDCl_3$): $\delta = 7.81$ (s, 2H, 2×=CH), 7.40 (d, 4H, Ar-H, $J = 8.70$ Hz), 6.98 (d, 4H, Ar-H, $J = 8.71$ Hz), 4.49 (d, 4H, 2×NCH_2, $J = 7.31$ Hz), 3.98 (m, 4H, 2×OCH_2), 3.88 (s, 6H, 2×Ar-OCH_3), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 186.92$, 160.56, 136.51, 132.47, 130.72, 130.68, 127.58,

114.31, 62.68, 62.64, 55.40, 55.32, 46.30, 46.27, 16.08, 16.03; MS (ESI): *m/z* 472.27 $[M+H]^+$; Anal. calcd for $C_{25}H_{30}NO_6P{\cdot}0.5\,H_2O$: C 62.43, H 6.24, N 2.91, found: C, 62.24, H 6.34, N 2.81.

[3,5-Bis-(3,4-dimethoxybenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4d): Yield: 58%; mp: 120 °C (EtOH); ^{1}H NMR ($CDCl_3$): $\delta = 7.79$ (s, 2H, 2×=CH), 7.05 (dd, 2H, Ar-H, $J = 1.52$, 8.32 Hz), 6.97 (d, 2H, Ar-H, $J = 1.53$ Hz), 6.95 (d, 2H, Ar-H, $J = 8.35$ Hz), 4.51 (d, 4H, 2×NCH_2, $J = 7.70$ Hz), 4.02 (m, 4H, 2×OCH_2), 3.95 (s, 6H, 2×Ar-OCH_3), 3.93 (s, 6H, 2×Ar-OCH_3), 1.22 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 186.75$, 150.27, 148.93, 136.79, 130.99, 130.96, 127.82, 123.97, 113.85, 111.17, 62.69, 62.65, 55.99, 55.98, 46.31, 46.28, 16.11, 16.05; MS (ESI): *m/z* 532.25 $[M+H]^+$; Anal. calcd for $C_{27}H_{34}NO_8P{\cdot}0.25\,H_2O$: C 60.44, H 6.34, N 2.61, found: C, 60.31, H 6.40, N 2.48.

[3,5-Bis-(3,4,5-trimethoxybenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4e): Yield: 54%; mp: 129 °C (MeOH); ^{1}H NMR ($CDCl_3$): $\delta = 7.78$ (s, 2H, 2×=CH), 6.66 (s, 4H, Ar-H), 4.52 (d, 4H, 2×NCH_2, $J = 8.40$ Hz), 3.97 (m, 4H, 2×OCH_2), 3.93 (s, 6H, 2×Ar-OCH_3), 3.91 (s, 12H, 4×Ar-OCH_3), 1.23 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta =$ 186.66, 153.26, 139.43, 137.17, 131.89, 131.86, 130.27, 107.89, 62.73, 62.69, 61.01, 56.27, 56.20, 46.27, 46.24, 16.12, 16.06; MS (ESI): *m/z* 592.24 $[M+H]^+$; Anal. calcd for $C_{29}H_{38}NO_{10}P{\cdot}0.25\,H_2O$: C 58.38, H 6.37, N 2.34, found: C, 58.01, H 6.44, N 2.21.

[3,5-Bis-(4-chlorobenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4f): Yield: 67%; mp: 132 °C (MeOH); ^{1}H NMR ($CDCl_3$): $\delta =$ 7.78 (s, 2H, 2×=CH), 7.44 (d, 4H, Ar-H, $J = 8.42$ Hz), 7.36 (d, 4H, Ar-H, $J = 8.45$ Hz), 4.45 (d, 4H, 2×NCH_2, $J = 7.54$ Hz), 3.97 (m, 4H, 2×OCH_2), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 186.57$, 135.73, 135.57, 133.12, 132.87, 132.83, 131.64, 129.13, 62.84, 62.79, 46.19, 46.17, 16.07, 16.02; MS (ESI): *m/z* 480.10 $[M+H]^+$; Anal. calcd for $C_{23}H_{24}Cl_2NO_4P{\cdot}0.25\,H_2O$: C 56.92, H 4.95, N 2.88, found: C, 56.91, H 5.05, N 2.78.

[3,5-Bis-(2-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4g): Yield: 42%; mp: 177 °C (EtOH); ^{1}H NMR ($CDCl_3$): $\delta = 8.23$ (d, 2H, Ar-H, $J = 8.22$ Hz), 8.11 (s, 2H, 2×=CH), 7.73 (t, 2H, Ar-H), 7.60 (t, 2H, Ar-H), 7.41 (d, 2H, Ar-H, $J = 7.61$ Hz), 4.18 (d, 4H, 2×NCH_2, $J = 9.67$ Hz), 3.95 (m, 4H, 2×OCH_2), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 185.49$, 147.93, 134.64, 133.75, 133.09, 133.06, 130.89, 130.82, 129.78, 125.35, 62.87, 62.82, 16.04, 15.99; MS (ESI): *m/z* 502.30 $[M+H]^+$; Anal. calcd for $C_{23}H_{24}N_3O_8P{\cdot}0.25\,H_2O$: C 54.51, H 4.77, N 8.20, found: C, 54.55, H 4.74, N 8.30.

[3,5-Bis-(3-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4h): Yield: 52%; mp: 147 °C (EtOH); ^{1}H NMR ($CDCl_3$): $\delta = 8.29$ (d, 2H, Ar-H, $J = 8.71$ Hz), 8.27 (s, 2H, Ar-H), 7.88 (s, 2H, 2×=CH), 7.76 (d, 2H, Ar-H, $J = 7.69$ Hz), 7.66 (t, 2H, Ar-H), 4.51 (d, 4H, 2×NCH_2, $J = 7.88$ Hz), 4.00 (m, 4H, 2×OCH_2), 1.22 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 185.89$, 136.14, 135.79, 134.56, 134.51, 130.00, 124.62, 123.98, 63.03, 62.98, 46.19, 46.17, 16.08, 16.03; MS (ESI): *m/z* 524.23 $[M+Na]^+$, 502.37 $[M+H]^+$; Anal. calcd for $C_{23}H_{24}N_3O_8P$: C 55.09, H 4.82, N 8.38, found: C, 54.79, H 4.76, N 8.33.

[3,5-Bis-(4-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid diethyl ester (4i): Yield: 55%; mp: 188 °C (EtOH); ^{1}H NMR ($CDCl_3$): $\delta = 8.33$ (d, 4H, Ar-H, $J = 8.64$ Hz), 7.86 (s, 2H, 2×=CH), 7.58 (d, 4H, Ar-H, $J = 8.57$ Hz), 4.48 (d, 4H, 2×NCH_2, $J = 8.37$ Hz), 3.98 (m, 4H, 2×OCH_2), 1.21 (t, 6H, 2×CH_3); ^{13}C NMR ($CDCl_3$): $\delta = 185.98$, 147.82, 140.79, 135.12, 135.08, 134.66, 130.86, 124.05, 63.05, 63.01, 46.21, 46.18, 16.10, 16.04; MS (ESI): *m/z* 500.59 $[M-H]^-$; Anal. calcd for $C_{23}H_{24}N_3O_8P$: C 55.09, H 4.82, N 8.38, found: C, 55.13, H 4.87, N 8.13.

Synthesis of [3,5-bis(arylidene)-4-oxo-1-yl]phosphonic acids (5a–i): *General procedure:* $Si(CH_3)_3Br$ (7.65 g, 0.05 mol) was added to a solution of **4a–i** (0.01 mol) in CH_3CN (30 mL) under N_2 atmosphere at room temperature, and the reaction was allowed to continue for 12–15 h. Reaction progress was monitored by TLC (solvent: MeOH/$CHCl_3$ 10:90 *v/v*). The solvent was evaporated under reduced pressure at 45–50 °C. H_2O (30 mL) was added to the crude mass and stirred for 2–3 h. The solid was filtered off, dried, and crystallized from $CHCl_3$/MeOH (2:8 *v/v*).

[3,5-Bis(benzylidene)-4-oxo-1-yl]phosphonic acid (5a): Yield: 41%; mp: 258 °C (dec.); ^{1}H NMR (DMSO): $\delta = 9.39$ (brs, 1H, OH), 9.33 (brs, 1H, OH), 7.91 (s, 2H, 2×=CH), 7.55 (m, 10H, Ar-H), 4.54 (s, 4H, 2×NCH_2); ^{13}C NMR (DMSO): $\delta = 182.84$, 139.79, 134.15, 131.04, 130.63, 129.47, 128.26, 44.58, 44.55; MS (ESI): *m/z* 276.29 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{18}NO_4P{\cdot}3\,H_2O$: C 55.70, H 4.39, N 3.42, found: C, 55.57, H 4.48, N 3.40.

[3,5-Bis-(4-methylbenzylidene)-4-oxo-1-yl]phosphonic acid (5b): Yield: 46%; mp: 254 °C (dec.); ^{1}H NMR (DMSO): $\delta = 9.29$ (brs, 2H, 2×OH), 7.87 (s, 2H, 2×=CH), 7.45 (d, 4H, Ar-H, $J = 8.04$ Hz), 7.36 (d, 4H, Ar-H, $J = 7.97$ Hz), 4.53 (s, 4H, 2×NCH_2), 2.39 (s, 6H, 2×Ar-CH_3); ^{13}C NMR (DMSO): $\delta = 182.72$, 140.82, 139.76, 131.40, 131.19, 130.09, 127.40, 44.63, 21.53; MS (ESI): *m/z* 304.33 $[M-HPO_3+H]^+$; Anal. calcd for $C_{21}H_{22}NO_4P{\cdot}1.25\,H_2O$: C 55.70, H 4.39, N 3.42, found: C, 55.57, H 4.48, N 3.40.

[3,5-Bis-(4-methoxybenzylidene)-4-oxo-1-yl]phosphonic acid (5c): Yield: 51%; mp: 256 °C; ^{1}H NMR (DMSO): $\delta = 9.29$ (brs, 2H, 2×OH), 7.86 (s, 2H, 2×=CH), 7.53 (d, 4H, Ar-H, $J = 8.72$ Hz), 7.11 (d, 4H, Ar-H, $J = 8.73$ Hz), 4.52 (s, 4H, 2×NCH_2), 3.85 (s, 6H, 2×Ar-OCH_3); ^{13}C NMR (DMSO): $\delta = 182.52$, 161.31, 139.43, 133.28, 126.73, 125.98, 115.05, 55.97, 44.65; MS (ESI): *m/z* 336.14 $[M-HPO_3+H]^+$; Anal. calcd for $C_{21}H_{22}NO_6P{\cdot}0.5\,H_2O$: C 59.38, H 5.18, N 3.29, found: C, 59.09, H 5.35, N 3.18.

[3,5-Bis-(3,4-dimethoxybenzylidene)-4-oxo-1-yl]phosphonic acid (5d): Yield: 53%; mp: 249 °C; ^{1}H NMR (DMSO): $\delta = 9.29$ (brs, 2H, 2×OH), 7.87 (s, 2H, 2×=CH), 7.17 (s, 2H, Ar-H), 7.14 (d, 4H, Ar-H, $J = 7.90$ Hz), 4.57 (s, 4H, 2×NCH_2), 3.85 (s, 6H, 2×Ar-OCH_3), 3.83 (s, 6H, 2×Ar-OCH_3); ^{13}C NMR (DMSO): $\delta = 182.44$, 151.15, 149.20, 139.86, 126.91, 126.07, 124.85, 114.86, 112.29, 56.19, 56.16, 44.67; MS (ESI): *m/z* 396.25 $[M-HPO_3+H]^+$; Anal. calcd for $C_{23}H_{26}NO_8P{\cdot}3\,H_2O$: C 52.13, H 4.91, N 2.64, found: C, 52.32, H 5.05, N 2.53.

[3,5-Bis-(3,4,5-trimethoxybenzylidene)-4-oxo-1-yl]phosphonic acid (5e): Yield: 61%; mp: 245 °C; ^{1}H NMR (DMSO): $\delta = 9.29$ (brs, 2H, 2×OH), 7.87 (s, 2H, 2×=CH), 6.87 (s, 4H, Ar-H), 4.63 (s, 4H, 2×NCH_2), 3.86 (s, 12H, 4×Ar-OCH_3), 3.75 (s, 6H, 2×Ar-OCH_3); ^{13}C NMR (DMSO): $\delta = 182.65$, 153.42, 140.07, 139.79, 129.65, 127.46, 108.98, 60.69, 56.66, 44.64; MS (ESI): *m/z* 456.22 $[M-HPO_3+H]^+$; Anal. calcd for $C_{25}H_{30}NO_{10}P{\cdot}0.5\,H_2O$: C 55.09, H 5.50, N 2.57, found: C, 55.06, H 5.66, N 2.52.

[3,5-Bis-(4-chlorobenzylidene)-4-oxo-1-yl]phosphonic acid (5f): Yield: 62%; mp: 264 °C (dec.); ^{1}H NMR (DMSO): $\delta = 9.34$ (brs, 2H, 2×OH), 7.89 (s, 2H, 2×=CH), 7.62 (d, 4H, Ar-H, $J = 8.58$ Hz), 7.58 (d, 4H, Ar-H, $J = 8.64$ Hz), 4.52 (s, 4H, 2×NCH_2); ^{13}C NMR (DMSO): $\delta =$ 182.67, 138.53, 135.35, 133.01, 132.80, 129.50, 128.81, 44.49; MS (ESI): *m/z* 344.14 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{16}Cl_2NO_4P{\cdot}4\,H_2O$: C 45.94, H 3.22, N 2.82, found: C, 45.59, H 3.31, N 2.58.

[3,5-Bis-(2-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid (5g): Yield: 34%; mp: 259 °C (dec.); ^{1}H NMR (DMSO): $\delta = 9.35$ (s, 2H, 2×OH), 8.42 (d, 2H, Ar-H, $J = 8.15$ Hz), 8.27 (s, 2H, 2×=CH), 7.89 (t,

2H, Ar-H), 7.76 (t, 2H, Ar-H), 7.58 (d, 2H, Ar-H, $J=7.60$ Hz), 4.31 (s, 4H, $2\times NCH_2$); ^{13}C NMR (DMSO): $\delta=182.50$, 146.53, 137.64, 137.11, 134.34, 129.80, 128.51, 128.13, 124.52, 44.46; MS (ESI): *m/z* 365.99 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{16}N_3O_8P\cdot0.5\,H_2O$: C 50.18, H 3.52, N 10.39, found: C, 50.45, H 3.87, N 10.76.

[3,5-Bis-(3-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid (5h): Yield: 52%; mp: 254 °C; ^1H NMR (DMSO): $\delta=9.35$ (br s, 2H, $2\times$OH), 8.38 (s, 2H, Ar-H), 8.35 (d, 2H, Ar-H, $J=8.20$ Hz), 8.03 (s, 2H, $2\times$=CH), 8.01 (d, 2H, Ar-H, $J=8.30$ Hz), 7.85 (t, 2H, Ar-H), 4.61 (s, 4H, $2\times NCH_2$); ^{13}C NMR (DMSO): $\delta=182.55$, 148.55, 137.69, 137.13, 135.67, 131.00, 130.35, 125.13, 124.89, 44.38; MS (ESI): *m/z* 366.18 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{16}N_3O_8P\cdot0.5\,H_2O$: C 50.18, H 3.52, N 10.39, found: C, 50.22, H 3.76, N 10.03.

[3,5-Bis-(4-nitrobenzylidene)-4-oxo-1-yl]phosphonic acid (5i): Yield: 42%; mp: 204 °C; ^1H NMR (DMSO): $\delta=9.36$ (br s, 2H, $2\times$OH), 8.37 (d, 4H, Ar-H, $J=8.70$ Hz), 8.00 (s, 2H, $2\times$=CH), 7.83 (d, 4H, Ar-H, $J=8.68$ Hz), 4.55 (s, 4H, $2\times NCH_2$); ^{13}C NMR (DMSO): $\delta=182.61$, 148.15, 140.55, 137.63, 132.05, 131.09, 124.35, 44.49; MS (ESI): *m/z* 366.06 $[M-HPO_3+H]^+$; Anal. calcd for $C_{19}H_{16}N_3O_8P\cdot0.5\,H_2O$: C 50.18, H 3.52, N 10.39, found: C, 50.32, H 3.67, N 10.48.

Determination of Clog P values

The Clog *P* values of the compounds in series **3**–**5** were determined with a commercial software package.[25] The Clog *P* values for the compounds in series **3** are as follows: **3a**: 3.29 ± 0.43; **3b**: 4.10 ± 0.55; **3c**: 3.25 ± 0.60; **3d**: 2.83 ± 0.73; **3e**: 2.62 ± 1.22; **3f**: 4.46 ± 0.39; **3g**: 3.05 ± 0.47; **3h**: 3.13 ± 0.54; **3i**: 3.07 ± 0.41.

Determination of QSARs

The σ, π, and MR values were obtained from Hansch and Leo,[26] whereas the σ^* value was taken from Taft.[27] Linear, semilogarithmic, and logarithmic plots were made with SPSS v. 14.0.0.[28]

Molecular modeling

Models of the compounds in series **4** and **5** were constructed using BioMedCache v. 6.1 software.[29] The lowest-energy conformations were generated with the MOPAC system and were optimized by PM3 parameters.

X-ray crystallography of 4g

Apart from the structure factors, CCDC 733192 (**4g**) contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Cytotoxicity assays

The evaluation of **3d**,**e**,**g**,**h**, **4a**–**i**, and **5a**–**i** as candidate cytotoxins using human Molt 4/C8 and CEM T-lymphocytes as well as murine L1210 cells was carried out by following published procedures.[30] Briefly, various concentrations of compounds were incubated with cells in RPMI 1640 medium for 72 h at 37 °C (Molt 4/C8 and CEM T-lymphocytes), whereas a 48 h incubation was used in the L1210 assay. The methodology with which **4a**,**c**,**d**, **5c**, melphalan, and 5-fluorouracil were assayed by using 58 or 59 human tumor cell lines was described previously.[18] The compounds were evaluated at concentrations of 0.10 mM–10 nM (**4a**,**c**,**d**, and **5c**), 0.25 mM–25 nM (melphalan), and 2.5 mM–250 nM (5-fluorouracil). The number of cell lines for which IC_{50} values lay outside the range of concentrations employed are: 1/58 (**4a**), 1/59 (**4c**), 0/59 (**4d**), 4/58 (**5c**), 0/59 (melphalan) and 6/58 (5-fluorouracil).

Determination of MDR reverting properties

The ability of compounds **4a**–**i** and **5a**–**i** to reverse MDR was evaluated by a published procedure,[31] which was summarized recently.[9] Briefly, the compounds were dissolved in DMSO, added to L-5178 MDR and parental cells, and incubated at room temperature for 10 min. After the addition of a solution of rhodamine 123 in DMSO, the cells were incubated at 37 °C for 20 min. The fluorescence was measured in treated MDR cells (F1), untreated MDR cells (F2), treated parental cells (F3), and untreated parental cells (F4), and the FAR values were calculated from the equation: FAR = (F1/F2)/(F3/F4). In these experiments, the FAR value of DMSO was 0.89.

Evaluation of 5g on cell proliferation and HT29 cell cycle

HT29 cells were obtained from the American Type Culture Collection (ATCC) and grown in DMEM and 10% fetal calf serum. Cell cultures were maintained at 37 °C under an atmosphere of humidified air and 5% CO_2.[32] The cells were subsequently dissociated from culture flask surfaces with a solution of trypsin (2.5 g L^{-1}) and resuspended in DMEM to give a concentration of 1×10^5 cells mL^{-1}. The cells were added to 96-well plates (9000 cells per plate) and allowed to attach for 24 h, after which time various concentrations of **5g** were added. After incubation for 96 h, cell proliferation was estimated by the MTT assay using a microplate reader ($\lambda=$ 540 nM).[33]

For cell cycle studies, HT29 cells were plated and grown for 48 h to reach 50–60% confluency.[34] Cells were treated with various concentrations of **5g**, and after 48 h the cells were treated with trypsin, washed with PBS, and fixed overnight in 70% EtOH at 4 °C. At the time of harvest, the cultures were 70–90% confluent. After removing the EtOH by centrifugation, the cells were resuspended in buffer containing Tris (10 mM, pH 7.5), sucrose (125 mM), $MgCl_2$ (2.5 mM), NP40 (0.185%), RNase A (0.02 mg mL^{-1}), sodium citrate (0.05%), and propidium iodide (25 μg mL^{-1}). After incubation on ice for 1 h, the cells were subjected to DNA content analysis using a FACScan cytometer (Becton Dickinson).

Effect of 5d and 5g on respiration in HT29 cells

The effect of **5d**, **5g**, and 5-fluorouracil on oxygen consumption in human HT29 colon cancer cells was measured by polarography[35] of 1×10^5 cells in air-saturated DMEM at 37 °C.

Acknowledgements

The authors thank the Canadian Institutes of Health Research for grants to J.R.D. and R.K.S., and the Natural Sciences and Engineering Research Council for a grant to B.B.. The Belgian Fonds voor Wetenschappelijk Onderzoek (Vlaanderen) provided funds for the Molt 4/C8, CEM, and L1210 assays; in this regard the excellent technical assistance by Mrs. L. van Berckelaer is greatly appreciated. The National Cancer Institute (USA) is thanked for providing the data presented in Table 5. The Szeged Foundation of Cancer Research (Hungary) kindly provided funds to J.M..

Keywords: 4-piperidones · cytotoxicity · multi-drug resistance · phosphono derivatives · unsaturated ketones

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Received: July 15, 2009
Revised: August 20, 2009
Published online on October 2, 2009

