European Journal of Medicinal Chemistry 51 (2012) 193–199

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Dimeric 3,5-bis(benzylidene)-4-piperidones: A novel cluster of tumour-selective cytotoxins possessing multidrug-resistant properties

Swagatika Das [a], Umashankar Das [a,*], Hiroshi Sakagami [b], Naoki Umemura [b], Shoko Iwamoto [c], Tomohiko Matsuta [c], Masami Kawase [d], Joseph Molnár [e], Julianna Serly [e], Dennis K.J. Gorecki [a], Jonathan R. Dimmock [a,*]

[a] Drug Design and Discovery Research Group, College of Pharmacy and Nutrition, University of Saskatchewan, 110 Science Place, Saskatoon, Saskatchewan S7N 5C9, Canada
[b] Division of Pharmacology, Meikai University School of Dentistry, Saitama 350-0238, Japan
[c] MPL Meikai University School of Dentistry, Saitama 350-0238, Japan
[d] Faculty of Pharmaceutical Services, Matsuyama University, 4-2 Bunkyo-cho, Matsuyama, Ehime 790-8578, Japan
[e] Institute of Medical Microbiology and Immunology, University of Szeged, Szeged 6720, Hungary

ARTICLE INFO

Article history:
Received 2 December 2011
Received in revised form
17 February 2012
Accepted 20 February 2012
Available online 27 February 2012

Keywords:
Cytotoxicity
Tumour-selectivity
MDR-revertants
Structure–activity relationships
α,β-Unsaturated ketones

ABSTRACT

A series of bis[3,5-bis(benzylidene)-4-oxo-1-piperidinyl]amides **1** display potent cytotoxic properties towards a wide range of tumours. A number of the CC_{50} and IC_{50} values are in the range of 10^{-8} M. Specifically, these compounds have the following important properties. First, greater toxicity was demonstrated towards certain tumours than various non-malignant cells. Second, various compounds in series **1** are toxic to a number of human colon cancer and leukaemic cells. Third, these compounds reverse P-gp mediated multidrug resistance. Various prototypic molecules such as **1a**,**b** and **1i** were identified as lead molecules for further studies. A representative lead molecule **1b** induces apoptosis via internucleosomal DNA fragmentation and PARP cleavage in HSC-2 and HL-60 cells while flow cytometry revealed that this compound blocked the G2/M and S-phases in the cell cycle of human colon cancer HCT-116 cells.

1. Introduction

The principal aim of this laboratory is the synthesis of antineoplastic conjugated unsaturated ketones which are designed as thiol alkylators [1,2]. One of the reasons for the interest in these compounds is that α,β-unsaturated ketones often react readily with thiols but have little or no affinity for hydroxyl and amino groups [3,4]; these latter functionalities are found in nucleic acids. Hence the genotoxic effects associated with a number of alkylating agents used in cancer chemotherapy [5] may be avoided. Furthermore, one or more thiol groups are found in a variety of cellular constituents such as thioredoxin, glutathione and cysteine. Thus thiol alkylators have the potential to be multitargeted ligands and the perceived importance of such pleiotropy has been documented recently [1,6–8].

Initially a number of cytotoxins were designed possessing a single 1-oxo-2-propenyl group (–CH=CH–CO–) [9,10]. However a number of studies revealed that an initial lowering of the concentrations of cellular thiols followed by a second chemical attack was more detrimental to various neoplasms than normal cells [11,12]. Such observations led to the decision to prepare series **1**. These compounds have the potential to display dual functions, namely to cause greater chemosensitivity to neoplasms than normal cells and also to cause toxicity *per se*. For example, an initial reaction of **1a**–**j** with a chemoprotectant thiol at one olefinic carbon atom could sensitize cells to subsequent alkylation at the remaining olefinic carbon atoms. Alternatively, reactions at two or three olefinic carbon atoms may reduce the concentration of chemoprotectant thiols significantly causing cancer cells to be particularly vulnerable to subsequent chemical insults. The linker group between the amidic carbonyl groups was absent (**1a**) or consisted of saturated alkyl chains (**1b**–**e**), unsaturated alkyl groups (**1f**–**h**) and an aryl ring (**1i**, **j**). In addition, the nature of the linker itself may contribute to potency and selectivity. Thus the hypothesis to be evaluated is that the design of series **1** leads to tumour-selective cytotoxins. A preliminary investigation revealed that **1a**–**j** inhibited the growth of human Molt4/C8 and CEM T-lymphocytes as well as murine L1210 lymphoid leukaemic cells while a representative molecule **1a** was cytotoxic to a number of cancer cell lines [13].

* Corresponding authors. Tel.: +1 306 966 6358; fax: +1 306 966 6377.
E-mail addresses: umashankar.das@usask.ca (U. Das), jr.dimmock@usask.ca (J.R. Dimmock).

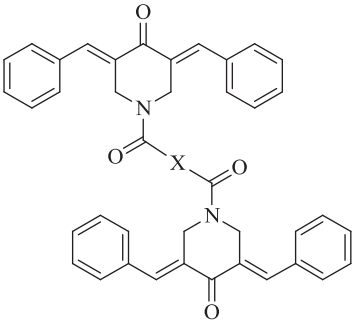
0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2012.02.042

The aims of the present investigations are fourfold. First, the question needs to be addressed whether the compounds in series **1** display greater toxicity to neoplasms than non-malignant cells. Second, in view of the interest in our laboratories of compounds which inhibit the growth of colon cancer and leukaemic cells [14,15], the evaluation of representative compounds in series **1** against these neoplasms was planned. Third, further probing regarding the mode of action of one of the representative potent cytotoxins was considered of importance. Finally, a major problem in cancer chemotherapy is the development of multidrug resistance (MDR) in malignant cells. Hence compounds which are MDR-revertants may be co-administered with an anticancer drug or those which possess both MDR-revertant and antineoplastic properties have immense clinical potential. Since a number of cytotoxic 1-acyl-3,5-bis(benzylidene)-4-piperidone hydrochlorides are potent revertants of MDR [16], an evaluation of **1a**–**j** for this property was considered. Fig. 1

2. Results

The synthesis of series **1** was accomplished by acid-catalyzed condensation of 4-piperidone hydrochloride with benzaldehyde which led to the formation of 3,5-bis(benzylidene)-4-piperidone which in turn reacted with a variety of acid chlorides to form **1a**–**j** [13]. In addition, acylation of 3,5-bis(benzylidene)-4-piperidone with phthaloyl chloride produced the corresponding bisamide which is a structural isomer of **1i** and **1j**. However, its lack of adequate solubility in different solvents precludes any discussion of its cytotoxic properties.

All of the compounds **1a**–**j** were evaluated against the following neoplasms namely human HSC-2, HSC-3 and HSC-4 squamous cell carcinomas and HL-60 promyelocytic leukaemic cells and non-malignant HGF gingival fibroblasts, HPC pulp cells and HPLF periodontal ligament fibroblasts. These data are presented in Table 1. In addition, **1a**–**c**, **f**, **g**, **i**, **j** were examined against a number of colon cancer and leukaemic cell lines and the results are summarized in Table 2. A representative lead cytotoxin **1b** was examined for its



1

a: X= –
b: X= -CH_2-
c: X= -$(CH_2)_2$-
d: X= -$(CH_2)_3$-
e: X= -$(CH_2)_6$-
f: X= –C≡C–
g: X= –CH=CH–
h: X= –(CH=CH)$_2$–
i: X= 
j: X= 

Fig. 1. The structures of the compounds in series **1**.

Table 1
Evaluation of **1a**–**j** against various human neoplasms and human normal cells.

Compound	Human tumour cell lines, CC_{50} (μM)[a]									Human normal cells, CC_{50} (μM)[a]				
	HSC-2	SI[b]	HSC-3	SI[b]	HSC-4	SI[b]	HL-60	SI[b]	Average CC_{50}	Average SI[b]	HGF	HPC	HPLF	Average CC_{50}	PSE[c]
1a	0.085 ± 0.013	38.4	0.15 ± 0.023	21.7	0.20 ± 0.011	16.3	0.11 ± 0.009	29.6	0.136	26.5	3.8 ± 0.52	0.68 ± 0.073	5.3 ± 0.50	3.26	195
1b	0.050 ± 0.001	18.4	0.046 ± 0.026	20.0	0.084 ± 0.005	11.0	0.046 ± 0.009	20.0	0.057	17.4	1.1 ± 0.01	0.36 ± 0.10	1.3 ± 0.33	0.92	305
1c	0.099 ± 0.008	92.6	0.23 ± 0.027	39.9	0.24 ± 0.013	38.2	0.41 ± 0.20	22.4	0.245	48.3	10 ± 0.89	1.5 ± 0.075	16 ± 1.0	9.17	197
1d	0.071 ± 0.023	34.8	0.17 ± 0.004	14.5	0.19 ± 0.038	13.0	0.39 ± 0.10	6.33	0.205	17.2	2.9 ± 0.22	0.61 ± 0.058	3.9 ± 0.01	2.47	83.9
1e	0.17 ± 0.008	61.2	0.21 ± 0.025	49.5	0.62 ± 0.12	16.8	1.3 ± 0.12	8.00	0.575	33.9	13 ± 1.6	1.1 ± 0.041	17 ± 2.5	10.4	59.0
1f	0.19 ± 0.004	41.4	0.49 ± 0.008	16.1	0.93 ± 0.012	8.46	0.87 ± 0.16	9.05	0.620	18.8	8.5 ± 0.78	2.1 ± 0.29	13 ± 0.94	7.87	30.3
1g	0.078 ± 0.010	191	0.62 ± 0.052	24.0	0.58 ± 0.054	25.7	1.2 ± 0.05	12.4	0.620	63.3	17 ± 2.8	5.7 ± 0.35	22 ± 2.7	14.9	102
1h	2.6 ± 0.17	13.5	8.5 ± 0.34	4.12	6.4 ± 0.34	5.47	7.4 ± 0.77	4.73	6.23	7.00	39 ± 0.54	19 ± 0.10	47 ± 0.51	35.0	1.12
1i	0.026 ± 0.001	189	0.076 ± 0.001	64.7	0.082 ± 0.019	60.0	0.11 ± 0.017	44.7	0.074	89.6	5.7 ± 1.7	0.56 ± 0.049	8.5 ± 0.89	4.92	1211
1j	3.2 ± 0.49	11.3	6.3 ± 0.27	5.76	8.0 ± 0.92	4.54	6.6 ± 1.0	5.50	6.03	6.78	42 ± 0.81	21 ± 0.05	46 ± 3.0	36.3	1.12
Average	0.657	69.2	1.68	26.0	1.73	20.0	1.84	16.3	1.48	32.9	14.3	5.26	18.0	12.5	22.2
Melphalan	8.7 ± 4.2	24.1	25 ± 7.7	8.40	32 ± 8.8	6.56	1.4 ± 1.2	150	16.8	47.3	161 ± 27	269 ± 153	199 ± 60	210	2.82

[a] The CC_{50} values are the concentrations of compound required to kill 50% of the cells.
[b] The letters SI refer to the selectivity index which is obtained by dividing the average CC_{50} value of the compound towards the normal cells by the CC_{50} figure of the compound towards a specific cancer cell line.
[c] The letters PSE refer to the potency-selectivity expression. These figures are calculated by multiplying the reciprocal of the average CC_{50} value of the compound towards the four tumour cell lines by the average selectivity index of these neoplasms.

Table 2
Evaluation of **1a**–**c**, **f**, **g**, **i**, **j** against various human tumour cell lines.

Compound	All all cell lines		Colon cancer cells, IC_{50} (μM)								Leukaemic cells, IC_{50} (μM)				
	[a]GI_{50} (μM)	SI	COLO205	HCC2998	HCT116	HCT15	HT29	KM12	SW620	Ave	HL-60 (TB)	K-562	RPMI8226	SR	Ave
1a	0.31	95.5	0.36	0.19	0.03	0.30	0.17	0.04	0.07	0.17	0.20	0.20	0.25	0.02	0.17
1b	0.24	1191	0.16	0.17	0.03	0.17	0.05	0.13	0.05	0.11	0.29	0.05	0.13	0.03	0.13
1c	0.69	3316	0.39	0.23	0.14	0.38	0.24	0.30	0.26	0.28	0.27	0.22	0.06	0.05	0.15
1f	0.48	38.9	0.25	0.41	0.12	0.39	0.20	0.28	0.20	0.26	0.25	0.30	0.08	0.11	0.19
1g	1.12	11950	0.56	0.22	0.13	0.83	0.25	0.21	0.22	0.35	0.45	0.32	0.05	0.23	0.26
1i	0.36	135	0.21	0.20	0.03	0.35	0.17	0.21	0.30	0.21	0.21	0.21	0.06	0.03	0.13
1j	2.69	661	2.40	–	0.20	1.70	0.55	0.35	0.36	0.93	2.04	1.55	0.73	0.15	1.12
Average	0.84	484	0.62	0.24	0.10	0.59	0.23	0.22	0.21	0.33	0.53	0.41	0.19	0.09	0.31
5-Fluorouracil	>56.2	>2239	4.17	13.5	8.51	9.71	10.2	8.32	380	62.1	>2512	2.40	–	1.12	>839
Melphalan	26.9	1118	66.1	41.7	30.2	36.3	46.8	43.7	38.9	43.4	2.04	43.7	66.1	1.86	28.4

[a] The term GI_{50} rather than IC_{50} is used since in the case of **1g**, **1j** and 5-fluorouracil, 50% inhibition of the growth of a cell line was not achieved at the maximum concentration in a few instances.

ability to cause internucleosomal DNA fragmentation, caspase-3 activation and PARP cleavage in HSC-2 and HL-60 cells. In addition, **1b** was examined for its effect on the cell cycle of human HCT-116 colon cancer cells. These results are presented in Figs. 2–5. The unsaturated ketones **1a**–**j** were evaluated for their potential to reverse P-glycoprotein (P-gp) multidrug resistance in murine L-5178Y cells which were transfected with the human mdr1 gene. These results are summarized in Table 3.

3. Discussion

All of the compounds in series **1** were evaluated against HSC-2, HSC-3, HSC-4 and HL-60 neoplastic cells and these results are portrayed in Table 1.These data reveal that in general **1a**–**j** are a cluster of highly potent cytotoxins. In fact 75% of the CC_{50} values (the concentrations to kill 50% of the cells) are submicromolar and 28% are in the double digit nanomolar range. The average CC_{50} figures, which are listed in Table 1, are lowest towards HSC-2 cells while the other three cell lines are approximately one-third less sensitive. Comparisons were made between the potencies of **1a**–**j** and melphalan which is an alkylating agent used in cancer chemotherapy. All of the compounds have lower CC_{50} values than melphalan towards HSC-2, HSC-3 and HSC-4 cells while **1a**, **b**, **i** are more potent than melphalan in the HL-60 bioassay, i.e., in 83% of the comparisons made. The average CC_{50} figures of series **1** against HSC-2, HSC-3 and HSC-4 cells are 13, 15 and 19 times lower than the CC_{50} values of melphalan in these three bioassays. In many cases, the greater toxicity than melphalan is huge, e.g., **1i** is 335, 329 and 390 times more potent than this drug in the HSC-2, HSC-3 and HSC-4 screens, respectively.

Structure–activity relationships were noted based on the average CC_{50} values for **1a**–**j** against HSC-2, HSC-3, HSC-4 and HL-60 cells which are presented in Table 1. There is no spacer group X in **1a** which is a potent cytotoxin. The addition of one methylene group produced **1b** having 2.4 times the potency of **1a**. However, increasing the number of methylene groups in **1c**–**e** gave rise to analogues with higher average CC_{50} values than both **1a** and **1b**. A comparison of the CC_{50} figures of **1c** with the unsaturated analogues **1f** and **1g** revealed a 2.5-fold reduction in potencies. The addition of a second unsaturated linkage to **1g** forming **1h** results in a 10-fold drop in potency. A comparison between the CC_{50} values of the structural isomers **1i** and **1j** revealed an 82-fold difference in the average CC_{50} data. One may summarize the SAR by stating that in general the distance between the two amidic carbonyl groups should be small; increasing this span causes a reduction in potencies.

In addition to potency, an issue of major importance is whether compounds exert greater toxic effects towards neoplasms than normal cells. In order to address this issue, the compounds in series **1** were also evaluated against HGF, HPC and HPLF non-malignant cells and the results are presented in Table 1. Under clinical conditions, neoplasms are surrounded by different types of normal cells. Hence the selectivity index (SI) figures were computed by dividing the average CC_{50} value of the compound against the three normal cells by the CC_{50} figure generated towards a particular tumour cell line. These SI values are displayed in Table 1.

All of the compounds in series **1** have SI values greater than 1. In many cases the selectivity is enormous, e.g., the average SI figures for series **1** is 32.9 and for **1c**, **g**, **i** they are 92.6, 191 and 189, respectively, against HSC-2 cells. The enones **1c**, **g**, **i** display the

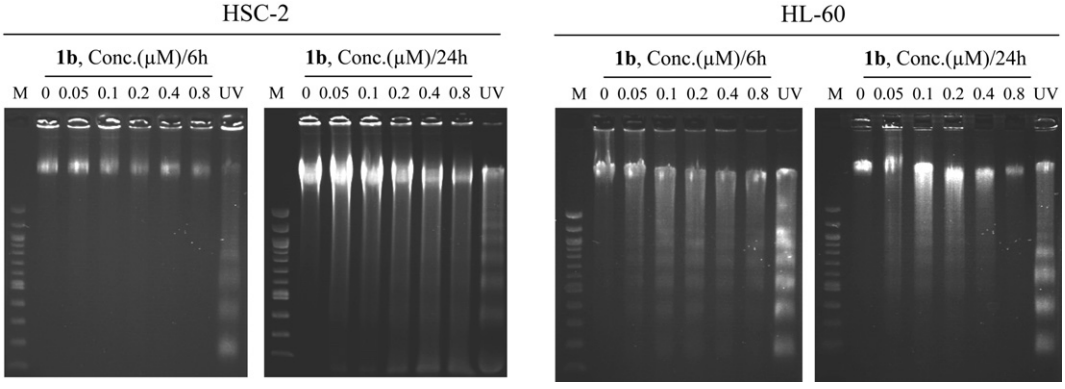


Fig. 2. The effect of **1b** on internucleosomal DNA fragmentation using HSC-2 and HL-60 cells. M is a 100 bp DNA ladder marker, the figures refer to the concentrations of **1b** in μM and cells irradiated with UV are the positive control.

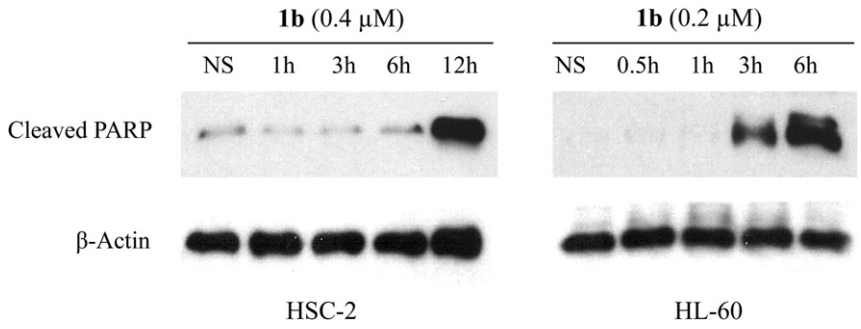


Fig. 3. A western blot analysis of the effect of **1b** on the cleavage of PARP in HSC-2 and HL-60 cells. NS means no stimulation.

highest average SI values of 48.3, 63.3 and 89.6, respectively, which are similar to or greater than the figure of 47.3 for melphalan. This observation indicates that **1c**,**g**,**i** are important lead molecules and it is of interest that there is a two carbon spacer between the amidic carbonyl groups in these three compounds. Other correlations between the average SI figures and the nature of the spacer group were noted. First, the degree of saturation and unsaturation in the two carbon spacer in **1c** (48.3), **1f** (18.8) and **1g** (63.3) have substantial effects on the extent of selectivity. Second, while the presence of one olefinic group in the spacer led to **1g** (63.3) with excellent selectivity, the placement of a second ethylenic moiety produced **1h** (7.00) which has a greatly reduced SI value. Third, there is a 13.2-fold difference between the average SI figures of the structural isomers **1i** (89.6) and **1j** (6.78). Thus one may conclude that series **1** is a novel group of tumour-selective cytotoxins.

Since both potency and selectivity are important features in identifying lead molecules, a potency-selectivity expression (PSE) was calculated for each compound. These values are the product of the reciprocal of the average CC_{50} values against four tumour cell lines and the average SI figures; these results are presented in Table 1. All of the compounds have higher PSE values than melphalan except **1h**, **j**. The compounds with the highest PSE figures are **1b** and **1i** which are 108 and 429 times greater than the value for melphalan. One may note that a short methylene chain is preferred (**1a**–**c** > **1d**, **e**) and the nature of the two carbon spacer affects the PSE figures considerably (**1c** > **1g** > **1f**).Furthermore, **1i** has a much more favourable PSE value than **1j**. From these data, **1a**–**c**, **g**, **i**, which have PSE figures in excess of 100, serve as prototypic molecules for further development.

The next phase of the investigation of the potential of these compounds as candidate anticancer drugs was the examination of representative members of series **1** for their ability to inhibit the growth of neoplasms of different somatic origins. Of particular interest was the question of whether cytotoxicity towards colon cancers and leukaemic cells would be observed. In order to address this issue, **1a**–**c**, **f**, **g**, **i**, **j** were examined against 59 ± 3 human tumour cell lines including not only colon cancers and leukaemia but also melanoma and non-small cell lung, central nervous system, ovarian, renal, prostate and breast cancers [17]. These data are presented in Table 2.

The biodata in Table 2 reveal that the compounds are potent cytotoxins to a wide variety of neoplasms. This conclusion may be drawn from the average GI_{50} values obtained for all cell lines which are submicromolar (**1a**–**c**, **f**, **i**) or in the low micromolar range (**1g**, **j**). In particular, the average GI_{50} figures for **1a**, **b**, **i** are impressive and serve as lead molecules. All of these compounds are substantially more potent than melphalan e.g., the average GI_{50} value of **1b** is 112 times lower than the figure of the established drug.

A review of the mean graphs [18], revealed that these compounds are particularly toxic towards colon cancers and leukaemic cells. In regard to colon cancers, all of the GI_{50} values are submicromolar except **1j** towards COLO 205 and HCT15 cells, i.e., in 95% of the GI_{50} values. In addition, double digit nanomolar values were obtained against HT-116 (**1a**, **b**, **i**), HT29 (**1b**), KM12 (**1a**) and SW620 (**1a**, **b**) cells. These observations establish **1a**, **b**, **i** as important lead molecules for further development of chemical agents against colon cancers. The anticancer drug 5-fluorouracil is used in treating colonic neoplasms and the huge differential in

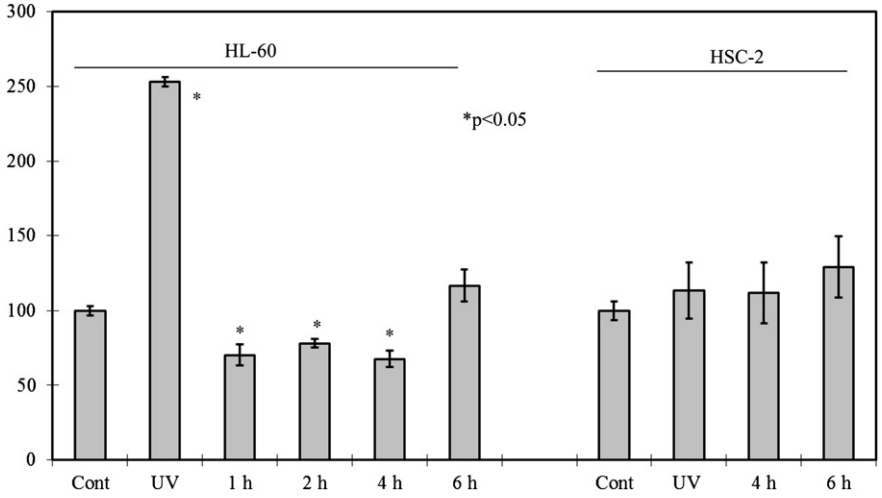


Fig. 4. Evaluation of **1b** as an activator of caspase-3. The bars are the mean determinations and the standard deviations ($n = 3$) are also indicated.

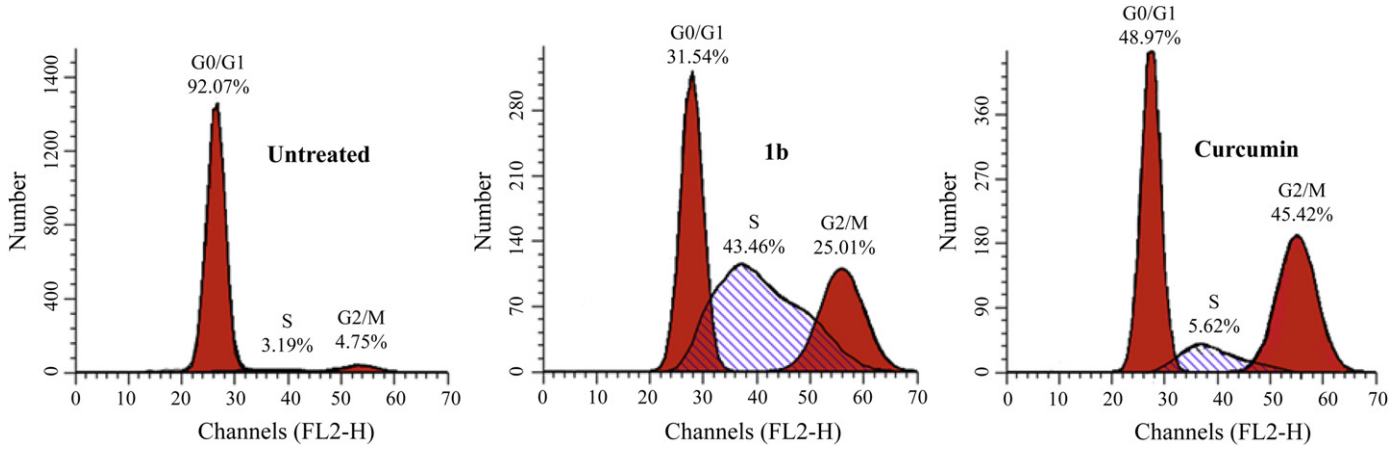


Fig. 5. Effect of **1b** (1 μM) and curcumin (16 μM) on the cell cycle of HCT-116 cells as determined by flow cytometry. Curcumin was taken as the positive control.

potencies between this drug and representative compounds in series **1** is most noteworthy. For example, the IC_{50} value of **1b** towards HCT 116 cells is 284 times lower than the figure for 5-fluorouracil.

The IC_{50} values of **1a**–**c**, **f**, **g**, **i**, **j** towards four leukaemic cell lines are presented in Table 2. These figures are submicromolar except for **1j** towards HL-60 (TB) and K-562 cells, i.e., in 93% of the determinations. Double digit nanomolar IC_{50} figures were noted in approximately one-third of the evaluations in the K-562 (**1b**), RPMI8226 (**1c**, **f**, **g**, **i**) and SR (**1a**–**c**, **i**) assays. The average IC_{50} data of these seven compounds towards the leukaemic cell lines are substantially greater than melphalan which is used in treating certain leukaemias. The biodata summarized in Table 2 reveals clearly that representative unsaturated ketones in series **1** inhibit the growth of a wide variety of human tumour cell lines especially colonic cancer and leukaemic cells.

An attempt was made to determine the mechanism of action of the most potent compound **1b**. Previously **1a** caused apoptosis in various neoplastic cells while very little necrosis was observed [13]. The structural similarity of **1a** and **1b** suggested that induction of apoptosis was an important way whereby **1b** also exerted its lethal effects. A characteristic feature of apoptosis is the formation of internucleosomal DNA fragmentation [19]. The unsaturated ketone **1b** caused DNA cleavage in both HSC-2 and HL-60 cells as indicated in Fig. 2. After 6 h, DNA fragmentation was noted in HL-60 cells but not in HSC-2 neoplasms while **1b** produced this effect in both cell lines after 24 h. This result indicates that apoptosis is one way in which **1b** displays its bioactivity and that this compound exerts its efficacy more rapidly in HL-60 than HSC-2 cells.

When DNA breaks, poly(ADP-ribose)polymerase 1 (PARP1) is activated almost immediately [20] since its principal action is the sensing and repair of DNA single-stranded breaks [21]. Hence compounds which cleave PARP1 may have an important role to play in cancer chemotherapy [22–24]. The results portrayed in Fig. 3 reveal that **1b** caused extensive PARP1 cleavage in HL-60 cells after 6 h and in HSC-2 cells after 12 h. These results correlate well with the DNA fragmentation experiments in which lower concentrations and shorter times of incubation with **1b** were noted using HL-60 cells.

Caspases, especially caspase-3, play important roles in apoptotic cell death induced by anticancer agents [25–27]. Compound **1b** was incubated with HSC-2 and HL-60 cells up to 6 h and the effect on caspase-3 activation is portrayed in Fig. 4. The data generated reveal that only a statistically insignificant activation of caspase-3 took place after 6 h; hence the cytocidal effect of **1b** on neoplastic cell lines is not principally due to caspase-3 activation. One may note that while in some cases PARP1 cleavage can be initiated by caspase-3 activation during apoptosis [28–30], the effect of **1b** on PARP1 cleavage is brought about by mechanisms other than caspase activation. In summary, the mode of action of **1b** includes apoptosis mediated by DNA fragmentation and PARP1 cleavage.

The effect of **1b** and curcumin on the cell cycle progression of HCT-116 cells was investigated by flow cytometry (Fig. 5). For cell cycle analysis, the cells were treated with 1 μM concentration of **1b** and 16 μM concentration of curcumin for 48 h. Curcumin, a potent cytotoxic agent [31] that possesses structural similarity with **1b** and known to display dose dependant G2/M phase arrest in HCT-116 cells [32] was considered as a positive control for the cell cycle analysis. **1b** induced an increase in the cell population in S-phase and G2/M phase by 40 and 20%, respectively as compared to the untreated cells. Upon treatment with **1b**, the cell population in G0/G1 phase reduced significantly to 32% as compared to 92% in untreated cells. These results suggest that **1b** displays growth inhibition of HCT-116 cells by inducing a G2/M and S block in the cell cycle. Our study also shows that curcumin induces G2/M phase arrest in HCT-116 cells which is in agreement with the previous report [32].

Finally, a major problem in cancer chemotherapy is the ability of various neoplasms to display multidrug-resistant (MDR) properties which reduces the efficacy of antineoplastic agents by different

Table 3
Fluorescence activity ratio values of **1a**–**j** in murine L-5178Y cells transfected with the human mdr1 gene.

Compound	FAR value[a]	
	4 μM	40 μM
1a	19.3	41.1
1b	35.3	53.3
1c	35.0	60.0
1d	50.2	73.4
1e	49.2	55.7
1f	42.0	45.7
1g	37.2	52.9
1h	48.6	44.3
1i	46.5	51.5
1j	38.9	61.3
Average	40.2	53.9

[a] The letters FAR refer to the fluorescence activity ratio. A reference drug verapamil has a FAR value of 8.71 when a concentration of 5.2 μM was employed.

means including accelerating the exodus of the anticancer drug by the membrane transporter P-glycoprotein (P-gp) [33]. Thus novel compounds which overcome MDR may find use as dual agents (possessing anticancer and MDR-revertant properties) or as compounds to be co-administered with established anticancer drugs. Previous work from this laboratory revealed that in addition to significant cytotoxic properties, various 1-acyl-3,5-bis(benzylidene)-4-piperidones reversed P-gp associated MDR [16]. Thus assessment of the compounds in series **1** for this property was considered appropriate.

The assay utilized employs murine L-5187Y lymphoma cells which have been transfected with the human mdr1 gene [34]. The concentrations of the dye rhodamine 123 in treated and untreated transfected and parental cells were obtained and the fluorescence measured. The results are expressed as fluorescence activity ratio (FAR) figures and a FAR value of greater than 1 indicates that reversal of MDR has occurred. The biodata will be discussed principally in terms of the FAR values obtained using a concentration of 4 μM since this figure is closer to the CC_{50} and IC_{50} cytotoxicity data. All of the compounds have MDR-revertant properties and are substantially greater than a reference MDR-modulator verapamil. With the exception of **1a**, the FAR values are in the range of 35–50. The compounds with the highest FAR values are **1d**, **e**, **h**, **i** which are clearly prototypic molecules for analogue development.

Molecular features which are found in a number of MDR-modulators are the presence of hydrogen bond acceptor atoms such as nitrogen and oxygen as well as hydrophobic aryl rings [35]. The compounds in series **1** have four oxygen atoms which permit hydrogen bonding to take place. The amidic groups *per se* may be important contributors to MDR-reversal since excision of the linker [−C(=O)−X−C(=O)−] leads to 3,5-bis(benzylidene)-4-piperidone which is virtually bereft of Pgp-mediated MDR-reversal [16]. Series **1** has four (**1a**–**h**) or five (**1i**, **j**) aryl rings and in order to determine if the hydrophobicity of the molecules was correlated with the extent of MDR-reversal, linear, semilogarithmic and logarithmic plots between the logP figures of **1a**–**j** and their FAR values were made. The semilogarithmic plot revealed a trend towards a positive correlation ($p = 0.12$) suggesting that MDR-revertant properties increase as the hydrophobicity of the molecules is raised.

The data in Table 3 indicate that, in general, by increasing the concentration of the compounds tenfold, the FAR values rise only by one-third approximately. This observation suggests that while MDR-reversal is enhanced as the concentration of the compounds is raised, biochemical processes which counteract MDR-revertant properties are activated. For example, the binding area of P-gp where these compounds interact may have become saturated. This phenomenon has been noted previously [16,36].

4. Conclusions

The bis[3,5-bis(benzylidene)-4-oxo-1-piperidinyl]amides **1a**–**j** are a cluster of potent cytotoxins which demonstrates greater toxicity to neoplasms than normal cells. This study revealed a number of promising lead molecules **1b**, **i** (in terms of potency and PCE figures) and **1c**, **g**, **i** (the most favourable SI values) for further development. Various compounds in series **1** inhibit the growth of a number of human colon cancer and leukaemic cells. Especially, three prototypic molecules were identified namely **1a**, **b**, **i.** An investigation of the mode of action of the most potent compound **1b** reveals that **1b** displays cytotoxicity by inducing apoptosis which acts through DNA damage and PARP cleavage in HSC-2 and HL-60 cells. **1b** also trigger G_2/M and S-phase arrest in cell cycle. Finally, the discovery of the MDR-revertant properties of series **1** indicates their being dual agents in the warfare against cancer insofar as they couple significant toxic effects towards neoplastic cells with inhibition of their extrusion from tumours by P-gp.

5. Experimental section

5.1. Synthesis of *1a–j*

The synthesis of **1a**–**j** has been described previously [13]. The details of synthesis and their characterization data are given in the supplementary section. In brief, 3,5-bis(benzylidene)-4-piperidone (0.02 mol) which was prepared using a literature procedure [37] was condensed with the appropriate dicarboxylic acid chloride (0.0 l mol) in the presence of triethylamine (0.01 mol) in dichloroethane at room temperature. After the reaction was complete, aqueous potassium carbonate solution was added to the mixture and the product was collected, dried and recrystallized from a suitable solvent.

5.2. Bioevaluations

5.2.1. Cytotoxic assays

The evaluation of **1a**–**j** against HSC-2, HSC-3, HSC-4, HL-60, HGF, HPC and HPLF cells was carried out using a literature method [38] except that the time of incubation was 48 h. The CC_{50} values were obtained from a dose–response curve.

The data in Table 2 was generated using a literature procedure [17]. The concentrations of each compound were 10^{-4} to 10^{-8} M (**1a**–**c**, **f**, **g**, **i**, **j**), $10^{-2.6}$ to $10^{-6.6}$M (5-fluorouracil) and $10^{-3.6}$ to $10^{-7.6}$M (melphalan). The compounds were evaluated against 50 (5-fluorouracil), 56 (**1j**), 57 (melphalan), 59 (**1b**) and 60 (**1a**, **c**, **f**, **g**, **i**) cell lines. The number of cell lines for which the maximum concentration of compound did not inhibit growth by 50% was 2/60 (**1g**), 5/56 (**1j**) and 6/50 (5-fluorouracil).

5.2.2. Internucleosomal DNA fragmentation

The evaluation of **1b** as an inducer of internucleosomal DNA fragmentation in HSC-2 and HL-60 cells was undertaken by a literature procedure [39]. In brief, cells were incubated with 0.05, 0.1, 0.2, 0.4 and 0.8 μM of **1b** for 6 h. As a positive control, cells were exposed to UV light (6J/m^2/min) for 1 min and incubated for 3 h. Subsequently the cells were harvested for evidence of DNA fragmentation.

5.2.3. Caspase-3 activation

The enone **1b** was examined for its ability to activate caspase-3 in both HSC-2 and HL-60 cells using a reported method [40]. In brief, cells were lysed and mixed with a substrate, namely DEVD-*p*-nitroanilide. After incubation at 37 °C for 2 h, the absorbance at 405 nm of the liberated chromophore *p*-nitroanilide was measured using a microplate reader.

5.2.4. PARP cleavage

HSC-2 and HL-60 cells were treated with 0.4 μM and 0.2 μM of **1b**, respectively. The cleavage of PARP was measured using a Promega PARP (Asp 214) human specific antibody [41]. In brief, cells were washed in ice-cold PBS, scraped, collected in lysis buffer (20 mM HEPES pH7.4, 1% Triton-X 100, 150 mM NaCl, 1.5 mM $MgCl_2$, 12.5 mM β-glycerophosphate, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF plus 1x protease inhibitor).The cell lysates were applied to an 8% SDS-PAGE and the protein bands in the gels were transferred onto polyvinylidene difluoride membranes. The membranes blocked with 5% (w/v) nonfat dry milk were incubated with primary antibody [anti-cleaved PARP1 (Cell Signalling Technology, Beverly, MA), anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA)], and then with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies.

5.2.5. Cell cycle analysis

Cell Cycle analysis was carried out by modifying a literature procedure [42]. HCT-116 cells were maintained in McCoys'5A Modified media (ATCC) supplemented with 10% Foetal Bovine Serum (Fischer Scientific) and 1% antibiotic-antimycotic solution (Sigma). Cells (1×10^6 cells) were seeded in 75 cm^2 flasks and incubated for 24 h in a humidified incubator at 37 °C with 5% CO_2. Each flask containing cells was treated with the samples and untreated control for 48 h in a humidified incubator at 37 °C with 5% CO_2. Floating cells were collected and adherent cells were harvested with trypsin-EDTA (0.2%) and pooled. The samples were washed with cold PBS, fixed in 70% ethanol and left on ice for 2 h. Further samples were washed after 2 h with PBS and resuspended in PBS containing RNase (300 μg/ml). The samples were incubated for 20 min in the dark with propidium iodide (20 μg/ml) and RNase (300 μg/ml). The samples were analyzed by a FACScalibur (BD) flow cytotmeter. The data were analyzed using Modfit LT free trial version 3.3 available from Variety software house. Cells were gated to include G0/G1, S-phase, and G2/M populations.

5.2.6. Multidrug resistance reversal assay

The ability of **1a**–**j** to reverse MDR in murine L-5178Y cells transfected with the mdr1 gene followed a published procedure [34]. In brief, solutions of the compounds in dimethylsulfoxide were added to MDR and parenteral cells at room temperature. After 10 min, rhodamine 123 was added and the cells were incubated at 37 °C for 20 min. In these experiments, the FAR value of 1% dimethylsulfoxide is 0.82.

5.3. Determination of log P values

The logP values of **1a**–**j** which were determined using a software programme [43] are as follows, namely **1a** : 5.40, **1b** : 5.34, **1c** : 5.29, **1d** : 5.71, **1e** : 6.96, **1f** : 5.67, **1g** : 5.57, **1h** : 6.08, **1i**: 7.08 and **1j** : 7.08. Linear, semilogarithmic and logarithmic plots were made using a commercial statistical package [44].

Acknowledgements

The authors thank the following agencies for financial support of this study, namely the Canadian Institutes of Health Research and the Saskatchewan Health Research Foundation for a grant to J.R. Dimmock and U. Das, the Ministry of Education, Science, Sports and Culture for a grant-in-aid to H. Sakagami, and the Szeged Foundation of Cancer Research to J. Molnár. The measurement of the FAR values was undertaken by Imre Ocsovszki which is gratefully acknowledged. The National Cancer Institute (USA) kindly provided the data presented in Table 2 and S.G. Dimmock, Department of Finance, Nanyang Technological University, Singapore, is thanked for discussions on the PSE expression. D. Michel kindly assisted with the flow cytometry experiment. Ms. B. McCullough and Ms. C. Sutton typed various drafts of this report who are gratefully acknowledged.

Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.02.042.

References

[1] U. Das, R.K. Sharma, J.R. Dimmock, Curr. Med. Chem. 16 (2009) 2001–2020.
[2] H.N. Pati, U. Das, R.K. Sharma, J.R. Dimmock, Mini-Rev. Med. Chem. 7 (2007) 131–139.
[3] B. Mutus, J.D. Wagner, C.J. Talpas, J.R. Dimmock, O.A. Phillips, R.S. Reid, Anal. Biochem. 177 (1989) 237–243.
[4] G. Baluja, A.M. Municio, S. Vega, Chem. Ind. (1964) 2053–2054.
[5] A.B. Okey, P.A. Harper, in: H. Kalant, D.M. Grant, J. Mitchell (Eds.), Principles of Medical Pharmacology, seventh ed. Elsevier, Toronto, 2007, p. 902.
[6] M. Galanski, B.K. Keppler, Anti-Cancer Agents Med. Chem. 7 (2007) 55–73.
[7] S. Frantz, Nature 437 (2005) 942–943.
[8] L.M. Espinoza-Fonseca, Bioorg. Med. Chem. 14 (2006) 896–897.
[9] J.R. Dimmock, P.L. Carter, P.D. Ralph, J. Chem. Soc. Sect. B (1968) 698–703.
[10] J.R. Dimmock, M.L.C. Wong, Can. J. Pharm. Sci. 11 (1976) 35–53.
[11] G. Chen, D.J. Waxman, Biochem. Pharmacol. 47 (1994) 1079–1087.
[12] K. Tsutsui, C. Komuro, K. Ono, T. Nishidia, Y. Shibamoto, M. Takahashi, M. Abe, Int. J. Radiat. Oncol. Biol. Phys. 12 (1986) 1183–1186.
[13] S. Das, U. Das, A. Varela-Ramírez, C. Lema, R.J. Aguilera, J. Balzarini, E. De Clercq, S.G. Dimmock, D.K.J. Gorecki, J.R. Dimmock, ChemMedChem. 6 (2011) 1892–1899.
[14] U. Das, H.N. Pati, H. Sakagami, K. Hashimoto, M. Kawase, J. Balzarini, E. De Clercq, J.R. Dimmock, J. Med. Chem. 54 (2011) 3445–3449.
[15] S. Das, U. Das, P. Selvakumar, R.K. Sharma, J. Balzarini, E. De Clercq, J. Molnár, J. Serby, Z. Baráth, G. Schatte, B. Bandy, D.K.J. Gorecki, J.R. Dimmock, ChemMedChem. 4 (2009) 1831–1840.
[16] U. Das, J. Molnár, Z. Baráth, Z. Bata, J.R. Dimmock, Bioorg. Med. Chem. Lett. 18 (2008) 3484–3487.
[17] M.R. Boyd, K.D. Paull, Drug Dev. Res. 34 (1995) 91–109.
[18] M.R. Grever, S.A. Schepartz, B.A. Chabner, Semin. Oncol. 19 (1992) 622–638.
[19] H. Gunji, S. Kharbanda, D. Kufe, Cancer Res. 51 (1991) 741–743.
[20] J.F. Haince, D. McDonald, A. Rodrigue, U. Dery, J.Y. Masson, M.J. Hendzel, G.G. Poirier, J. Biol. Chem. 283 (2008) 1197–1208.
[21] M. Malanga, F.R. Althaus, Biochem. Cell Biol. 83 (2005) 354–364.
[22] Y. Drew, H. Calvert, N.Y. Ann, Acad. Sci. 1138 (2008) 136–145.
[23] A. Peralta-Leal, M.I. Rodriguez, F.J. Oliver, Clin. Transl. Oncol. 10 (2008) 318–323.
[24] E.T. Sakamoto-Hojo, A.S. Balajee, Anticancer Agents Med. Chem. 8 (2008) 402–416.
[25] D.W. Nicholson, N.A. Thornberry, Trends Biochem. Sci. 22 (1997) 299–306.
[26] R.U. Janicke, M.L. Sprengart, M.R. Wati, A.G. Porter, J. Biol. Chem. 273 (1998) 9357–9360.
[27] S.A. Susin, E. Douglas, L. Ravagnan, et al., J. Exp. Med. 192 (2000) 571–579.
[28] D.A. Le, Y. Wu, Z. Huang, K. Matsushita, N. Plesnila, J.C. Augustinack, B.T. Hyman, J. Juan, K. Kuida, R.A. Flavell, M.A. Moskowitz, Proc. Natl. Acad. Sci. USA 99 (2002) 15188–15193.
[29] D. D'Amours, S. Desnoyers, I. D'Silva, G.G. Poirier, Biochem. J. 342 (1999) 249–268.
[30] K.K. Wang, Trends Neurosci. 23 (2000) 20–26.
[31] B.B. Aggarwal, A. Kumar, A.C. Bharati, Anticancer Res. 23 (2003) 363–398.
[32] L. Moragoda, R. Jaszewski, AP.N. Majumdar, Anticancer Res. 21 (2001) 873–878.
[33] B.C. Baguley, Mol. Biotechnol. 46 (2010) 308–316.
[34] M. Kawase, H. Sakagami, N. Motohashi, H. Hauer, S.S. Chatterjee, G. Spengler, A.V. Vigyikanne, A. Molnár, J. Molnár, In Vivo 19 (2005) 705–712.
[35] X. Shen, G. Chen, G. Zhu, W.-F. Fong, Bioorg. Med. Chem. 14 (2006) 7138–7145.
[36] J.R. Dimmock, U. Das, H.I. Gul, M. Kawase, H. Sakagami, Z. Baráth, I. Ocsovsky, J. Molnár, Bioorg. Med. Chem. Lett. 15 (2005) 1633–1636.
[37] J.R. Dimmock, M.P. Padmanilayam, R.N. Puthucode, A.J. Nazarali, N.L. Motaganahalli, G.A. Zello, J.W. Quail, E.O. Oloo, H.B. Kraatz, J.A. Prisciak, T.M. Allen, C.L. Santos, J. Balzarini, E. De Clercq, E.K. Manavathu, J. Med. Chem. 44 (2001) 586–593.
[38] N. Motohashi, W. Wakabayashi, T. Kurihara, H. Fukushima, T. Yamada, M. Kawase, Y. Sohara, S. Tani, Y. Shirataki, H. Sakagami, K. Satoh, H. Nakashima, A. Molnár, G. Spengler, N. Gyémént, K. Ugocsai, J. Molnár, Phytother. Res. 18 (2004) 212–223.
[39] F. Yanagisawa-Shiota, H. Sakagami, N. Kuribayashi, M. Iida, T. Sakagami, M. Takeda, Anticancer Res. 15 (1995) 259–266.
[40] R. Takeuchu, H. Hoshijima, N. Onuki, H. Nagasaka, S.A. Chowdhury, M. Kawase, H. Sakagami, Anticancer Res. 25 (2005) 4037–4041.
[41] Distributed by Cell Signaling Technology, Inc.
[42] A. Lakshmikuttyamma, E. Pastural, N. Takahashi, K. Sawada, D.P. Sheridan, J.F. DeCoteau, C.R. Geyer, Oncogene 27 (2008) 3831–3844.
[43] Verchor 12.0, ChemBioOffice, 2010.www.cambridgesoft.com.
[44] Statistical Package for Social Sciences, SPSS for Windows, Release 17.0, SPSS Inc, Chicago, 2008.