Bioorganic & Medicinal Chemistry Letters 20 (2010) 6464–6468

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Sequential cytotoxicity: A theory examined using a series of 3,5-bis(benzylidene)-1-diethylphosphono-4-oxopiperidines and related phosphonic acids

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ARTICLE INFO

Article history:
Received 25 August 2010
Revised 9 September 2010
Accepted 10 September 2010
Available online 17 September 2010

Keywords:
Sequential cytotoxicity
Selective toxicity
Neoplasms
QSAR
Caspase activation

ABSTRACT

The concept of sequential cytotoxicity, which states that successive chemical attacks on cellular constituents can be more deleterious to neoplasms than normal cells, was evaluated using a series of 3,5-bis(benzylidene)-1-diethylphosphono-4-oxopiperidines **1** and related phosphonic acids **2**, which were screened against a panel of malignant and normal cell lines. The compounds proved to be not only potent cytotoxins (71% of the CC_{50} figures are submicromolar) but to display greater cytotoxicity to the neoplastic cells. QSAR revealed that both cytotoxic potencies and selective toxicity were increased by a rise in the electron-withdrawing properties and a decrease in the hydrophobicity of the aryl substituents. Utilisation of the PL10 concept and evaluation of druglike properties revealed **1c** as the lead tumour-specific cytotoxin. This molecule activated caspase-3 in HL-60 cells but not in the HSC-2 cell line. While **1c** caused internucleosomal DNA fragmentation in HL-60 cells, it did not elicit this effect in either HSC-2 and HSC-4 cells. Clearly **1c** exerts its cytotoxic potencies by different mechanisms and such pleiotropy is likely the principal reason for the remarkable display of preferential toxicity towards malignant cells of the compounds in series **1** and **2**.

The theory of sequential cytotoxicity was originally defined as the successive release of two or more cytotoxic compounds thereby causing greater toxicity to neoplasms than normal cells.[1] In the present study, this hypothesis is simplified to refer to compounds which are designed to cause successive chemical attacks on cellular constituents. This concept is based on the observation that on occasions an initial chemosensitisation followed by a subsequent chemical attack is more deleterious to tumours than non-malignant tissues.[2,3]

In order to examine this hypothesis further, a series of 3,5-bis (benzylidene)-1-diethylphosphono-4-oxopiperidines **1** and related phosphonic acids **2** were designed for the following reasons. First, conjugated enones have a marked affinity for thiols in contrast to amino and hydroxyl groups.[4,5] Hence interactions with the amino and hydroxyl groups of nucleic acids, which may lead to genotoxic effects,[6] should be absent in these compounds. Second, series **1** and **2** contain the dienone motif which permits sequential interactions at the olefinic carbon atoms with cellular thiols. Third, previous studies revealed that various cytotoxic 3,5-bis(benzylidene)-4-piperidones **3** demonstrated greater toxicity to certain neoplasms than various non-malignant cell lines.[7] N-acylation of series **3** with acryloyl chloride led to series **4** which was accompanied by increases in both potency and selective toxicity to malignant cells.[7] However the hydrophobicity of **4** increases compared to **3** which may lead to pharmacokinetic and formulation problems. Hence the acryloyl group of **4** was replaced by the hydrophilic phosphono substituent leading to series **2**.[8] Since there are two acidic protons in **2**, which may impede penetration of cell membranes, the corresponding diethyl esters **1** were synthesised.[8] The structures of the compounds in series **1**–**4** are presented in Figure 1.

A preliminary investigation revealed that most of the compounds in series **1** and **2** display potent cytotoxicity towards several malignant and transformed cell lines.[8] The aims of the present investigation are (1) to determine whether the theory of sequential cytotoxicity is validated from the biodata generated in series **1** and **2**, (2) to identify a lead molecule that possesses potent cytotoxicity and selective toxicity to neoplasms compared to normal cells for future in vivo pharmacokinetic and pharmacodynamic evaluations, (3) to investigate the mode of action of the lead molecule in order to gain some insight into the possible ways bioactivity is mediated and (4) to develop suitable QSAR models which will enable the design of potent, tumour-selective cytotoxins for future development.

All of the compounds in series **1** and **2** were evaluated against four human neoplastic cell lines namely HL-60 promyelocytic

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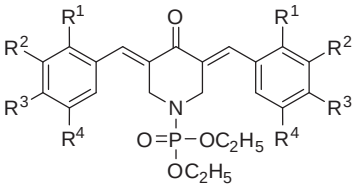
doi:10.1016/j.bmcl.2010.09.051



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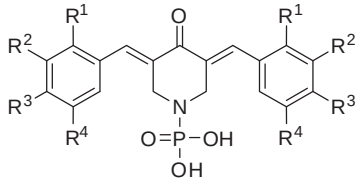

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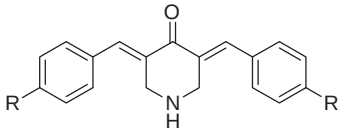

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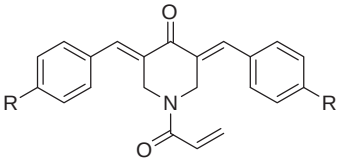

4

1 and **2** **a**: $R^1=R^2=R^3=R^4=H$; **b**: $R^1=R^2=R^4=H$; $R^3=CH_3$; **c**: $R^1=R^2=R^4=H$; $R^3=OCH_3$; **d**: $R^1=R^4=H$; $R^2=R^3=OCH_3$; **e**: $R^1=H$, $R^2=R^3=R^4=OCH_3$; **f**: $R^1=R^2=R^4=H$, $R^3=NO_2$; **g**: $R^1=R^2=R^4=H$; $R^3=Cl$; **h**: $R^1=NO_2$, $R^2=R^3=R^4=H$; **i**: $R^1=R^3=R^4=H$, $R_2=NO_2$

3 and **4** R=H, Cl, NO_2, CH_3

Figure 1. Structures of the compounds in series **1**–**4**.

leukemic cells as well as HSC-2, HSC-3 and HSC-4 oral squamous cell carcinomas. In addition, these dienones were screened against three human normal cells viz HGF gingival fibroblasts, HPC pulp cells and HPLF periodontal ligament fibroblasts.[9] These data are presented in Table 1.

The biodata displayed in Table 1 indicate that the compounds in series **1** and **2** are potent cytotoxins. In fact, 71% of the CC_{50} values of **1a–i** and **2a–i** are submicromolar while **1i**, **2e**, **h**, **i** have double digit nanomolar CC_{50} figures towards HL-60 cells. The potencies of virtually all of the compounds towards the three squamous cells carcinomas compare very favourably with melphalan, for example, **2h** possesses 51, 78 and 320 times the potency of this drug towards HSC-2, HSC-3 and HSC-4 cell lines, respectively.

In order to address the issue of whether the compounds display greater toxicity to neoplasms than normal cells, selectivity index (SI) figures were calculated for **1a–i** and **2a–i**. Under clinical conditions, tumours are surrounded by a number of different types of cells and in order to simulate in vivo situations, the average CC_{50}

Table 1
Evaluation of **1a**–**i**, **2a**–**i** and melphalan against human tumour and normal cell lines

	Human tumour cell lines[a] (CC_{50}, μM)								Human normal cell lines[a] (CC_{50}, μM)			
	HL-60	SI[b]	HSC-2	SI[b]	HSC-3	SI[b]	HSC-4	SI[b]	HGF	HPC	HPLF	Ave[c]
1a	0.61 ± 0.05	15	2.4 ± 0.13	3.8	2.5 ± 0.11	3.7	1.1 ± 0.11	8.4	11 ± 0.55	6.5 ± 0.91	10 ± 0.2	9.2
1b	3.3 ± 0.54	6.7	4.9 ± 0.05	4.5	4.1 ± 0.19	5.4	4.6 ± 0.09	4.8	28 ± 7.50	18 ± 1.9	21 ± 0.7	22
1c	1.9 ± 0.18	25	0.92 ± 0.08	51	0.66 ± 0.07	71	1.3 ± 0.32	36	53 ± 3.20	41 ± 0.5	46 ± 3.2	47
1d	0.75 ± 0.12	8.4	1.7 ± 0.23	3.7	1.4 ± 0.38	4.5	0.50 ± 0.01	13	7.4 ± 2.60	6.2 ± 1.9	5.3 ± 0.5	6.3
1e	0.29 ± 0.03	6.2	0.33 ± 0.02	5.5	0.43 ± 0.09	4.2	0.16 ± 0.03	11	2.0 ± 0.07	1.3 ± 0.38	2.2 ± 0.11	1.8
1f	0.14 ± 0.02	17	0.33 ± 0.02	7.3	0.38 ± 0.09	6.3	0.72 ± 0.22	3.3	3.1 ± 0.75	1.9 ± 0.35	2.2 ± 0.35	2.4
1g	0.60 ± 0.04	6.7	1.5 ± 0.25	2.7	2.0 ± 0.05	2.0	0.64 ± 0.19	6.3	4.3 ± 0.16	2.8 ± 0.4	4.8 ± 0.22	4.0
1h	0.38 ± 0.04	37	1.6 ± 0.24	8.8	1.2 ± 0.01	12	1.8 ± 0.26	7.8	18 ± 1.40	8.3 ± 1.9	15 ± 4.9	14
1i	0.05 ± 0.01	54	0.76 ± 0.07	3.3	0.74 ± 0.18	3.4	0.26 ± 0.04	9.6	2.2 ± 0.05	1.5 ± 0.18	3.9 ± 4.9	2.5
2a	0.46 ± 0.11	12	0.77 ± 0.54	7.4	0.98 ± 0.03	5.8	0.57 ± 0.14	10	6.9 ± 0.81	3.2 ± 0.50	7.0 ± 1.8	5.7
2b	1.1 ± 0.23	4.6	0.76 ± 0.09	6.7	0.95 ± 0.05	5.4	0.96 ± 0.05	5.3	5.9 ± 1.70	3.3 ± 0.92	6.1 ± 0.25	5.1
2c	5.6 ± 1.30	12	1.7 ± 0.18	40	2.2 ± 0.15	31	3.9 ± 1.20	17	107 ± 7	54 ± 6.5	44 ± 6.30	68
2d	0.26 ± 0.01	8.1	0.40 ± 0.11	5.3	0.59 ± 0.11	3.6	0.42 ± 0.04	5.0	2.6 ± 0.45	1.3 ± 0.56	2.5 ± 0.05	2.1
2e	0.06 ± 0.01	12	0.13 ± 0.01	6.2	0.27 ± 0.01	3.0	0.12 ± 0.03	6.7	0.88 ± 0.04	0.59 ± 0.36	1.0 ± 0.05	0.8
2f	0.14 ± 0.03	21	0.18 ± 0.02	17	0.23 ± 0.02	13	0.13 ± 0.00	23	4.1 ± 0.61	1.6 ± 0.11	3.4 ± 0.85	3.0
2g	0.46 ± 0.02	9.8	0.42 ± 0.18	11	0.41 ± 0.10	11	0.72 ± 0.08	6.3	5.9 ± 0.50	2.6 ± 0.05	5.1 ± 0.23	4.5
2h	0.07 ± 0.02	30	0.17 ± 0.05	13	0.32 ± 0.18	6.9	0.10 ± 0.01	22	1.4 ± 0.57	2.1 ± 0.06	3.0 ± 0.09	2.2
2i	0.09 ± 0.01	30	0.20 ± 0.04	13	0.18 ± 0.05	14	0.14 ± 0.03	19	2.3 ± 1.20	2.2 ± 0.11	3.3 ± 0.83	2.6
Melphalan	1.4 ± 1.2	150	8.7 ± 4.20	24	25 ± 7.70	8.4	32 ± 8.80	6.6	161 ± 27	269 ± 153	199 ± 60	210

[a] The CC_{50} values are the concentrations of the compounds required to kill 50% of the cells.
[b] The letters SI refer to the selectivity index. These numbers are the quotients of the average CC_{50} value of the compound towards normal cells and the CC_{50} figure generated for each neoplastic cell line.
[c] These figures are the average CC_{50} values of the compounds towards HGF, HPC and HPLF cell lines.

value of the compounds towards the three normal cell lines was divided by the CC_{50} figure generated using a specific neoplasm. These data are presented in Table 1. All of the compounds have SI figures of greater than 1 which indicates that the theory of sequential cytotoxicity is worthy of further investigation.

An effort was made to identify lead molecules using the PL10 concept.[10] This approach seeks to identify *P*romising *L*ead compounds which have CC_{50} values of 10 μM or less and the SI figure is 10 or more. No less than 43% of the results obtained indicate the PL10 criteria has been achieved namely **1a**, **c**, **f**, **h**, **i**, **2a**, **c**, **e**, **f**, **h**, **i** (HL-60 screen), **1c**, **2c**, **f–i** (HSC-2 assay), **1c**, **h**, **2c**, **f**, **g**, **i** (HSC-3 test) and **1c–e**, **2a**, **c**, **f**, **h**, **i** (HSC-4 screen). Of particular note are **1c**, **2c**, **f**, **i** which have PL10 status when assayed against all four tumour cell lines. The average SI values for these four compounds (average CC_{50} figures in μM against the four neoplastic cell lines are presented in parentheses) are 46(1.20), 25(3.35), 19(0.17) and 19(0.15), respectively, indicating their being important lead molecules, especially **1c**.

The next phase of the study was aimed at discerning any physicochemical properties of the aryl substituents which control the magnitude of the CC_{50} figures towards the HL-60, HSC-2, HSC-3 and HSC-4 malignant cell lines. Accordingly linear and semilogarithmic plots were made between the σ/σ^* and π constants of the aryl substituents of **1a–i**.[11] In addition, linear, semilogarithmic and logarithmic plots were constructed between the CC_{50} values and the molecular refractivity (MR) figures of the aryl groups. The process was then repeated for the compounds in series **2**. The following correlations (p <0.05) and trends towards significance (p <0.1) were noted. The CC_{50} figures of **1a–i** correlate negatively with the σ constants in the HL-60 screen and positively with the π values in the HSC-3 assay. Positive trends towards significance were noted between the π values in the HL-60 and HSC-2 screens and a negative trend with the MR constants in the HSC-4 test. In the case of series **2**, negative correlations were noted between the σ constants in all four bioassays. No other correlations or trends to significance were noted (p >0.1). Thus future development should include the placement of substituents in the aryl rings of both series **1** and **2** which are more electron-withdrawing while in series **1**, these groups should also be more hydrophilic.

In a further attempt to discern correlations between various physicochemical parameters and the cytotoxic potencies of the compounds in series **1** and **2**, multilinear regression analyses were undertaken.[12] Excellent correlations were noted using the biodata generated in the HSC-2 and HSC-3 assays as indicated in Eqs. 2 and 3, respectively, and the studied descriptors. However modest correlations were obtained for HL-60 and HSC-4 cell lines. Efforts to improve the statistical quality of the Eqs. 1 and 4 by changing or omitting the studied descriptors did not give any good results.

$$\begin{aligned}\mathrm{Log}_{10}(CC_{50\ \mathrm{HL-60}}) &= -1.93(\pm0.86) - 1.12(\pm0.31)\sum\sigma - 0.94(\pm0.76)\sum\pi + 0.66(\pm0.34)\\ &\quad \log P + 0.73(\pm0.49)I_{\mathrm{Et}}\\ &\quad n = 18, r = 0.795, r_{\mathrm{adj}} = 0.518, s = 0.425, F = 5.57, p = 0.008 \qquad (1)\\ \mathrm{Log}_{10}(CC_{50\ \mathrm{HSC-2}}) &= 1.05(\pm0.86) - 0.97(\pm0.49)\sum\sigma + 1.65(\pm0.88)\sum\pi - 0.62(\pm0.34)\\ &\quad \log P - 1.55(\pm0.58)I_{\mathrm{Et}} - 0.09(\pm0.03)\sum\mathrm{MR} + 0.011(\pm0.009)\mathrm{TPSA}\\ &\quad n = 18, r = 0.906, r_{\mathrm{adj}} = 0.722, s = 0.236, F = 8.37, p = 0.001 \qquad (2)\\ \mathrm{Log}_{10}(CC_{50\ \mathrm{HSC-3}}) &= 1.51(\pm0.84) - 0.31(\pm0.17)\sum\sigma + 0.90(\pm0.51)\sum\pi - 0.46(\pm0.27)\\ &\quad \log P - 0.95(\pm0.37)I_{\mathrm{Et}} - 0.05(\pm0.02)\sum\mathrm{MR}\\ &\quad n = 18, r = 0.863, r_{\mathrm{adj}} = 0.637, s = 0.234, F = 6.94, p = 0.003 \qquad (3)\\ \mathrm{Log}_{10}(CC_{50\ \mathrm{HSC-4}}) &= 0.23(\pm0.22) - 0.60(\pm0.19)\sum\sigma - 0.05(\pm0.01)\sum\mathrm{MR} + 0.32(\pm0.17)I_{\mathrm{Et}}\\ &\quad n = 18, r = 0.764, r_{\mathrm{adj}} = 0.494, s = 0.35, F = 6.53, p = 0.005 \qquad (4)\end{aligned}$$

In these equations, n is the number of determinations, r is the correlation coefficient, r_{adj} is the adjusted π value, s is the standard deviation of the regression equation, F is related to the F—statistic analysis (Fisher test) and I_{Et} is an indicator variable which is assigned a value of 1 or 0 depending upon the ethyl group being present or absent, respectively.

From these statistical analyses guidelines for expansion of this study have been achieved. Thus by inserting the appropriate physicochemical constants into Eqs. 1–4, a prediction of analogs with increased cytotoxic potencies can be made. The fact that quite different equations were generated depending on the cell line under consideration reinforces the conclusion that the compounds in series **1** and **2** have pleiotropic properties which give rise to the remarkable SI values observed.

In order to evaluate whether certain of the physicochemical properties of the aryl substituents in series **1** and **2** influence the SI values, the following statistical analysis was undertaken. Linear and semilogarithmic plots were made between the σ, π and MR constants and the SI figures. In the HL-60 screen a positive trend to significance with the σ constants and a negative correlation with the π values of **1a–i** were noted. For series **2**, a positive correlation between the σ and SI figures was observed in the HL-60 and HSC-4 screens and a negative correlation with the π constants in these screens. No other correlations were noted (p >0.1). Thus in considering analog development, greater cytotoxic potencies and selective toxicity to neoplasms are predicted to occur by placing substituents with increased electron-withdrawing and hydrophilic substituents in the aryl rings of series **1** and **2** such as forming the 3-nitro-4-acetoxy and 2-nitro-4-carboxy analogs.

A study was conducted to determine if any of the lead compounds identified by using the PL10 concept have favourable druglike properties. Hence **1c, 2c, f, i** were examined in terms of certain physicochemical parameters which govern intestinal absorption[13] as well as their predicted capacity for inducing various toxic symptoms. The results are portrayed in Table 2 which reveals that the most favourable ratings appear with **1c** and **2c**. However, **1c** is considered the primary lead molecule due its higher SI value and greater potency towards neoplastic cell lines than **2c** (Table 1).

A number of cytotoxic agents exert their bioactivity, at least in part, by inducing apoptosis.[14] There are two apoptotic mechanisms, namely one which operates via the intrinsic pathway in the mitochondria and also the extrinsic pathway involving death receptors on the cell surface.[15] Both mechanisms of action utilise the proteolytic enzymes known as caspases. In particular caspase-3 is an effector caspase. The lead compound **1c** was evaluated for its ability to activate caspase-3 using concentrations which are ×1, ×2 and ×4 of the CC_{50} values for the cells. The result is portrayed in Figure 2.[16] Clearly **1c** causes a concentration-dependent activation of caspase-3 in HL-60 cells not in HSC-2 cells. Therefore the cytotoxicity of this compound to HSC-2 cells is regulated by an alternate mechanism. This finding is further substantiated by the observation that **1c** induced internucleosomal DNA fragmentation[17] as revealed in Figure 3. In contrast, using the same concentrations of **1c** as indicated in Figure 3 did not lead to any DNA fragmentation in HSC-2 and HSC-4 cells. The fact that **1c** causes cell death by different mechanisms depending on the specific cell line is probably the reason for the remarkable SI values of this compound and others in series **1** and **2**.

In conclusion, this study was designed to explore the theory of sequential cytotoxicity and is validated when the 3,5-bis(benzylidene)-1-diethylphosphono-4-oxopiperidines **1** and related phosphonic acids **2** are considered. In addition the submicromolar CC_{50} values of many of the compounds are noteworthy. Series **1** and **2** are novel clusters of molecules which should be developed in three ways, namely (1) analog development based on the QSAR, (2) further explorations as to the mechanisms of action of these compounds, and (3) in vivo pharmacokinetic and pharmacodynamic evaluations of the lead compound **1c**.

Table 2
Evaluation of **1c**, **2c**, **f**, **i** for druglike properties

Compound	Physicochemical properties[a]						Toxicity[b]				
	Log *P*	MW	HBA	HBD	RB	TPSA	M	T	I	R	Ratings[c]
1c	4.09	471.49	7	0	9	74.32	–	–	–	–	10
2c	2.78	415.38	7	2	5	96.30	–	–	–	–	10
2f	2.59	445.32	11	2	5	169.48	+	+	–	–	6
2i	2.54	445.32	11	2	5	169.48	–	–	–	–	8
Druglike compound	<5	<500	<10	<5	<10	<140	–	–	–	–	10

[a] The physicochemical properties considered are the logarithm of the partition coefficient (log *P*), molecular weight (MW), the number of hydrogen bond acceptors (HBA), hydrogen bond donors (HBD) and rotatable bonds (RB) as well as total polar surface area (TPSA). These figures were obtained using the molinspiration Web explorer.[21]
[b] The possible induction of certain toxic effects are mutagenicity (M), tumour-induction (T), irritant effects (I) and impairment of reproduction (R). The assessment for toxicity used the Osiris Property Explorer tool.[22]
[c] One point was allocated for each positive result, that is, a favourable physical property or the absence of one of the toxic symptoms. There is a maximum of 10 points. The toxicity effects are classified as toxic (+), less toxic (±) and no toxicity (−).

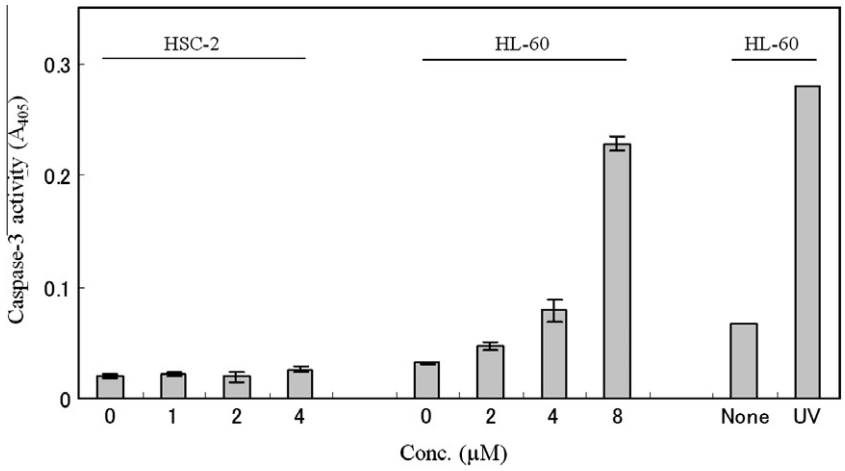

Figure 2. Evaluation of **1c** to activate caspase-3. Cells were incubated for 6 h and then harvested for caspase-3 activity. The bars are the mean determinations accompanied by standard deviations ($n = 3$).

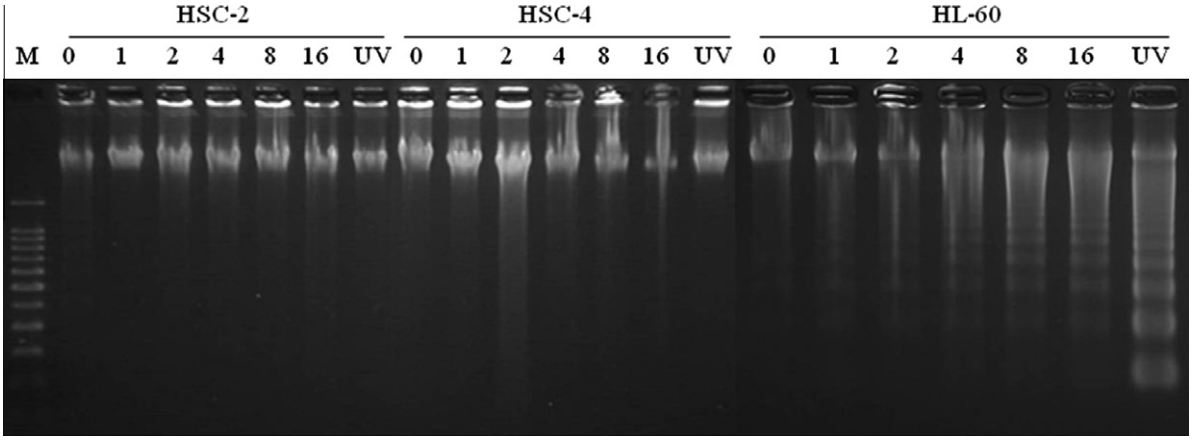

Figure 3. The effect of **1c** on internucleosomal DNA fragmentation in HSC-2, HSC-4 and HL-60 cells. The cells were incubated with different concentrations (0, 1, 2, 4, 8, 16 μM) of **1c** for 6 h and then harvested for DNA fragmentation. As a positive control, cells were exposed to UV irradiation for 1 min. followed by 3.5 h incubation. M is a 100 bp DNA ladder marker.

Acknowledgements

The authors thank the Canadian Institutes of Health Research for awarding an operating grant to J.R.D., the Ministry of Education, Science, Sports and Culture of Japan for a grant-in-aid to H.S. and Gwen Korte who typed various drafts of the manuscript.

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