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6-Benzylidene-2-[4-(pyridin-3-ylcarboxy)benzylidene]cyclohexanones: A novel cluster of tumour-selective cytotoxins

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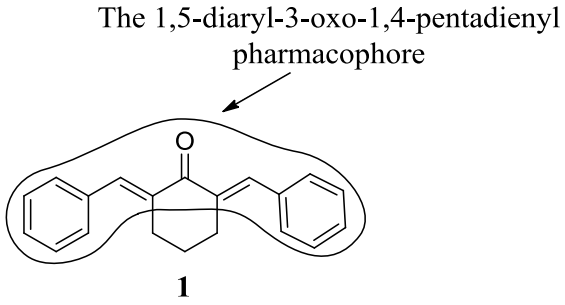
ABSTRACT

Novel cytotoxins **3-5** containing the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore are disclosed. The compounds in series **3** and **5** have the potential to liberate niacin which may reduce some of the side effects of antineoplastic compounds. **3a-c** emerged as the most potent cytotoxic compounds with IC_{50} values in the low micromolar range against human Molt4/C8 and CEM CD_4^+ T-lymphocytes as well as murine L1210 leukemia cells. QSAR studies revealed that cytotoxic potencies were negatively correlated with the magnitude of the Hammett sigma values of the aryl substituents. The compounds **3a-e** displayed tumour-selective toxicity against human HL-60, HSC-2, HSC-3 and HSC-4 neoplasms as compared to human HGF, HPC and HPLF nonmalignant cells. A representative potent compound **3a** caused PARP1 cleavage and G_0/G_1 cell cycle arrest in HSC-2 cells. These compounds are well tolerated in mice at doses up to and including 300 mg/kg of the compounds and no mortalities were noted after 4 hrs. The stability studies undertaken did not reveal that a representative compound **3a** underwent hydrolysis to the related phenol **2a**. Some guidelines for further analog development of the novel esters **3** were made.

Keywords; conjugated unsaturated ketones / cytotoxins / tumour-selective toxicity / structure-activity relationships / QSAR / niacin

One of the long-term objectives of this laboratory is the creation of molecules which are cytotoxic to neoplastic cells and yet have the capacity to liberate a compound which may diminish the pathological side effects which often occur in cancer chemotherapy. This goal is planned in various stages and the present report describes the concept and the initial studies.

The design of the candidate cytotoxins was based on the following observations. First, the choice was made for a conjugated unsaturated keto group being the cytotoxic pharmacophore, since various studies revealed that conjugated unsaturated ketones have a preferential or exclusive affinity for thiols, in contrast to hydroxyl and amino groups which are present in nucleic acids.[1-3] Hence, the genotoxic properties of a number of contemporary anticancer drugs which lead to tumour formation[4,5] should be avoided. Second, in the case of the 1,5-diaryl-3-oxo-1,4-pentadienes, sequential interaction with thiols can take place. In other words, a reaction occurs initially at the most electron-deficient olefinic carbon atom and subsequently at the remaining olefinic group. Several studies revealed that an initial lowering of cellular thiol concentrations followed by a second chemical insult is more detrimental to neoplasms than normal cells.[6,7] In the present study, the 1,5-diaryl-3-oxo-1,4-pentadienyl group was mounted on a cyclohexyl ring since a variety of 2,6-bis(benzylidene)cyclohexanones such as **1** (Figure 1) have demonstrated significant cytotoxic potencies.[8,9]

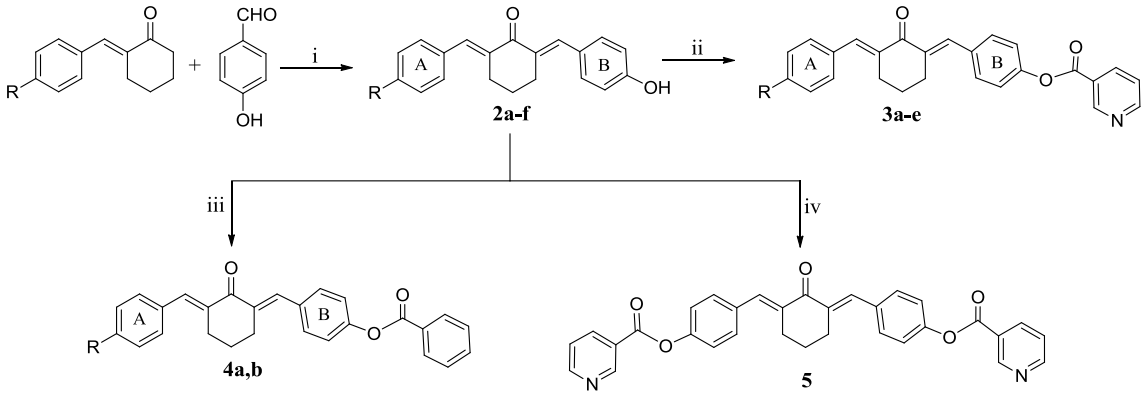


1

Figure 1. Compound **1** carrying a cytotoxic 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore.

One of the effects of cancer chemotherapy is a deficiency of niacin (pyridine-3-carboxylic acid, nicotinic acid, vitamin B_3)[10] which can lead to a number of adverse clinical conditions such as pellagra and dermatitis.[11] Conversely, niacin supplementation decreases the incidence of nonlymphocytic leukemia in rats treated with a carcinogenic nitrosourea.[12]

The problem therefore is to create molecules which possess a cytotoxic pharmacophore and yet have the capacity to ameliorate or eliminate the problem of niacin deficiency. Such considerations led to the designing of series **3** and **5** with the consideration that niacin will be released upon hydrolysis *in situ* (Scheme 1). In the case of series **3**, the rate of niacin release will be controlled by the electronic nature of the substituents in ring A. On the other hand, if the compounds are stable any bioactivity observed will be due to the intact molecules *per se*. In this case, the effect on cytotoxic potencies of replacing the pyridyl group of **3a,b** by a phenyl ring to give **4a,b** was considered of interest. In addition, if the release of niacin leads to beneficial results such as increases in selective toxicity for neoplasms, then compound **5** may prove to be a useful antineoplastic agent. The objective of the present study is to examine whether the novel compounds in series **3**-**5** (1) display favourable cytotoxic properties, (2) demonstrate tumour-selective toxicity as compared to non-malignant cells, (3) release niacin upon hydrolysis, and (4) are tolerated in mice. In addition if potent cytotoxicity is demonstrated, some of the ways whereby bioactivity is mediated will be investigated.



Scheme 1. The syntheses of the compounds in series **2** – **5**. The aryl substituents are as follows: **a**: R = H; **b** : R = CH_3; **c**: R = OCH_3; **d** : R = Cl; **e**: R = NO_2; **f**: R = OH. The reagents used in these syntheses are i = HCl / CH_3COOH; ii = **2a-e** / nicotinoyl chloride; iii = **2a**,**b** / benzoyl chloride; iv = **2f** / nicotinoyl chloride.

The compounds in series **3** – **5** were synthesised by the route indicated in Scheme 1. Various 2-benzylidenecyclohexanones, which have been described previously[9,13,14], were condensed with 4-hydroxybenzaldehyde to produce **2a-d**, **f**[9] and **2e**.[15] Acylation of **2a-e** with nicotinoyl chloride led to the esters **3a-e**. Reaction of **2a**, **b** with benzoyl chloride produced the corresponding esters **4a**, **b,** while reaction of **2f** with nicotinoyl chloride led to the formation of **5**.

Table 1
Evaluation of **3a-e, 4a,b** and **5** for their cytotoxic activity against Molt 4/C8, CEM and L1210 cells

Compound	Molt 4/C8		CEM		L1210		Average IC_{50}(μM)	
	IC_{50}(μM)	$\Delta_{2a-e/3a-e}$[b]	IC_{50}(μM)	$\Delta_{2a-e/3a-e}$[b]	IC_{50}(μM)	$\Delta_{2a-e/3a-e}$[b]	**3-5**	**2**
2a[a]	5.17±1.83	---	5.82±0.83	---	7.32±0.87	---	---	---
2b[a]	5.22±1.72	---	4.31±1.16	---	7.41±0.86	---	---	---
2c[a]	4.18±1.92	---	5.68±1.65	---	6.85±1.18	---	---	---
2d[a]	4.90±0.67	---	2.69±0.39	---	6.44±1.14	---	---	---
2e[a]	9.41±0.18	---	7.27±0.93	---	9.95±0.35	---	---	---
2f[a]	5.52±2.12	---	6.84±0.14	---	13.5±2.3	---	---	---
3a	1.62±0.26	3.19	7.30±0.91	0.80[c]	8.71±0.5	0.84	5.88	6.10
3b	2.88±1.51	1.82[c]	9.49±1.14	0.45	12.8±10.3	0.58[c]	8.39	5.65
3c	1.19±0.78	3.51	9.85±2.33	0.58	7.63±0.83	0.90[c]	6.22	5.57
3d	7.89±1.70	0.62	17.4±9.3	0.16	29.6±1.3	0.22	18.3	4.68
3e	38.1±9.3	0.25	48.1±18.5	0.15	61.1±10.2	0.16	49.1	8.88
4a	10.4±0.6	---	40.4±6.3	---	46.7±2.0	---	32.5	---
4b	5.70±1.56	---	10.6±2.2	---	37.9±7.6	---	18.1	---
5	22.6±15.3	---	169±58	---	213±42	---	135	---
Niacin	>500	---	>500	---	>500	---	>500	---
Melphalan	3.24±0.56	---	2.47±0.21	---	2.13±0.02	---	2.61	---

[a]Data reported previously.[9,15] [b]The $\Delta_{2a-e/3a-e}$ values are the quotients of the IC_{50} figures towards a specific cell line of the members of series **2** and **3** which have the same aryl substituents in ring A. [c]The difference between the IC_{50} values is not statistically significant.

The compounds in series **3-5** were evaluated against human Molt 4/C8 and CEM CD4+ T-lymphocytes as well as murine L1210 leukemic cells. The data are presented in Table 1. A number of the compounds have IC_{50} values in the low micromolar range with 42% of these figures being less than 10 μM. The cell lines vary in their sensitivity to these dienones. Thus the average IC_{50} values of the compounds in series **3 -5** towards Molt4/C8, CEM and L1210 cells are 11.3, 39.0 and 52.2 μM, respectively. This cell line specificity may indicate compounds with greater toxicities towards neoplasms than nonmalignant cells, i.e., they may display tumour-specific toxicity and this possibility was examined, *vide infra*.

Melphalan is an alkylating agent used in cancer chemotherapy and a comparison was made between its potencies and the compounds in series **3** – **5** which were designed as thiol alkylators. The biodata generated in the Molt4/C8 bioassay revealed that the enones **3a** and **3c** are 2.00 and 2.72 times more potent than melphalan, while **3b** is equipotent with this established drug.

An examination was made of the biodata in terms of finding how cytotoxic potencies varied with alterations in the structure of the compounds. Previous disclosures revealed the IC_{50} values of **2a-d**[9] and **2e**[15] in the Molt4/C8, CEM and L1210 evaluations. These values are presented in the supplemental section. A comparison was made between the potencies of **2a-e** with the analog in series **3** which has the same aryl substituent in ring A. Thus the IC_{50} value of **2a** was compared with **3a** in the Molt4/C8 bioassay and so forth with standard deviations being taken into account. These comparisons are expressed as $\Delta_{\mathbf{2a-e}}$ / $\Delta_{\mathbf{3a-e}}$ figures in Table 1. The introduction of a 3-pyridylcarbonyl moiety to the compounds in series **2** leads to the analogs **3a-e** with retention of cytotoxic potencies. In the Molt4/C8 bioassay, **3a** and **3c** have increased potency compared to **2a** and **2c** in this screen while **3b** (Molt4/C8 assay), **3a** (CEM screen) and **3b**, **c** (L1210 bioassay) are equipotent with the analogs in series **2**. Clearly **3a-c** are more potent than **3d**, **e** and, as indicated in Table 1, **3a-c** have average IC_{50} values similar to **2a-c**.

The question arises whether the relative potencies of **3a-e**, **4a**,**b** and **5** are the same in each of the three bioassays. If this is the case, then the average IC_{50} values may be calculated and comparisons made between the potencies of the dienones. Alternatively if the relative potencies vary, then comments on structure-activity relationships need to be made for each cell line. In order to address this problem, Kendall's coefficient of concordance[16] was used. In this analysis, if the relative potencies are identical, then Kendall's coefficient of concordance will be 1; conversely if there is no correlation, the coefficient will be 0. In this investigation, the coefficient of concordance is 0.947 ($p = 0.006$). Hence the conclusion drawn is despite the variation in sensitivities of the cell lines to the compounds, the chemical and physicochemical properties of **3a-e**, **4a**,**b** and **5** influence potencies in a similar fashion.

Replacement of the 3-pyridyl group of **3a**, **b** by a phenyl ring led to **4a**, **b**. The aryl ester **4a** has lower potency than **3a** in the three screens, **3b** and **4b** are equipotent in the Molt4/C8 and CEM bioassays and **4b** has a higher IC_{50} value than **3b** in the L1210 screen. The logP values of **3a**, **b** and **4a, b** are 5.26, 5.71, 6.84 and 7.28, respectively. Hence the lower IC_{50} values of **3a**, **b** in general may have been due, at least partially, to their lower lipophilicity. Hence future structural modifications should retain the 3-pyridylcarbonyl group in aryl ring B in this cluster of compounds. The replacement of the small groups attached to ring A of **3a-e** by a 3-pyridylcarboxy group present in **5** lowered potencies. Hence among series **3 -5**, it is the niacin esters **3** which should be pursued further and in particular **3a-c**. Subsequent discussion and experimentation therefore is focussed principally on series **3**.

The aryl substituents present in series **3** are found in all four quadrants of a Craig diagram for *para* substituents.[17] Linear and semilogarithmic plots were made between the Hammett σ, Hansch π and molar refractivity (MR) constants of the aryl substituents of **3a-e** and the IC_{50} values generated in the Molt4/C8, CEM and L1210 evaluations. Positive correlations ($p<0.05$) were noted between the σ values of the aryl substituents present in **3a-e** and the IC_{50} values in the Molt4/C8, CEM and L1210 evaluations. No correlations or trends to a correlation ($p<0.1$) were noted between the cytotoxic potencies and the π and MR constants. Hence in the future groups with strongly electron-donating

Table 2
Evaluation of the compounds in series **3** for their cytotoxicity against human tumour and human nonmalignant cells

Compound	Human Tumour Cell Lines										Human Non-malignant Cells, CC_{50} (μM)[a]			
	HL-60 CC_{50} (μM)[a]	SI[b]	HSC-2 CC_{50} (μM)[a]	SI[b]	HSC-3 CC_{50} (μM)[a]	SI[b]	HSC-4 CC_{50} (μM)[a]	SI[b]	Ave CC_{50}	Ave SI[b]	HGF	HPC	HPLF	Ave
3a	1.5±0.61	70.7	12.5±0.86	8.48	17.7±1.5	5.99	15.9±0.9	6.67	11.9	23.0	116±15.1	142±16	60±31	106
3b	1.9±0.31	>95.8	90.3±16.0	>2.02	98±2.5	>1.86	94.3±12.0	>1.93	71.1	>25.4	184±4.0	>200	163±15	>182
3c	2.3±0.21	>87.0	30.3±13.9	>6.60	16.0±1.0	>12.5	22.7±1.5	>8.81	17.8	>28.7	>200	>200	>200	>200
3d	10.5±1.1	16.6	30.5±10.0	5.71	33.7±1.5	5.16	37.3±1.5	4.67	28.0	8.04	190±10	179±18	154±9.3	174
3e	>200	~1.0	112±12.5	>1.79	>200	~1.0	>200	~1.0	>178	~1.20	>200	>200	>200	>200
Niacin	>200	~1.0	>200	~1.0	>200	~1.0	>200	~1.0	>200	~1.0	>200	>200	>200	>200
Melphalan	0.89±0.17	75.6	12±2.7	5.61	46±31	1.46	46±6.0	1.46	26.2	21.0	73±15	37±7.3	92±7.8	67.3

[a]The CC_{50} values represent the concentrations of the compounds which kill 50% of the cells.
[b]The letters SI refer to the selectivity index figures. The values are obtained by dividing the average CC_{50} figures of the non-malignant cells by the CC_{50} value of a specific malignant cell line.

properties should be placed in ring A such as the 4-dimethylamino and 4-isopropyloxy group which have σ values of -0.83 and -0.45, respectively.[18]

The next phase of the investigation was to determine whether the compounds in series **3** display tumour-selective cytotoxicity, i.e., do they have greater potency towards neoplasms than non-malignant cells? Hence **3a-e** were evaluated against HL-60, HSC-2, HSC-3 and HSC-4 neoplastic cell lines as well as HGF, HPC and HPLF nonmalignant cells. These data are presented in Table 2.

The biodata in Table 2 reveal that in general the compounds in series **3** are lethal to HL-60, HSC-2, HSC-3 and HSC-4 cells. The relative average potencies of **3a-e** are **3a** > **3c** > **3d** > **3b** > **3e** which are similar to the average potencies in Table 1 viz **3a** > **3c** > **3b** > **3d** > **3e**, i.e., the greatest cytotoxicity is displayed by **3a** and **3c** while **3e** is the least potent. In particular, **3a**, **c**, **d** are more potent than melphalan towards HSC-4 cells while the following esters are equipotent with this drug (cell line in parentheses) namely **3a**, **b** (HL-60), **3a** (HSC-2) and **3a**, **c**, **d** (HSC-3).

The compounds in series **3** were also evaluated against HGF, HPC and HPLF non-malignant cells. The low average CC_{50} values of **3b-e** are noteworthy and reveal that in general, series **3** is well tolerated by nonmalignant cells. In fact, in 47 % of the bioassays, the CC_{50} values are in excess of 200 μM. All of these esters are less toxic than melphalan for these three cell lines except **3a** and melphalan have the same toxicity towards HPLF cells. In order to quantify the greater toxicity of **3a-e** for neoplasms than normal cells, selectivity index (SI) figures were calculated. Individual tumours are surrounded by different nonmalignant cells and hence the SI values are the quotients of the average CC_{50} value towards HGF, HPC and HPLF cells and the CC_{50} figure of the compound against a specific neoplastic cell line. The SI figures of **3a-c** generated using HL-60 cells are huge and in addition these three compounds have SI values in excess of 1 against HSC-2, HSC-3 and HSC-4 cells. The average SI values of **3a-c** are greater than is displayed by melphalan. Furthermore the virtual lack of potency of the 4-nitro analog **3e** to both neoplastic and nonmalignant cells is noteworthy.

In order to identify compounds which have both good potencies and are selectively toxic to neoplasms, the potency-selectivity expression (PSE) values of the compounds in series **3** were calculated. This property is the product of the reciprocal of the average CC_{50} value and the average SI figure multiplied by 100.[19] Thus the PSE values of **3a-e** are 193, >35.7, >161, 28.7 and >0.67, respectively, in contrast to a figure of 80.2 for melphalan. These data reinforce the identification of **3a** and **3c** as important lead molecules.

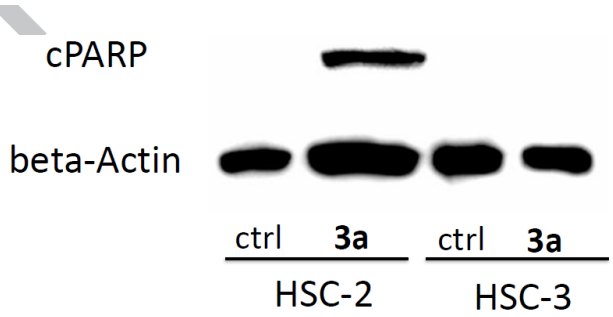


Figure 2. Evaluation of **3a** (7μM) for PARP1 cleavage in HSC-2 and HSC-3 cells for 24 h.

An investigation was launched to gain some idea of the way in which a lead compound **3a** exerts its lethal effect on various neoplasms. The enzyme poly(ADP-ribose)polymerase 1 (PARP1) repairs DNA single-stranded breaks[20] and hence compounds which cleave PARP1 may be of considerable value in cancer chemotherapy. The ability of **3a** to cleave PARP1 in HSC-2 and HSC-3 cells was investigated and the results are depicted in Figure 2. This compound caused PARP1 cleavage in HSC-2 cells and hence its antineoplastic effect against this cell line is partially due to this mode of action. Under the same conditions, **3a** did not cause cleavage of PARP1 in HSC-3 cells which may contribute to this compound displaying greater toxicity to HSC-2 cells than to the HSC-3 neoplasms.

Another way in which anticancer drugs exert their bioactivity is by interfering with different stages of the cell cycle. Consequently HSC-2 cells were incubated with **3a** (7μM) for 24 hours and the distribution of the cells determined at the end of the time period. The percentage of cells in the G_0/G_1, S and G_2/M phases (the figures for untreated cells are in parentheses) are 86 (73), 9.7 (13) and 1.5 (11), respectively. Thus another way that **3a** exerts its antineoplastic effect on HSC-2 cells is by causing G_0/G_1 phase arrest. In summary, the cytotoxicity of **3a**, and probably structurally related compounds, includes PARP1 cleavage and cell cycle arrest.

A major problem in treating cancer patients with drugs is the resultant toxicity of these compounds.[21] Hence the stimulus for developing these compounds will be enhanced if they are well-tolerated in mammals. Consequently short-term toxicology experiments were initiated. All of the compounds in series **3** – **5** were administered intraperitoneally to mice using doses of 30, 100 and 300 mg/kg and the animals were examined for survival and neurological deficit after 0.5 and 4 hours. No mortalities were observed and after 0.5 hour only **3a** caused neurotoxicity in less than half of the animals receiving a dose of 300 mg/kg. These symptoms were absent after 4 hours. Doses of 50 and 30 mg/kg of **3a** and **3b,** respectively, were administered orally to rats and the animals examined after 0.25, 0.5, 1, 2 and 4 hours did not reveal any overt toxicity. Bearing in mind that the LD_{50} value of melphalan is 21.7 mg/kg in mice[21], these animal data for series **3**–**5** are encouraging.

Table 3

Evaluation of **3a-c** for drug-like properties

Compound	Physicochemical parameter[a]					
	M.W.	logP	HBA	HBD	RB	TPSA
3a	395.46	5.26	4	0	5	56.27
3b	409.48	5.21	4	0	6	56.27
3c	425.48	5.32	5	0	7	65.50
Ideal compound	<500	<5.00	<10	<5	<10	<140

[a]MW: molecular weight, log P: (the logarithm of the partition coefficient), HBA: the number of hydrogen bond acceptor atoms, HBD: the number of hydrogen bond donor atoms, RB: the number of rotatable bonds (RB) and TPSA: the total polar surface area (TPSA). The characteristics of an ideal compound are based on guidelines proposed in the literature.[22,23]

In view of the promising biodata, drug-likeness properties of **3a-c** were assessed. Various studies have revealed that in general for good oral absorption, organic molecules should have a molecular weight of less than 500, a logP value below 5, less than 10 hydrogen bond acceptor atoms and fewer than 5 hydrogen bond donor atoms.[22] In addition, the number of rotatable bonds should be less than 10 and the total polar surface area should be below 140Å.[23] The relevant physicochemical parameters of **3a-c** are presented in Table 3 which reveals that the compounds meet the guidelines except the logP values are above 5. In fact logP figures of 0-3 are considered to be optimal for oral bioavailability.[24] Hence future development will need to introduce hydrophilic groups into the molecule as well as electron-repelling groups *vide supra*. Examples of suitable aryl substituents which have both hydrophilic and electron-donating properties (π and σ constants in parentheses) are hydroxyl (-0.67, -0.37), amino (-1.23, -0.66) and methylamino (-0.47, -0.84).[25]

Finally, various experiments were performed to assess the likelihood of the compounds in series **3** undergoing hydrolysis. A representative compound **3a** was incubated under different conditions for 48 hours and at 37° C which are the time and temperature used in the in vitro assays. First, a solution of **3a** was incubated in a mixture of deuterium oxide and deuterated dimethylsulfoxide. However the ^{1}H NMR spectrum revealed the presence of unreacted **3a**. Second, **3a** was incubated in a mixture of buffer

pH 7.4 and dimethylsulfoxide and with the aid of a water suppressor technique, the ^{1}H NMR spectrum revealed only the presence of intact **3a**. Third, **3a** was incubated in a mixture of rat serum and dimethylsulfoxide and subsequently extracted with ethyl acetate. The experiment was repeated in the absence of **3a** and both extracts were examined by ^{1}H NMR spectroscopy. By creating difference spectra, unreacted **3a** was detected but not the hydrolysis product 6-benzylidene-2-(4-hydroxybenzylidene)cyclohexanone. One or more products were also observed which may have been due to the reaction of **3a** with compounds in the rat serum. While an exhaustive probe as to the stability of **3a** and related compounds is beyond the span of this study, the evidence to hand suggests that in the future electron attracting substituents should be placed in the aryl ring B to enhance hydrolysis.

In conclusion, three novel series of compounds **3-5** were developed as candidate antineoplastic agents and among these derivatives **3a-c** display excellent cytotoxic properties towards Molt4/C8, CEM and L1210 cells. In addition, these dienones have CC_{50} values of approximately 2 μM towards human HL-60 leukemia cells and substantially lower toxicity to HGF, HPC and HPLF nonmalignant cells. SAR and QSAR studies reveal that cytotoxic potencies are enhanced by the incorporation of electron-donating groups into ring A of series **3**. A representative potent compound **3a** cleaved PARP1 and caused cell cycle arrest in HSC-2 cells. The compounds in series **3** as well as **4** and **5** are well tolerated in mice and in general possess drug-like properties. Three different stability studies of a representative compound **3a** revealed that it did not undergo hydrolysis to the corresponding phenol **2a**. Various suggestions for future analog development have been outlined.

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Supplementary data

Supplementary data are available which describes the syntheses of **3a-e**, **4a**,**b** and **5**, the methods for determining physicochemical properties of the compounds and bioevaluations.

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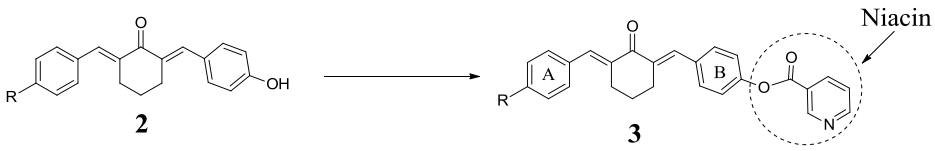
GRAPHICAL ABSTRACT



2a (R=H): IC_{50} 5.17 μM (Molt 4/C8)

3a (R=H): IC_{50} 1.62 μM (Molt 4/C8)

A novel series of cytotoxins **3** was designed to liberate the chemoprotectant niacin. The compounds in series **3** bearing electron-releasing aryl substituents (R) in ring A display good cytotoxic potencies and tumour-selective properties as well as being well tolerated in mice.

Highlights

- Novel series of cytotoxins displaying tumour-selective toxicity.
- Development of SAR and QSAR correlations.
- The compounds possess drug-like properties.
- The compounds are well tolerated in a short term screen in mice.
- The mode of actions include PARP1 cleavage and G_0/G_1 cell cycle arrest.