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Synthesis and cytotoxic activities of usnic acid derivatives

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ABSTRACT

Nine usnic acid–amine conjugates were evaluated on murine and human cancer cell lines. The polyamine derivatives showed significant cytotoxicity in L1210 cells. Their activities appeared to be independent of the polyamine transport system (PTS). Indeed, their activities were similar in chinese hamster ovary (CHO) and in the PTS deficient CHO-MG cells. In addition, α-difluoromethylornithine, an ornithine decarboxylase inhibitor known to indirectly enhance the activity of the PTS and consequently increase the cytotoxicity of cytotoxic drugs entering cells via the PTS, had no effect on the activity of the polyamine derivatives. The more active derivative (1,8-diaminooctane derivative) displayed similar activities on all cancer cell lines studied and induced apoptosis.

1. Introduction

Usnic acid (Scheme 1) is a dibenzofuran derivative uniquely found in lichens. It is especially abundant in genera such as *Alectoria*, *Cladonia*, *Usnea, Lecanora*, *Ramalina* and *Evernia*.[1] Usnic acid displays a wide range of pharmacological activities including antibiotic, antiviral, antiprotozoal, anti-inflammatory, analgesic and antiproliferative activities.[1,2] Regarding the antiproliferative activity, both (−) and (+) isomers of usnic acid showed in vitro moderate to strong cytotoxicity against a wide variety of murine and human cancer cell lines.[3–7] (−) Usnic acid also induced apoptosis of the murine leukaemia L1210 cells in a dose and time-dependent manner.[4]

A few attempts have been made to improve the antitumour activity of usnic acid. In particular, the disruption of the strong intramolecular hydrogen bonds of (−) usnic acid, which increases the overall hydrosolubility of the molecule, failed to generate more active compounds, but revealed the importance of the β-triketone function for the activity of the parent compound.[3] Also improving the delivery of acid usnic into the cells, for example, by complexation with 2-hydroxypropyl-β-cyclodextrin, greatly enhance its activity.[8]

Recent reports have demonstrated that targeting anti-cancer drugs to the polyamine transport system (PTS) may improve their activity.[9–19] Because of the broad tolerance of the PTS and its high activity in rapidly dividing cancer cells, the vectorization of a chemotherapeutic agent by a natural or a synthetic polyamine chain may improve its delivery into cancer cells, and consequently its activity and selectivity.[11,17] In addition, the activity of the drug can be levelled up when given in combination with α-difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase, which induces a depletion of intracellular polyamine pools, in reaction to which the cell enhances the PTS activity.[20,21] Detailed studies have started to reveal the structural requirements associated with the delivery of polyamine conjugates via the PTS in murine leukaemia (L1210) and chinese hamster ovary (CHO) cells. These studies have emphasized the influence of the size of the substituent, the length of the spacer and the structure of the polyamine chain. In particular, the homospermidine chain (*N*-(4-aminobutyl)-1,4-diaminobutane) with a methylene spacer was identified as an excellent vector to deliver arenyl systems as bulky as anthracene and pyrene.[12–15] Dihydroquinolin systems targetted the PTS when conjugated to a spermidine (*N*-(3-aminopropyl)-1,4-diaminobutane) chain linked via an amidine bond.[17]

New pharmacomodulations consisting in amine conjugation were envisaged to improve the activity of usnic acid. Nine usnic acid derivatives conjugated to various amine moieties were evaluated for their cytotoxicity on murine and human cancer cell lines. Amines included the three natural polyamines (putrescine, spermidine, spermine (Fig. 1)); nonylamine and 1,8-diaminooctane, lipophilic mono- and diamine homologous to spermidine; aminobutanol, an hydroxylated monoamine homologous to putrescine; three amino acids (L-isoleucine, L-leucine ester, L-phenylalanine

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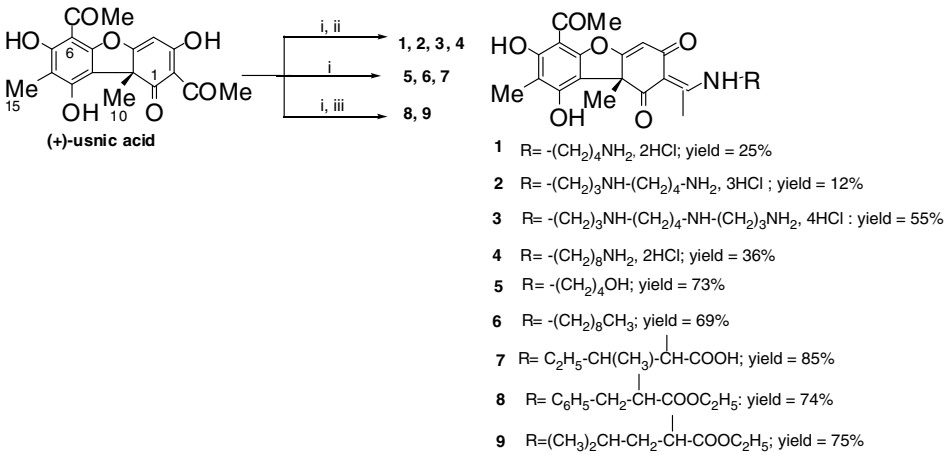

Scheme 1. Synthesis of usnic acid derivatives **1**–**9**. Reagents and condition: (i) RNH_2 1 equiv, refluxing EtOH/THF 80:20, 4 h; (ii) 2.4 N HCl gas, AcOEt; (iii) Et_3N, 1 equiv.

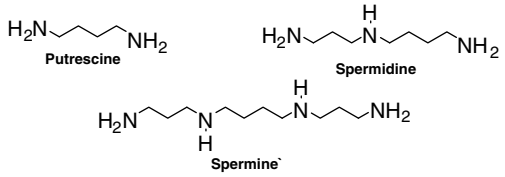

Figure 1. Structure of natural polyamines.

ester) whose hydrophobicity may improve cell delivery of the conjugates.

The ability of the polyamine derivatives to be delivered into cells via the PTS was also determined by assessing the differential cytotoxicity of the conjugates in CHO versus CHO-MG cells, as well as in L1210 versus DFMO-treated L1210 cells.

In addition, ability to induce apoptosis in L1210 cells was analyzed for the most active compound in order to gain mechanistic information on these potential antitumour agents.

2. Results

2.1. Chemistry

The (+) and (−) isomers of usnic acid were reported to have similar activities against a wide variety of cancer cell lines (Table 1).[4] The (+) isomer was selected in our study because it was commercially available. Our strategy was based on the condensation of the highly reactive methyl ketone[22] of the (+) usnic acid with various selectively protected polyamines, amines and amino acids leading to enamine compounds as previously reported[23] (Scheme 1).

The condensation of usnic acid (1 equiv) with each amine moiety (1 equiv) in refluxing EtOH/THF during 5 h afforded nine derivatives in good yields (55–85%). The preparation of compounds **1**–**4** required the use of the corresponding mono-Boc-protected polyamines (*N*-*tert*-butoxycarbonyl-1,4-diaminobutane[24] for **1**, N^4,N^8-di-*tert*-butoxycarbonylspermidine[25] for **2**, N^1, N^4, N^9-tri-*tert*-butoxycarbonylspermine[26] for **3**, *N*-*tert*-butoxycarbonyl-1,8-diami-

Table 1
Cytotoxic activity of (+) and (−)-usnic acid and of compounds **1**–**9** on various cell lines[a]

Compound[c]	IC_{50}[b] (μM)							
	L1210	CHO	CHO-MG	3LL	DU145	MCF7	K-562	U251
(−) UA	17.4 ± 1.4	nd	nd	35.1 ± 10.7	45.9 ± 7.0	51.7 ± 7.3	21.8 ± 3.8	19.7 ± 4.6
(+) UA	26.4 ± 8.5	31.3 ± 16	30 ± 12	23 ± 6.6	57.4 ± 2.1	105.4 ± 16	52.8 ± 8.7	19.5 ± 1.6
Put	≫100	≫100	≫100	nd	nd	nd	nd	nd
Spd	≫100	≫100	≫100	nd	nd	nd	nd	nd
Spm	54.5 ± 9.2	≫100	≫100	nd	nd	nd	nd	nd
DAO	≫100	≫100	nd	nd	nd	nd	nd	nd
(+) UA + 100 μM Put	20.5 ± 3.8	nd	nd	nd	nd	nd	nd	nd
(+) UA + 100 μM Spd	23.2 ± 4.3	nd	nd	nd	nd	nd	nd	nd
(+) UA + 50 μM Spm	14.2 ± 6.4	nd	nd	nd	nd	nd	nd	nd
(+) UA + 25 μM Spm	20.0 ± 7.4	nd	nd	nd	nd	nd	nd	nd
(+) UA + 100 μM DAO	14.7 ± 5.2	nd	nd	nd	nd	nd	nd	nd
1	12.0 ± 2.8	10.7 ± 2.0	12 ± 1.0	nd	nd	nd	nd	nd
2	**8.4 ± 1.4**	29.3 ± 1.0	28.0 ± 3.0	nd	nd	**9.9 ± 0.2**	nd	12.5 ± 4.9
3	15.3 ± 2.6	48 ± 20	59 ± 9.0	nd	nd	11.2 ± 2.3	nd	**4.4 ± 1.1**
4	**2.7 ± 0.8**	**3.5 ± 1.2**	**5 ± 2.0**	**8.2 ± 0.7**	14.1 ± 2.1	**5.8 ± 0.4**	**7.2 ± 0.8**	**8.2 ± 0.6**
5	19.9 ± 1.5	19.5 ± 5.0	nd	nd	nd	nd	nd	nd
6	>500	nd	nd	nd	nd	nd	nd	nd
7	54.6 ± 18.5	nd	nd	nd	nd	>100	56.7 ± 28.9	nd
8	74.4 ± 1.3	nd	nd	nd	nd	>100	>100	>100
9	48.9 ± 10.5	nd	nd	nd	nd	>100	60.7 ± 37.0	>100
Etoposide	**0.5 ± 0.25**	nd	nd	**4.0 ± 1.3**	**1.5 ± 0.4**	**12.2 ± 0.5**	**4.9 ± 0.8**	**0.5 ± 0.1**

Values in bold character correspond to IC_{50} < 10μM.

[a] Murine lymphocytic leukaemia L1210 (ATCC CCL 219), 3LL, murine Lewis lung carcinoma (CRL-1642), K-562, human chronic myelogenous leukaemia (ATCC CCL 243), human prostate carcinoma brain metastasis DU145 (ATCC HTB 81), human breast adenocarcinoma MCF7 (ATCC HTB 22), U251, human glioblastoma U251 (RCB0461), Chinese Hamster Ovary CHO, Polyamine-transport deficient CHO-MG.[32]

[b] Cells were cultured 48 h or 72 h in presence of various concentrations of the compounds as indicated in Section 4. All results are the means of at least three independent experiments ± SD. nd: not determined.

[c] UA, usnic acid; Put, Putrescine; Spd, spermidine; Spm, spermine; DAO, diaminooctane.

nooctane[27] for **4**). These final products were then afforded in good yields as hydrochloride salts (**1**–**4**) after a deprotective step using 2.4 N HCl gas in ethyl acetate. An additional purification step, necessary to eliminate free polyamine along with deprotection side products, was carried on by simple washing (for **1**, **4**) or column chromatography on a sephadex LH20 column (for **2**, **3**). The **1**–**4** compounds were finally prepared with a yield range from 12% to 55%. The amino acid derivatives (**7**–**9**) were prepared with good yields (74–85%). Among them, the conjugates **8** and **9** were generated as ethyl ester derivatives by the use of ethanol in presence of triethylamine (1 equiv) from L-phenylalanine and L-leucine methyl ester hydrochloride, respectively.

2.2. Biological activity

The cytotoxicity of the derivatives against a variety of murine and human cancer cell lines was evaluated using the MTT assay (Table 1).[28]

The introduction of a (poly)aminoalkyl or aminoacid side chain on usnic acid greatly influenced its cytotoxicity. The natural polyamine derivatives (**1**–**3**) were more active on L1210 cells (IC_{50} below or equal to 12 μM) than (+) usnic acid (IC_{50} = 26 μM). This improved cytotoxicity did not result from the additive cytotoxicity of the two components of the conjugates. Indeed, free polyamines have no or very low cytotoxicity. In addition, the co-administration of (+) usnic acid with free polyamines did not modify its cytotoxicity. These observations demonstrate that the improved cytotoxicity of polyamine–usnic acid conjugates is an intrinsic characteristic of these molecules.

In contrast, the aminobutanol **5** and amino acid (**7**–**9**) derivatives had lesser activity on L1210 cells than their parent compound. In addition, while the nonylamine **6** derivative was completely devoid of any activity on L1210 cells, the diaminooctane derivative **4** was the more active compound (IC_{50} = 2.7μM). This derivative displayed significant cytotoxicity (3 μM < IC_{50} < 14 μM) against all the cancer cell lines studied showing 3- to 18-fold more activity than the parent (+) usnic acid. This compound, however, displayed similar cytotoxicity towards the CHO cells that are nontransformed epithelials. This suggests that this compound has probably no selectivity towards cancer cell lines. It has to be noted that the spermine derivative **3** showed also a significant activity against glioblastoma U251 cell lines (IC_{50} = 4.4 μM).

Some polyamine–drug conjugates can be selectively delivered into cells via the PTS.[19] We have previously reported the use of comparative cytotoxicity to investigate this possibility, PTS selective drugs exhibiting a higher cytotoxicity on CHO than on PTS deficient CHO-MG cells.[11–17] None of four polyamine derivatives (**1**–**4**) displayed differential cytoxicities against CHO and CHO-MG cell lines (Table 1), indicating that none of the conjugates used the PTS for cellular entry. This conclusion is supported by the absence of DFMO effect on the cytotoxicity of those derivatives on L1210 cells as well as on CHO cells (data not shown).

While usnic acid does not interact directly with DNA[29] it has been shown to affect some key steps in cell division,[6] perhaps by inhibiting mitotic spindle formation.[30] We showed recently that both (−) and (+) usnic acid isomers induced apoptosis of L1210-treated cells.[4] We then compared the apoptosis-inducing activity of (+) usnic acid and of its most active derivative **4** in L1210 cells. For each compounds, two concentrations (close and higher to their respective IC_{50}) were assayed. Apoptosis was determined by microscopic evaluation of Hoechst-stained cell nuclei 24–48 h after treatment (Fig. 2). Both compounds induced a dose-dependent and time-dependent induction of apoptosis in L1210. Apoptosis induction was further confirmed by measuring caspase-3 activity in L1210 cells treated for, 48 h with (+) usnic acid, compound **4** and etoposide. As displayed in Table 2, an increase in caspase-3 activity, a landmark of apoptosis, was observed in all treated cells.

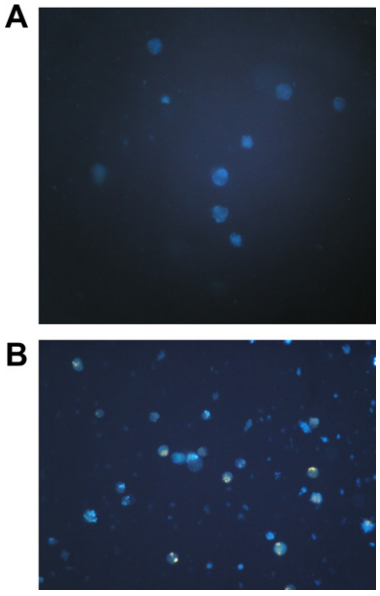

Figure 2. Apoptotic cell morphology evaluation by Hoechst 33342 staining. Cells were treated and stained with Hoechst 33342. The morphology of untreated cells (A) and compound **4** (3 μM) treated cells (B) is shown.

Table 2
Induction of apoptosis by (+)-usnic acid and derivative **4** in L1210 cells[a]

Compound[a]	Conc (μM)	Apoptotic cells[b] (%)		Caspase-3 activity[c]
		24 h	48 h	48 h
None		1.0 ± 0.3[d]	1.8 ± 0.8	1.0 ± 0.1
(+) Usnic acid	17	30 ± 9	33 ± 3	3.4 ± 0.3
(+) Usnic acid	26	32 ± 7	38 ± 3	7.4 ± 0.7
(+) Usnic acid	35	35 ± 9	42 ± 5	21.9 ± 3
4	3	37 ± 5	37 ± 3	1.4 ± 0.2
4	4.5	38 ± 10	42 ± 4	1.7 ± 0.1
4	6	35 ± 6	48 ± 5	1.7 ± 0.2
Etoposide	1.7	47 ± 7	53 ± 7	19.5 ± 1.5

[a] L1210 cells were cultured in presence of the compounds as indicated.
[b] After washings, the cells were stained with Hoechst 33342. The percentage of cells with fragmented cell nuclei was determined under fluorescence microscopy.
[c] Cells were collected after 48 h treatment and the caspase-3 activity was measured as described in Section 4 and the activity was expressed. The results display the mean caspase-3 activity relative to the basal activity measured in untreated cells.
[d] Data represent means of three independent experiments ± SD.

3. Discussion and conclusion

In this study, we demonstrate that the cytotoxic activity of usnic acid against cancer cell lines can be improved by its conjugation to a polyamine chain. Thus, conjugated compounds **1**–**3** showed better activities (4.4 < IC_{50} < 15.3 μM) towards all cancer cell lines studied here than (+) usnic acid (19.5 < IC_{50} < 105.4 μM). However, the targeting to the PTS was unsuccessful: none of the conjugates with natural polyamines showed a preferential activity in a cell line with a high PTS activity. This failure may be due to an unfavourable architecture of the conjugates: (1) the absence of a linker between the polyamine chain and the usnic acid bulby moiety may impair the targeting: the presence and the size of a linker were crucial parameters for the targeting of aryl systems[15] or (2) an unfavourable polyamine vector: in other systems, the homospermidine chain has been shown to be the more efficient vector.[12–15]

The conjugation of usnic acid to diaminooctane generated the more active compound **4** in terms of cytotoxicity (3 μM < IC_{50} < 14 μM). Compound **4** is then 3- to 18-fold more active than (+) usnic acid on the cancer cell lines studied here. The lower activity of its

spermidine analogue **2** (IC_{50} around 10 μM), which bears a secondary amine group in place of a methylene group, points the importance of the long lipophilic alkyl chain. Such hydrophobic chain may favour the cell membrane crossing. However, the presence of a primary amino group at the end of the alkyl chain is also essential as suggested by the absence of activity of the nonylamine derivative **6** (IC_{50} on L1210 > 500 μM). This observation also indicates that the terminal amino group may be involved in the cytotoxic activity of the derivative. The oxidation of this amine, which could generate a cytotoxic aldehyde group, may be considered. However, the amine conjugation may not alter fundamentally the mode of action of usnic acid since both the parent compound and its derivative appeared to be apoptosis-inducing agents.

Amine conjugation was initially explored to improve the selectivity of usnic acid. This goal may not be fulfilled since none of our new derivatives targeted the PTS. However, we identified that the diaminooctane chain was a good vector to improve the cytotoxicity of usnic acid. Further investigations are needed to determine whether this functionalization results in an improved activity of usnic acid in vivo.

4. Experimental

4.1. General

4.1.1. Chemistry

Dry tetrahydrofuran (THF) was distilled under sodium and benzophenone. Other solvents and reactants of high quality and (+) usnic acid were purchased from commercial suppliers. Triethylamine, aminoalcohols and diamines were distilled under N_2 from KOH. ^1H NMR and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker DMX 500 WB or at 270 and 67.5 MHz, respectively, on a Jeol GSX 270 WB, using $CDCl_3$ or DMSO-d_6 (and TMS as internal standard) or D_2O (NMR spectra were then reported in ppm relative to the nondeuterated solvent peak). Two-dimensional NMR experiments were acquired on a Jeol GSX 270 WB. δ values are given in parts per million (ppm), coupling constants (J values) are given in Hertz (Hz) and multiplicities of ^1H NMR signals are reported as follows: s, singlet; d, doublet; t, triplet; m, multiplet; bs, broad signal; Ar, aromatic. Values with asterisk (*) can be inverted. High-resolution mass (HRMS) measurements for exact mass determination were performed on a ZABSpec TOF Micromass at the Centre Régional de Mesures Physiques de l'Ouest. Samples were ionized either with an electrospray ion source (ESI = electrospray ionization) or by LSIMS (liquid secondary ion mass spectrometry) ionization. IRFT spectra were run on a Perkin Elmer 16 PC spectrometer (KBr pellets, v: cm^{-1}). Thin layer chromatography was performed out on silica gel 60F254 plates (Merck). Column chromatography was performed using silica gel (63–200 μm, Kieselgel 60, Merck) and using Sephadex LH20 Fluka ref. [9041-37-6]. Optical rotations were recorded with a Perkin Elmer 341 automatic polarimeter at 20 °C at the sodium line; $[\alpha]_D$ are given in 10^{-1} deg cm^2 g^{-1}.

4.1.2. General experimental procedures

4.1.2.1. Synthesis of mono-Bocprotected polyamines. The protected polyamines, *N-tert*-butoxycarbonyl-1,4-diaminobutane[24], N^4,N^8-di-*tert*-butoxycarbonylspermidine[25], N^1,N^4, N^9-tri-*tert*-butoxycarbonylspermine,[26] *N-tert*-butoxycarbonyl-1,8-diaminooctane[27] were prepared as previously reported.

4.1.2.2. General procedure for usnic acid derivatives synthesis (A). To a solution of aminobutanol, nonylamine, amino acid or protected polyamine (1 equiv) at 80°C was added dropwise usnic acid (1 equiv) in THF/EtOH (20:80) (10 mL). After stirring at 80 °C for 4 h, the mixture was concentrated under reduced pressure. The obtained residue was purified by column chromatography on silica gel.

4.1.2.3. General procedure (B): removal of Boc groups and preparation of hydrochlorides (1–4). The conjugate was stirred at 0 °C during 12 h in a 2.4 N solution of HCl gas in ethyl acetate (1.2 equiv per amino group). After evaporation of the ethanol, the residue was triturated in anhydrous ether to give a pale yellow hygroscopic solid. To remove free polyamines, the diamino derivatives dissolved in CH_2Cl_2 (8 mL) were washed with water (2 × 150 μL). The organic layers were dried under Na_2SO_4 and evaporated under reduced pressure to give final products **1** and **4**. The triamino derivatives were chromatographed on a sephadex LH20 column using MeOH as eluent. The residues were then evaporated under reduced pressure to give final products **2**–**3**.

As indicated in Figure 3, the compounds were numbered for the heterocyclic moiety using the IUPAC rules and for the polyamine moiety with letters a–j.

4.1.3. Biological studies

4.1.3.1. Reagents. Unless otherwise stated, usual laboratory chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA). DFMO was obtained from Ilex Oncology (San Antonio, TX, USA).

4.1.3.2. Cell culture. Murine cancer cell lines: L1210 (lymphocytic leukaemia-ATCC CCL 219) and 3LL (murine Lewis lung carcinoma-CRL-1642), human cancer cell lines: K-562 (chronic myeologenous leukaemia-ATCC CCL-243), DU145 (brain metastasis of a prostate carcinoma-ATCC HTB-81), MCF7 (breast adenocarcinoma-ATCC HTB-22) and U251 (glioblastoma-RCB-0641) and hamster cell lines: CHO (chinese hamster ovary) and CHO-MG (a polyamine transport-deficient mutant CHO cell line) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 U/mL), streptomycin (50 μg/mL) (Eurobio, Les Ulis, France). L-proline (2 μg/mL) was added to the culture medium for CHO-MG cells. The culture medium of K-562 cells contained 10 mM Hepes (Biomedia, France). Cells were grown at 37 °C under a humidified 5% CO_2 atmosphere. Aminoguanidine (2 mM) was added to the culture medium to prevent the oxidation of the drugs by the serum amine oxidase present in the calf serum.

4.2. Chemistry

4.2.1. 6-Acetyl-2-[1-(4-aminobutylamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyldi-benzo-furan-1,3(2*H*,9b*H*)-dione (1) (hydrochloride salt)

General procedure A from *N-tert*-butoxycarbonyl-1,4-diaminobutane (0.275 g, 1.45 mmol) and usnic acid (0.500 g, 1 equiv) in THF/EtOH (20:80). Column chromatography using CH_2Cl_2/AcOEt 90:10, then CH_2Cl_2/MeOH/NH_4OH 95:5:1. Yellow oil; 79%. R_f 0.43 (CH_2Cl_2/AcOEt 80:20). General procedure B from 203 mg. Pale yellow solid; 32%. R_f = 0.39 (CH_2Cl_2/MeOH/isopropylamine, 4:4:0.2); $[\alpha]_D^{20} = +141$ (c 0.20, MeOH); ^1H NMR (270 MHz, DMSO-d_6) δ 1.63–1.74 (m, 7H, H-b, H-c, CH_3-10), 1.96 (s, 3H, CH_3-15), 2.59 (s, 3H, CH_3-12), 2.63 (s, 3H, CH_3-14), 2.83 (m, 2H, H-d), 3.58 (m, 2H, H-a), 5.88 (s, 1H, H-4), 12.25 (bs, 1H, OH-9), 13.04 (bs, 1H, NHC(CH_3)=C), 13.41 (s, 1H, OH-7); ^{13}C NMR (67.5 MHz,

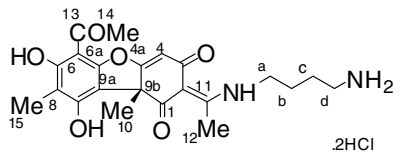

Figure 3. Example of atom numbering used for NMR assignments.

DMSO-d_6) δ 8.12 (*C*H_3-15), 18.84 (*C*H_3-12), 24.91 (C-c), 25.91 (C-b), 31.65 (*C*H_3-14), 32.34 (*C*H_3-10), 38.87 (C-d), 43.41 (C-a), 56.93 (C-9b), 101.50 (C-6), 102.19 (C-2), 102.96 (C-4), 105.73 (C-9a), 106.94 (C-8), 156.38 (C-6a), 158.26 (C-9), 163.12 (C-7), 173.68 (C-4a), 175.61 (C-11), 189.58 (C-3), 197.90 (C-1), 201.56 (C-13); FTIR 2400 to 3600 (NH^+, OH), 1699 (CO-1), 1624 (CO-13, C=C enamine, C=C Ar), 1554 (CO-3); HRMS (LSIMS) (*m/z*) calcd for $C_{22}H_{27}N_2O_6$ $(M+H)^+$ 415.1869; found 415.1870. Anal. ($C_{22}H_{26}N_2O_6$, 2HCl) calcd: C, 54.22; H, 5.79; N, 5.75; found: C, 55.47; H, 5.95; N, 5.81.

4.2.2. 6-Acetyl-2-{1-[3-(4-aminobutylamino)propylamino]-ethylidene}-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2*H*, 9b*H*)-dione (2) (hydrochloride salt)

General procedure A from N^4, N^8-di-*tert*-butoxycarbonylspermidine (0.485 g, 1.45 mmol) and usnic acid (0.500 g, 1 equiv) in THF/EtOH (20:80). Column chromatography using CH_2Cl_2/AcOEt 90:10, then CH_2Cl_2/MeOH/NH_4OH 98:2:1. Yellow oil; 55%. R_f 0.39 (CH_2Cl_2/AcOEt 80:20). General procedure B from 198 mg. Pale yellow solid; 22%. R_f = 0.62 (CH_2Cl_2/MeOH/isopropylamine, 4:4:2); $[\alpha]_D^{20}$ + 186 (*c* 0.25, MeOH); ^{1}H NMR (270 MHz, DMSO-d_6)δ 1.57–1.74 (m, 7H, H-e, H-f, C*H*$_3$-10), 1.96–2.09 (m, 5H, H-b, C*H*$_3$-15), 2.60 (s, 3H, C*H*$_3$-12), 2.65 (s, 3H, C*H*$_3$-14), 2.79 (t, *J* = 7 Hz, 2H, H-g), 2.87–3.00 (m, 4H, H-c, H-d), 3.70 (m, 2H, H-a), 5.88 (s, 1H, H-4), 7.90 (bs, 2H, NH_2), 8.78 (bs, 1H, NH), 12.30 (s, 1H, OH-9), 13.28 (bs, 1H, N*H*C(CH$_3$)=C), 13.76 (s, 1H, OH-7); ^{13}C NMR (67.5 MHz, DMSO-d_6) δ 8.11 (*C*H_3-15), 18.91 (*C*H_3-12), 23.14 (C-f), 24.68 (C-e), 25.77 (C-b), 31.64 (*C*H_3-14), 32.30 (*C*H_3-10), 38.63 (C-g), 41.51 (C-a), 44.63 (C-c), 46.52 (C-d), 56.82 (C-9b), 101.48 (C-6), 102.35 (C-2), 102.96 (C-4), 105.73 (C-9a), 106.93 (C-8), 156.35 (C-6a), 158.24 (C-9), 163.11 (C-7), 173.66 (C-4a), 175.84 (C-11), 189.54 (C-3), 197.90 (C-1), 201.54 (C-13); FTIR 2350 to 3600 (NH^+, OH), 1699 (CO-1), 1616 (CO-13, C=C enamine, C=C Ar), 1558 (CO-3); HRMS (LSIMS) (*m/z*) calcd for $C_{25}H_{34}N_3O_6$ $(M+H)^+$ 472.2447; found 472.2448. Anal. ($C_{25}H_{33}N_3O_6$, 3HCl) calcd: C, 51.69; H, 6.25; N, 7.23; found: C, 51.81; H, 6.50; N, 7.25.

4.2.3. 6-Acetyl-2-{1-{3-[4-(3-aminopropylamino)butylamino]-propylamino}ethylidene}-7,9-dihydroxy-8,9b-dimethyldibenzofuran-1,3(2*H*,9b*H*)-dione (3) (hydrochloride salt)

General procedure A from N^1, N^4,N^9-tri-*tert*-butoxycarbonylspermine (0.672 g, 1.33 mmol) and usnic acid (0.460 g, 1 equiv) in THF/EtOH (20:80). Column chromatography using CH_2Cl_2/AcOEt 90:10, then CH_2Cl_2/MeOH/NH_4OH 95:5:1. Yellow oil; 81%. R_f 0.21 (CH_2Cl_2/AcOEt 90:10). General procedure B from 296 mg. Pale yellow solid; 68%. R_f = 0.29 (CH_2Cl_2/MeOH/isopropylamine, 4:4:2); $[\alpha]_D^{20}$ = +175 (*c* 0.30, MeOH); ^{1}H NMR (500 MHz, DMSO-d_6)δ 1.67 (s, 3H, C*H*$_3$-10), 1.71 (bs, 4H, H-e, H-f), 1.94–2.05 (m, 7H, H-b, H-i, C*H*$_3$-15), 2.61 (s, 3H, C*H*$_3$-12), 2.64 (s, 3H, C*H*$_3$-14), 2.79 (t, *J* = 7 Hz, 2H, H-g), 2.89–3.23 (m, 8H, H-c, H-d, H-h, H-i), 3.69 (t, *J* = 6 Hz, 2H, H-a), 5.90 (s, 1H, H-4), 8.03 (bs, 2H, NH_2), 9.07 (bs, 1H, NH), 9.11 (bs, 1H, NH), 12.27 (s, 1H, OH-9), 13.01 (bs, 1H, N*H*C(CH$_3$)=C), 13.40 (s, 1H, OH-7); ^{13}C NMR (67.5 MHz, DMSO-d_6) δ 7.46 (*C*H_3-15), 18.27 (*C*H_3-12), 22.54 and 22.63 (C-e, C-f), 23.60* (C-b), 25.15* (C-i), 30.99 (*C*H_3-14), 31.68 (*C*H_3-10), 36.11 (C-j), 40.93 (C-a), 43.74* (C-c), 44.01* (C-h), 45.93 (C-d, C-g), 56.31 (C-9b), 100.83 (C-6), 101.71 (C-2), 102.29 (C-4), 105.09 (C-9a), 106.29 (C-8), 155.69 (C-6a), 157.61 (C-9), 162.49 (C-7), 172.98 (C-4a), 175.20 (C-11), 188.90 (C-3), 197.27 (C-1), 200.86 (C-13); FTIR 2350 to 3550 (NH^+, OH), 1699 (CO-1), 1624 (CO-13, C=C enamine, C=C Ar), 1559 (CO-3); HRMS (LSIMS) (*m/z*) calcd for $C_{28}H_{41}N_4O_6$ $(M+H)^+$ 529.3026; found 529.3027. Anal. ($C_{28}H_{40}N_4O_6$, 4HCl, 2H_2O) calcd: C, 47.33; H, 6.81; N, 7.89; found: C, 47.39; H, 6.97; N, 8.12.

4.2.4. 6-Acetyl-2-[1-(8-aminooctylamino)ethylidene]-7,9-dihydroxy-8,9b-dimethyldiben-zofuran-1,3(2*H*,9b*H*)-dione (4) (hydrochloride salt)

General procedure A from *N*-*tert*-butoxycarbonyl-1,8-diaminooctane (0.380 g, 1.55 mmol) and usnic acid (0.530 g, 1 equiv) in THF/EtOH (20:80). Column chromatography using CH_2Cl_2/AcOEt 90:10 then CH_2Cl_2/MeOH/NH_4OH 95:5:1. Yellow oil; 72%. R_f 0.56 (CH_2Cl_2/AcOEt 90:10). General procedure B from 253 mg. Pale yellow solid; 50%. R_f = 0.40 (CH_2Cl_2/MeOH/isopropylamine, 4:4:0.2); $[\alpha]_D^{20}$ = +65 (*c* 0.25, MeOH); ^{1}H NMR (270 MHz, DMSO-d_6) δ 1.22–1.30 (m, 10H, H-c, H-d, H-e, H-f, H-g), 1.54–1.64 (m, 5H, H-b, C*H*$_3$-10), 1.97 (s, 3H, C*H*$_3$-15), 2.58 (s, 3H, C*H*$_3$-12), 2.64 (s, 3H, C*H*$_3$-14), 2.76 (t, *J* = 8 Hz, 2H, H-h), 3.53 (m, 2H, H-a), 5.87 (s, 1H, H-4), 7.89 (bs, 2H, NH_2), 12.33 (s, 1H, OH-9), 13.06 (bs, 1H, N*H*C(CH$_3$)=C), 13.41 (s, 1H, OH-7): ^{13}C NMR (67.5 MHz, DMSO-d_6) δ 7.81 (*C*H_3-15), 18.49 (*C*H_3-12), 26.02 (C-f), 26.38 (C-c), 27.24 (C-g), 28.44 (C-b), 28.64 (C-d, C-e), 31.33 (*C*H_3-14), 32.02 (*C*H_3-10), 38.88 (C-h), 43.76 (C-a), 56.61 (C-9b), 101.17 (C-6), 101.80 (C-2), 102.63 (C-4), 105.44 (C-9a), 106.60 (C-8), 156.06 (C-6a), 157.97 (C-9), 162.81 (C-7), 173.33 (C-4a), 175.09 (C-11), 189.32 (C-3), 197.53 (C-1), 201.22 (C-13); FTIR 2350 to 3500 (NH^+, OH), 1700 (CO-1), 1616 (CO-13, C=C enamine, C=C Ar), 1559 (CO-3); HRMS (LSIMS) (*m/z*) calcd for $C_{26}H_{35}N_2O_6$ $(M+H)^+$ 471.2495; found 471.2491. Anal. ($C_{26}H_{34}N_2O_6$, 2HCl, 0.5 H_2O) calcd: C, 56.52; H, 6.75; N, 5.07; found: C, 56.81; H, 6.59; N, 5.10.

4.2.5. 6-Acetyl-2-[1-(4-aminobutanol)ethylidene]-7,9-dihydroxy-8,9b-dimethyldibenzo-furan-1,3(2*H*,9b*H*)-dione (5)

General procedure A from aminobutanol (0.052 g, 0.58 mmol) and usnic acid (0.200 g, 1 equiv) in THF/EtOH (20:80). Column chromatography using CH_2Cl_2/AcOEt 90:10. Yellow solid; 73%. R_f 0.48 (CH_2Cl_2/AcOEt 90:10); $[\alpha]_D^{20}$ = +239 (*c* 0.43, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.71 (s, 2H, C*H*$_3$-10), 1.85 (m, 4H, H-b, H-c), 2.09 (s, 3H, C*H*$_3$-15), 2.64 (s, 3H, C*H*$_3$-12), 2.67 (s, 3H, C*H*$_3$-14), 3.45 (m, 2H, H-a), 4.25 (m, 2H, H-d), 5.79 (s, 1H, H-4), 11.97 (s, 1H, OH-9), 13.36 (s, 1H, OH-7), 13.60 (bs, 1H, N*H*C(CH$_3$)=C). ^{13}C NMR (125 MHz, $CDCl_3$) δ 7.47 (*C*H_3-15), 18.34 (*C*H_3-12), 25.64 (C-b), 29.59 (C-c), 31.24 (*C*H_3-14), 32.03 (*C*H_3-10), 43.83 (C-a), 57.04 (C-9b), 61.90 (C-d), 101.32 (C-6), 102.19 (C-2), 102.42 (C-4), 105.08 (C-9a), 107.91 (C-8), 155.87 (C-6a), 158.26 (C-9), 163.43 (C-7), 174.05 (C-4a), 174.87 (C-11), 190.10 (C-3), 198.20 (C-1), 200.71 (C-13); FTIR 2375 to 3300 (NH, OH), 1700 (CO-1), 1620 (CO-13, C=C enamine, C=C Ar), 1556 (CO-3); HRMS (LSIMS) (*m/z*) calcd for $C_{22}H_{26}NO_7$ $(M+H)^+$ 416.1709; found 416.1713. Anal. ($C_{22}H_{25}NO_7$) calcd: C, 63.61; H, 6.07; N, 3.37; found: C, 62.53; H, 6.10; N, 3.33.

4.2.6. 6-Acetyl-7,9-dihydroxy-8,9b-dimethyl-2-[1-octylamino)-ethylidene]-dibenzofuran-1,3(2*H*,9b*H*)-dione (6)

General procedure A from nonylamine (0.207 g, 1.45 mmol) and usnic acid (0.500 g, 1 equiv) in THF/EtOH (20:80). Column chromatography using CH_2Cl_2. Yellow oil; 69%. R_f 0.33 (CH_2Cl_2/AcOEt 90:10); $[\alpha]_D^{20}$ + 276 (*c* 0.37, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.90 (t, *J* = 7 Hz, 3H, C*H*$_3$), 1.27–1.35 (m, 10H, H-c, H-d, H-e, H-f, H-g), 1.44 (m, 2H, H-h), 1.70–1.75 (m, 5H, H-b, C*H*$_3$-10), 2.10 (s, 3H, C*H*$_3$-15), 2.66 (s, 3H, C*H*$_3$-12), 2.68 (s, 3H, C*H*$_3$-14), 3.48 (m, 2H, H-a), 5.98 (s, 1H, H-4), 12.00 (s, 1H, OH-9), 13.36 (s, 1H, OH-7), 13.38 (bs, 1H, N*H*C(CH$_3$)=C). ^{13}C NMR (125 MHz, $CDCl_3$) δ 7.47 (*C*H_3-15), 14.10 (*C*H_3), 18.34 (*C*H_3-12), 22.64 (C-h), 26.84, 28.94, 29.10, 29.14, 29.19, 31.81 (C-b, C-c, C-d, C-e, C-f, C-g), 31.26 (*C*H_3-14), 32.02 (*C*H_3-10), 44.07 (C-a), 57.07 (C-9b), 101.33 (C-6), 102.43 (C-2, C-4), 105.13 (C-9a), 107.89 (C-8), 155.91 (C-6a), 158.32 (C-9), 163.47 (C-7), 173.90 (C-4a), 174.73 (C-11), 190.70 (C-3), 198.17 (C-1), 200.66 (C-13); FTIR 2375 to 3300 (NH, OH), 1700 (CO-1), 1620 (CO-13, C=C enamine, C=C Ar), 1556 (CO-3); HRMS (LSIMS) (*m/z*) calcd for $C_{27}H_{36}NO_6$ $(M+H)^+$

470.2543; found 470.2539. Anal. ($C_{27}H_{35}NO_6$) calcd: C, 69.06; H, 7.51; N, 2.98; found: C, 69.30; H, 7.74; N, 3.04.

4.2.7. *N*-(6-Acetyl-2-ethylidene-7,9-dihydroxy-8,9b-dimethyl-dibenzofuran-1,3(2*H*,9b*H*)-dion-11-yl)-L-isoleucine (7)

General procedure A from L-isoleucine (0.114 g, 0.87 mmol) and usnic acid (0.300 g, 1 equiv) in THF/EtOH (20:80). Column chromatography using CH_2Cl_2/MeOH/NH_4OH (70:10:1). Yellow pale solid; 85%. R_f 0.17 CH_2Cl_2/MeOH/NH_4OH (70:10:1); $[\alpha]_D^{20}$ + 129 (*c* 0.18, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.98 (bs, 6H, 2 × CH_3), 1.37 (m, 1H, CH_2-CH_3), 1.59–1.72 (m, 4H, CH_2-CH_3, CH_3-10), 1.98 (m, 1H, CH_2CHCH_3), 2.08 (s, 3H, CH_3-15), 2.59 (s, 3H, CH_3-12), 2.65 (s, 3H, CH_3-14), 4.39 (s, 1H, NHCHCOOH), 5.84 (s, 1H, H-4), 11.75 (s, 1H, OH-9), 13.30 (s, 1H, OH-7), 13.71 (s, 1H, NHC(CH_3)═C). ^{13}C NMR (125 MHz, $CDCl_3$) δ 7.51 (CH_3-15), 12.03* (CH_3), 15·10 (CH_3), 19.34 (CH_3-12), 25.72 (CH_2-CH_3), 31.13 (CH_3-14), 31.75 (CH_3-10), 57.27 (C-9b), 62.67 (CH_2CHCH_3), 101.15 (C-6), 102.21 (C-2), 102.31 (C-4), 104.69 (C-9a), 108.24 (C-8), 155.56 (C-6a), 158.12 (C-9), 163.45 (C-7), 172.99 (C-4a), 174.73 (C-11, COOH), 190.43 (C-3), 198.23 (C-1), 200.66 (C-13); FTIR 2350 to 3680 (NH, OH, COOH), 1699 (CO-1, COOH), 1627 (CO-13, C═C enamine, C═C Ar), 1557 (CO-3); HRMS (LSIMS) (*m/z*) calcd for $C_{24}H_{28}NO_8$ (M+H)$^+$ 458.1815; found 458.1819.

4.2.8. *N*-(6-Acetyl-2-ethylidene-7,9-dihydroxy-8,9b-dimethyl-dibenzofuran-1,3(2*H*,9b*H*)-dion-11-yl)-L-phenylalanine ethyl ester (8)

General procedure A from L-phenylalanine methyl ester hydrochloride (0.188 g, 0.87 mmol) and usnic acid (0.300 g, 1 equiv) in THF/EtOH (20:80) in presence of triethylamine (0.088 g, 1 equiv). Column chromatography using CH_2Cl_2/MeOH (98:2). Yellow pale solid; 74%. R_f 0.74 CH_2Cl_2/MeOH (98:2); $[\alpha]_D^{20}$ = +263 (*c* 0.34, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.31 (m, 3H, OCH_2CH_3), 1.69 (s, 3H, CH_3-10), 2.09 (s, 3H, CH_3-15), 2.27 (s, 3H, CH_3-12), 2.68 (s, 3H, CH_3-14), 3.13 (m, 1H, CH_2-Ph), 3.33 (m, 1H, CH_2-Ph), 4.27 (m, 2H, OCH_2CH_3), 4.65 (s, 1H, NHCH), 5.82 (s, 1H, H-4), 7.18–7.38 (m, 5H, H-Ar), 11.80 (bs, 1H, OH-9), 13.36 (s, 1H, OH-7), 14.02 (s, 1H, NHC(CH_3)═C). ^{13}C NMR (125 MHz, $CDCl_3$) δ 7.50 (CH_3-15), 14.07 (OCH_2CH_3), 18.34 (CH_3-12), 31.40 (CH_3-14), 31.74 (CH_3-10), 39.49 (CH_2-Ph), 57.35 (C-9b), 58.38 (CH_2CH), 62.67 (OCH_2CH_3), 101.25 (C-6), 102.29 (C-2, C-4), 104.91 (C-9a), 107.94 (C-8), 127.74, 128.92, 129.29, 129.39 (CH-Ar), 134.68 (C-Ar), 155.75 (C-6a), 158.13 (C-9), 163.37 (C-7), 169.22 ($COOC_2H_5$), 174.23 (C-4a), 174.51 (C-11), 190.74 (C-3), 198.54 (C-1), 200.74 (C-13); FTIR 2300 to 3270 (NH, OH), 1751 ($COOC_2H_5$),1699 (CO-1), 1627 (CO-13, C═C enamine, C═C Ar), 1558 (CO-3); HRMS (LSIMS) (*m/z*) calcd for $C_{29}H_{30}NO_8$ (M+H)$^+$ 520.1971; found 520.1968. Anal. ($C_{29}H_{29}NO_8$) calcd: C, 67.04; H, 5.63; N, 2.70; found: C, 66.85; H, 5.67; N, 2.84.

4.2.9. *N*-(6-Acetyl-2-ethylidene-7,9-dihydroxy-8,9b-dimethyl-dibenzofuran-1,3(2*H*,9b*H*)-dion-11-yl)-L-leucine ethyl ester (9)

General procedure A from L-leucine methyl ester hydrochloride (0.158 g, 0.87 mmol) and usnic acid (0.300 g, 1 equiv) in THF/EtOH (20:80) in presence of triethylamine (0.088 g, 1 equiv). Column chromatography using CH_2Cl_2/MeOH (98:2). Yellow pale oil; 75%. R_f 0.76 CH_2Cl_2/MeOH (98:2); $[\alpha]_D^{20}$ = +161 (*c* 1, CH_2Cl_2); ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.95–1.05 (m, 6H, CH_3CHCH_3), 1.29–1.36 (m, 3H, OCH_2CH_3), 1.69 (s, 3H, CH_3-10), 1.78–1.87 (m, 3H, CH_3CHCH_3, $CHCH_2CH$), 2.10 (s, 3H, CH_3-15), 2.59 (s, 3H, CH_3-12), 2.68 (s, 3H, CH_3-14), 4.28 (m, 2H, OCH_2CH_3), 4.45 (m, CHCO), 5.81 (s, 1H, H-4), 11.80 (s, 1H, OH-9), 13.35 (s, 1H, OH-7), 14.00 (s, 1H, NHC(CH_3)═C). ^{13}C NMR (67.5 MHz, $CDCl_3$) δ 7.49 (CH_3-15), 14.14 (OCH_2CH_3), 18.70 (CH_3-12), 21.92, 24.89 (CH_3CHCH_3), 31.30 (CH_3-14), 31.83 (CH_3-10), 41.41 ($CHCH_2CH$), 55.42 ($CHCOOC_2H_5$), 57.46 (C-9b), 62.27 (OCH_2CH_3), 101.38 (C-6, C-2), 102.34 (C-4), 105.01 (C-9a), 108.06 (C-8), 155.84 (C-6a), 158.25 (C-9), 163.51 (C-7), 170.14 ($COOC_2H_5$), 174.36 (C-4a), 174.86 (C-11), 190.91 (C-3), 198.56 (C-1), 200.67 (C-13); FTIR 2350 to 3500 (NH, OH), 1750 ($COOC_2H_5$),1700 (CO-1), 1616 (CO-13, C═C enamine, C═C Ar), 1559 (CO-3); HRMS (LSIMS) (*m/z*) calcd for $C_{29}H_{30}NO_8$ (M+H)$^+$ 486.2127 found 486.2137.

4.3. Biological assays

4.3.1. In vitro evaluation of drugs cytotoxicity

Assays were performed in 96-wells microtitre plates (Costar). Cells (100 μL per well) were seeded at the concentration indicated: L1210, 2.5×10^4; K-562, 7×10^4; 3LL, 1×10^4; U251, 2×10^4; DU145, 4×10^4; MCF7, 5×10^4; CHO and CHO-MG, 2×10^3 (cells/mL of medium). Drug solutions (10 μL per well) of appropriate concentration were added at the time of seeding for suspension–growing cells and after an overnight incubation for attached cells. In some experiments, 5 mM DFMO was added to the culture medium at the time of drug addition. After exposure to the drug for 48 h for murine and hamster cell lines and 72 h for human cell lines, cell growth was determined by measuring formazan formation from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.[31] Absorbance (540 nm) was recorded on a Multiskan RC microplate reader (Labsystems, Cergy-Pontoise, France).

4.3.2. Apoptosis assays

L1210 cells were seeded at 2×10^5 cells/mL in 6-well dishes, and cultured in presence or absence of the drug at the appropriate concentration. After 24 and 48 h, cells were collected, washed twice in phosphate-buffered saline, then stained with the DNA-specific fluorochrome Hoechst 33342 for 15 min at room temperature in the dark. The count of apoptotic cells was performed by visual observations on a fluorescence Olympus BX60 microscope.

4.3.3. Caspase-3 activity assay

L1210 cells were seeded at 2.5×10^5 cells/mL in flask, and cultured in presence or absence of the drug at the appropriate concentration. After 48 h, cells were collected, washed twice in phosphate-buffered saline, then freezed at −20 °C. The caspase-3 activity was measured in cells using the caspase-3/CPP32 fluorimetric assay kit (Biovision, Mountain View, CA, USA).

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