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Original article

Design, synthesis and pro-apoptotic antitumour properties of indole-based 3,5-disubstituted oxadiazoles

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ABSTRACT

A series of new indole-based 3,5-disubstituted 1,2,4-oxadiazoles has been designed and synthesised as potential pro-apoptotic antitumour agents, via the base-catalysed condensation reaction between substituted amidoximes and indole esters. Evaluation of antiproliferative activity against the human cancer cell lines COLO 320 (colorectal) and MIA PACA-2 (pancreatic) revealed IC_{50} values in the low micromolar range. Selected compounds were able to trigger apoptosis in sensitive cell lines, for example via activation of caspase-3/7, demonstrating that indole-based oxadiazoles possess in vitro antitumour and pro-apoptotic activity.

1. Introduction

The fundamental roles of defective apoptotic pathways in a range of disease states are well characterised. Insufficient apoptosis is a characteristic feature of proliferative diseases such as cancer or rheumatoid arthritis, whereas too much apoptosis is known to contribute to the development of degenerative disease such as Alzheimer's disease and multiple sclerosis [1]. Apoptotic pathways are particularly well characterised in the cancer field, where resistance to apoptotic stimuli is known as one of the six hallmarks of cancer articulated by Hanahan and Weinberg [2].

Mitochondria play a prominent and central role in the execution of the cell death process via apoptosis, with mitochondrial membrane permeabilisation and subsequent release of apoptogenic factors usually marking the point-of-no-return for a cell destined to undergo apoptotic cell death. The discovery of small molecules able to selectively induce apoptosis in cancer cells via a variety of mechanisms including those centred on the mitochondrial membrane represents an enticing prospect for future cancer therapeutics [3]. A number of articles published in recent years suggest that the goal of selective pro-apoptotic therapy for cancer treatment might be attainable, although translation of promising pre-clinical drug candidates into registered drugs in this category has not yet been realised [4].

Although cell death through apoptosis is an indirect consequence of many, if not most, cancer therapeutic agents, selective pro-apoptotic antitumour agents such as those directly targeting cancer cell mitochondria would be expected to have a number of beneficial properties compared to existing cytotoxic and molecularly targeted drugs. For example, tumour cells eliminated by pro-apoptotic agents by definition will not contribute to tumour relapse, as may be the case for cytostatic agents. In addition, selective pro-apoptotic agents would likely bypass the severe toxicological side effects associated with cytotoxic cancer chemotherapy, since genotoxic effects would be mediated through processes such as production of Reactive Oxygen Species (ROS) and oxidative DNA damage rather than direct DNA interaction. Finally, apoptosis is a particularly efficient mode of cell death (compared to necrosis for example), and would not lead to local inflammation and damage to surrounding tissue [3]. A potential disadvantage of pro-apoptotic agents is their likely diverse mode of action, making optimisation through structure–activity relationships studies difficult; for this reason targeted therapies are often preferred in drug development.

Targeting the physiological process of apoptosis as a therapeutic strategy relies on the assumption that cancer cells, particularly cancer cell mitochondria, have fundamentally different properties compared to their normal tissue counterparts. The emerging field of

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tumour bioenergetics provides an important clue as to how cancer cells differ from their normal counterparts at the level of cell mitochondria. Alongside their role as the gateway to apoptosis, mitochondria perform a vital role as the "energy factory" of the cell through production of ATP orchestrated under normal conditions through the cycle of oxidative phosphorylation. It is well established that cancer cells exhibit a unique metabolic profile whereby aerobic glycolysis is the main source of energy compared to oxidative phosphorylation in normal cells, a phenomenon first observed back in the 1930's and now known as the "Warburg effect" [5].

In an intriguing study, Bonnet et al. demonstrated that a simple commercially available chemical, the sodium salt of dichloroacetate (DCA), selectively induced apoptosis, reduced proliferation and inhibited growth of cancer cells by reversing this metabolic remodeled process [6] through the inhibition of mitochondrial pyruvate dehydrogenase kinase (PDK2). *In vivo* studies demonstrated that using clinically relevant DCA doses in the drinking water of rats effectively led to the inhibition and prevention of tumour growth with no observed toxicity [6], raising hopes for a potent orally active and cost-effective antitumour agent.

More relevant to the present study was the identification of 3,5-diaryl-1,2,4-oxadiazoles such as compound **1** (Fig. 1) as novel inducers of apoptosis using a high-throughput caspase- and cell-based screening assay [7]. The pro-apoptotic activity of **1** was characterised in human SKBr3 cancer cells, where a reduction in pro-caspase levels and appearance of active caspases-3 and -7 were observed. Induction of apoptosis selectively in human cancer cells (such as breast T47D and ZR75-1, and colorectal DLD-1 and HT29) was found, with EC_{50} values in the range 0.44–3.34 μM. Photo-affinity-labelling studies led to the identification of the mannose-6-phosphate receptor trafficking protein TIP47 as a molecular target of the diaryl-oxadizoles [8]. Over-expression of TIP47 in several tumour types is known [9], illustrating the utility of this chemical genetics approach to cancer drug discovery. Further recent development of the 3,5-diaryl-1,2,4-oxadiazoles as apoptosis-inducing anticancer agents has led to the identification of more water soluble analogues with good in vivo efficacy in cancer xenograft models [10]. Other notable small molecules of current interest known to exert pro-apoptotic effects selectively in cancer cells include the anti-apoptotic Bcl-2-targeting Flex-Hets such as **2** [11], the mitochondria targeting jasmonates such as methyl jasmonate **3** [12], and the 4-anilinoquinazolines such as **4** [13] (Fig. 1).

In view of the utility of the oxadiazole scaffold for the discovery of novel pro-apoptotic antitumour agents [14], we chose to focus on the synthesis and evaluation of new disubstituted oxadiazoles in our own work, in order to design new antitumour agents and further understand structure–activity relationships within this series. In this study we report our antitumour and pro-apoptotic data on a new series of 3,5-diaryloxadiazoles containing an indol-2-yl ring in the oxadiazole 5-position. Indoles are an important heterocyclic component of a number of antitumour agents; for example, the use of indole derivatives as inhibitors of tubulin polymerisation has been reviewed [15]. The present work builds on our research interest and experience in the discovery of novel pro-apoptotic antitumour agents [3,16,17].

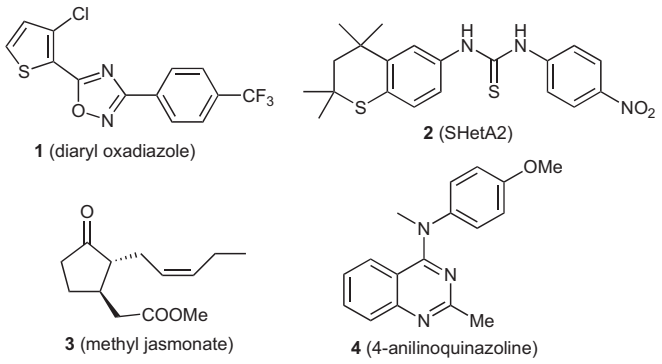


Fig. 1. Representative selective pro-apoptotic antitumor agents.

2. Chemistry results and discussion

Amidoximes **5a**–**d** were readily accessible in high isolated yields (76–99%) via the reaction between benzonitriles and hydroxylamine hydrochloride under basic conditions, according to the method of Quan and Kurth [18]. Indole-based starting materials (**6a**–**e**) for oxadiazole synthesis were readily available either commercially (ethyl-5-chloroindole-2-carboxylate **6e**) or via simple esterification of commercially available materials (in the case of methylindole-2-carboxylate **6a** and methyl-5-methoxyindole-2-carboxylate **6b**). Both ethyl-5-fluoroindole-2-carboxylate **6c** and ethyl-5-nitroindole-2-carboxylate **6d** were prepared from the corresponding anilines via the Fischer indole synthesis in 60% and 52% yield respectively [19].

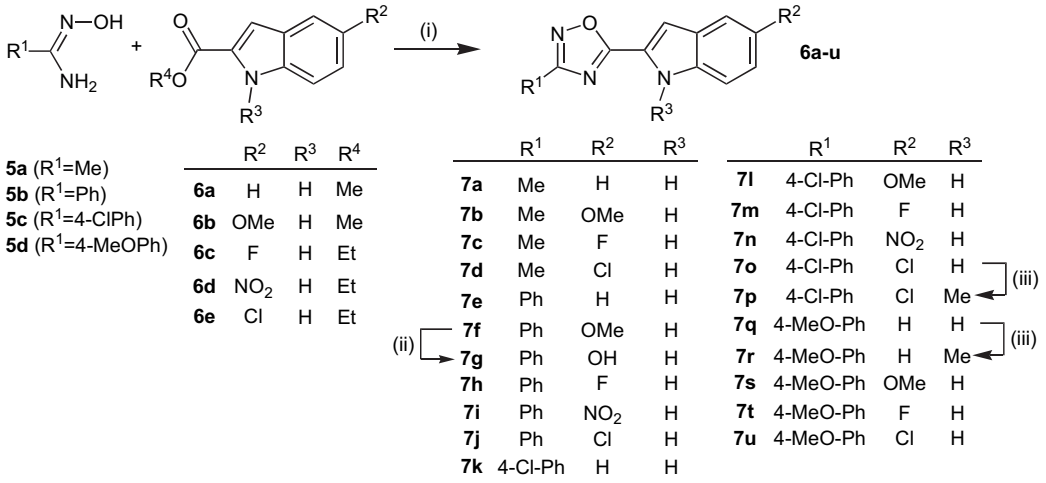
The key step in the synthesis of target structures **7a**–**u** was the base-catalysed condensation reaction between substituted amidoximes (**5a**–**d**) and indole esters (**6a**–**e**), using sodium ethoxide in refluxing ethanol (Scheme 1) to give concise access to oxadiazole products in low to moderate yields (11–47%) [20]. Oxadiazole **7g** containing a phenol group at the indole 5-position was obtained via demethylation of **7f** using boron tribromide in dichloromethane in 85% yield, and the two N-methylindole products (**7p** and **7r**) were accessible by sodium hydride-promoted N-methylation using iodomethane in DMF (83% and 80% yield respectively).

3-Amino-5-(indol-2-yl)-1,2,4-oxadiazole (**10**) was obtained in two steps from indole-2-carboxylic acid chloride **8** (Scheme 2) [21]. Base-promoted (NaH) reaction between the acyl chloride **8** and cyanamide in DMF gave the intermediate *N*-cyano-1*H*-indole-2-carboxamide **9** in 60% yield. Compound **9** was then used without further purification in reaction with hydroxylamine hydrochloride in pyridine/ethanol (1:2) to afford the desired product 3-amino-5-(indol-2-yl)-1,2,4-oxadiazole (**10**) in low yield (8%) following purification using column chromatography.

3. Biological results and discussion

3.1. Structure–activity relationship (SAR) analysis – in vitro inhibition of cellular proliferation

The antitumour effect in vitro of compounds **7a**–**u** and **10** was assessed against a panel of cancer cell lines derived from different human tissues including colon (COLO 320), pancreas (MiaPaCa-2), breast (MCF-7) and peripheral blood of a T cell leukemia patient (Jurkat). Antiproliferative data was compared with the previously reported pro-apoptotic oxadiazole **1** as control [7]. The screening was performed by using a cell proliferation assay based on the mitochondrial dehydrogenase-mediated cleavage of the tetrazolium salt WST-1 to formazan, following 72-h drug exposure. Tetrazolium-based assays (such as MTT, MTS or WST-1) represent one of the most commonly used methods for anticancer drug screening; of relevance here is their application in the assessment of antiproliferative activity of related pro-apoptotic agents [11,12]. Overall, the COLO 320 (colon) and MIA PaCa-2 (pancreatic) cell lines were most responsive to indolyl-oxadiazoles and exposure of the cells to compounds resulted in dose-dependent decrease in cell viability with mean 50% inhibition (IC_{50}) in the micromolar concentration range (Table 1). The most active compounds in the COLO 320 screen (**7n**, IC_{50} 7.7 μM; **7u**, IC_{50} 9.6 μM) were found to be



[a]Reagents and conditions: (i) NaOEt, EtOH, reflux; (ii) BBr_3, CH_2Cl_2; (iii) NaH, MeI, DMF.

Scheme 1. Reagents and conditions: (i) NaOEt, EtOH, reflux; (ii) BBr_3, CH_2Cl_2; (iii) NaH, MeI, DMF.

less active than the previously reported lead compound **1** (IC_{50} 1.2 μM), however a number of active compounds from the present series with low micromolar IC_{50} values compared favourably with compound **1** in the MiaPaCa-2, MCF-7 and Jurkat cancer cell lines where compound **1** was found to be inactive ($IC_{50} > 50$ μM).

In general, structure–activity relationships for cell growth inhibition are difficult to determine within the indolyl-oxadiazole series, and analyses of activity need to be examined individually on each cell line to give meaningful SAR insights. The most active compounds overall in the most responsive COLO 320 and MIA PaCa-2 cell lines were found to be compounds **7n** and **7u**.

3.2. Effect of R^1 (oxadiazole 3-substituent)

In the COLO 320 cell line, the methyl R^1 substituent (compounds **7a**–**d**) gave some of the most potent activity in the series (IC_{50} of 10–20 μM). SAR in the R^1 = (substituted)phenyl series was more variable and difficult to determine, where compounds **7n** and **7u** (R^1 = 4-Cl-Ph and 4-MeO-Ph respectively) were the most active compounds in this series. Similarly SAR's for different R^1 substituents in the MIA PaCa-2 cell line were more difficult to determine.

3.3. Effect of R^2 and R^3 (indole substituents)

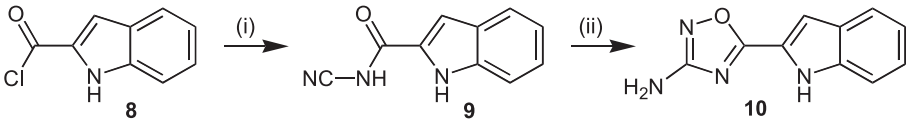
The data clearly demonstrates that both electron-withdrawing groups (e.g. $R^2 = NO_2$, compound **7n**) and electron-donating groups (e.g. R^2 = OMe, compounds **7l**, **7s**) are tolerated on the indole 5-position with respect to potency in COLO 320 and MIA PaCa-2 cell lines. The specific effect of different R^2 substituents is difficult to determine, with related compounds with the same R^2 group giving quite different results (e.g. compound **7u** is the most active of the series in COLO 320 and MIA PaCa-2, whereas the close analogue **7o** is amongst the least active). For most compounds in the series the indole nitrogen was unsubstituted, with the exception of compounds **7p** and **7r**, where R^3 = methyl. Compounds **7p** and **7r** were amongst the least active in the series, implying (albeit in this limited study) that methyl substitution at the indole nitrogen decreases activity.

3.4. In vitro assessment of cardiac cell viability

Potential side effects of test compounds could arise by affecting mitochondria in non-cancerous tissues, particularly those characterised by high abundance of mitochondria (e.g. cardiomyocytes). Therefore, the effect of test compounds was evaluated over 72 h against the H9c2 cardiac muscle cell line at the IC_{50} values determined on COLO320 cells (i.e. the most responsive cells to test compounds). The data presented in Fig. 2 suggests that indolyl-oxadiazole derivatives may exhibit some selectivity for cancer cells since, for all compounds except **7o**, cardiac cell viability was significantly higher than 50% ($P < 0.05$). Similarly, compound **1** was also found to exhibit no substantial effects on cardiac cell viability at the COLO 320 cell IC_{50} concentration. These results appear to be in line with evidence demonstrating that mitochondriotoxic effects at the tumour site may occur in the presence of minimal side effects and a complete lack of cardiotoxicity [22], probably due to differences in the mitochondrial contribution to the cancer cell physiology compared to their normal tissue counterparts.

3.5. In vitro apoptosis induction

Since the cytotoxicity assay did not indicate a specific cellular death mechanism, we investigated the ability of selected compounds to induce internucleosomal degradation of genomic DNA (a hallmark of apoptosis) against sensitive cancer cell lines. Overall, the exposure of COLO 320 cells to compounds resulted in apoptotic cell death; in particular, the active compounds, **7g** and **7h**, displayed similar abilities to induce DNA degradation and



[a]Reagents and conditions: (i) H_2N-CN, NaH, DMF; (ii) NH_2OH.HCl, pyridine/EtOH.

Scheme 2. Reagents and conditions: (i) H_2N–CN, NaH, DMF; (ii) NH_2OH·HCl, pyridine/EtOH.

Table 1
Activity of new substituted indolyl-oxadiazoles **6a**–**u** and **9** against human cancer cell lines.[a]

IC_{50} values (μM)[b] in cell lines[c]				
Compd.	COLO 320	MIA PaCa-2	MCF-7	Jurkat
1	1.2 ± 0.13	>50	>50	>50
7a	11.8 ± 0.8	30.5 ± 1.0	>50	>50
7b	10.4 ± 1.0	>50	16.7 ± 1.0	19.9 ± 1.0
7c	18.1 ± 1.2	19.4 ± 1.0	12.4 ± 1.0	22.9 ± 1.2
7d	19.5 ± 1.0	>50	34.4 ± 1.4	21.6 ± 1.3
7e	12.0 ± 0.9	22.0 ± 1.0	45.7 ± 2.2	>50
7f	18.7 ± 0.7	32.6 ± 1.1	>50	>50
7g	15.7 ± 0.5	13.0 ± 1.2	26.0 ± 1.7	26.9 ± 1.5
7h	10.8 ± 1.2	33.6 ± 1.1	35.0 ± 2.1	33.8 ± 1.2
7i	12.5 ± 1.0	>50	>50	>50
7j	16.5 ± 1.1	23.1 ± 1.3	29.1 ± 1.9	>50
7k	13.9 ± 1.3	19.2 ± 1.2	>50	>50
7l	12.8 ± 1.2	22.7 ± 1.0	>50	>50
7m	>50	>50	>50	>50
7n	7.7 ± 1.0	17.6 ± 1.1	>50	>50
7o	25.0 ± 0.8	>50	>50	>50
7p	16.9 ± 0.9	>50	>50	>50
7q	27.7 ± 0.9	>50	>50	>50
7r	>50	23.1 ± 1.0	>50	>50
7s	28.3 ± 1.2	5.6 ± 1.2	>50	>50
7t	14.8 ± 1.0	10.3 ± 1.0	>50	>50
7u	9.6 ± 1.0	7.8 ± 1.0	>50	>50
10	27.7 ± 1.1	18.7 ± 1.1	>50	>50

[a] Determined by WST-1 assay (72 h drug exposure), see Biological Experimental for details.
[b] Compounds tested in triplicate, data expressed as mean values of independent experiments ± standard error of the mean (SEM).
[c] Cancer cell line origin: COLO 320 (colon), MIA PaCa-2 (pancreas), MCF-7 (breast; ER +ve), Jurkat (leukemia). When the IC_{50} was not reached at 50 μM, compounds were considered to be inactive.

formation of oligonucleosomal fragments (4.3-fold and 5.3-fold respectively over control), whereas the amount of apoptotic DNA generated upon exposure to compounds **7e**, **7j**, **7k**, **7l** and **7n** to COLO 320 cells was from 1.8-fold to 3.0-fold higher than that of untreated control. On the contrary, the production of cytoplasmic oligonucleosomes induced by compounds **7c**, **7s**, **7t** and **7u** was negligible (Table 2). Notably, as observed for COLO 320 cells, treatment of MIA PaCa-2 cells with compounds **7g** and **7h** significantly increased the amount of cytoplasmic histone-associated DNA fragments (7.3-fold and 6.9-fold respectively over control) suggesting that apoptosis may be a major contributor to their

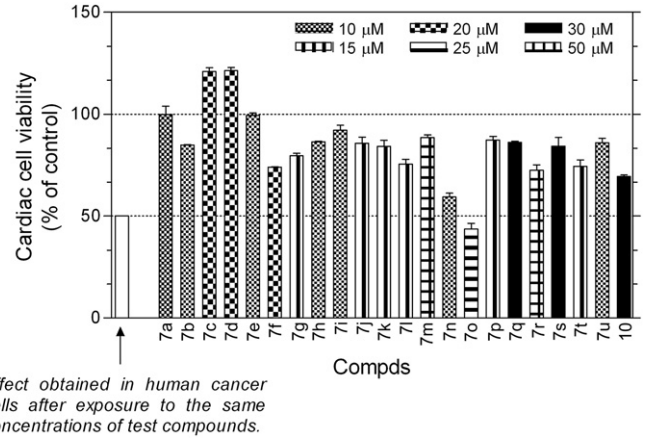


Fig. 2. H9c2 cardiac cell viability following 72 h exposure to test compound concentrations equal to their IC_{50} mean values obtained in the most responsive human cancer cell line (COLO 320). Data are the mean of three experiments and are reported as mean ± standard error of the mean (SEM).

Table 2
Induction of internucleosomal DNA fragmentation (enrichment factor) after treatment with new substituted indolyl-oxadiazoles in the sensitive cell lines.[a]

Compd.	Cell line	
	COLO 320	MIA PaCa-2
7c	1.0 ± 0.2	7.3 ± 0.8
7e	2.5 ± 0.1	1.4 ± 0.1
7g	4.3 ± 1.8	7.3 ± 0.8
7h	5.3 ± 0.6	6.9 ± 0.3
7j	2.1 ± 0.6	1.9 ± 0.1
7k	3.0 ± 0.7	1.0 ± 0.1
7l	1.8 ± 0.3	1.2 ± 0.2
7n	1.8 ± 0.2	2.7 ± 0.9
7s	1.2 ± 0.3	1.9 ± 0.1
7t	1.4 ± 0.4	2.2 ± 0.3
7u	1.2 ± 0.2	1.4 ± 0.1

[a] Determined by Cell Death Detection ELISA assay (100 μM for 24 h drug exposure); see Biological Experimental for details. Compounds were tested in duplicate, data expressed as mean ± SEM.

anticancer action. Otherwise, while the compound **7c** was inactive in the COLO 320 cell line, it was able to consistently induce apoptosis in the MIA PaCa-2 cells (Table 2). Treatment of MIA PaCa-2 cells with compounds **7j**, **7n**, **7s**, and **7t** was associated with an increase in the extent of DNA fragmentation from 1.9 to 2.7-fold, while the effect of compounds **7e**, **7k**, **7l** and **7u** was negligible (Table 2).

An intriguing aspect of this work is that some of test compounds that were found to be very active in the cytotoxicity assay (such as **7u**), did not induce apoptosis in MIA PaCa-2 cells. To clarify this apparent discrepancy, we evaluated whether the compound **7u** (taken as a paradigmatic example) was able to promote necrosis against MIA PaCa-2 cells. As a matter of fact, when given at 100 μM for 24 h, compound **7u** induced plasma membrane ruptures with the consequent accumulation of oligonucleosomes in the supernatant (necrosis), but not in the cytoplasmic fraction (apoptosis) of MIA PaCa-2 cells (Fig. 3).

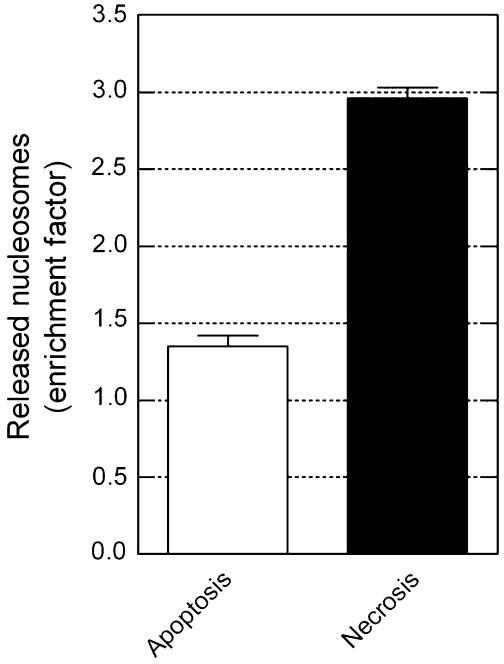


Fig. 3. Enrichment of nucleosomes in the cytoplasm (white bar) or in the supernatant (black bar) of MIA PaCa-2 cells treated with the compound **7u** at 100 μM for 24 h.

To explore in more detail the underlying mechanism of apoptosis induced by the indolyl-oxadiazole compounds, we assessed whether caspase-3 and/or caspase-7, two enzymes involved in the effector phase of apoptosis, were a target of the new indolyl-substituted oxadiazole compounds. Caspase activation (2.1- to 6.4-fold induction) was consistently observed after COLO 320 cells were incubated with compounds **7e**, **7g**, **7h**, **7l**, **7n**, **7t** and **7u** (Table 3). Of note, for most test compounds, such an effect was associated with DNA fragmentation (see Table 2) suggesting that, at least in this specific cell type, caspase activation may contribute to their pro-apoptotic action. Results in Table 3 show that caspase-3/7 activity was marginally altered in MIA PaCa-2 cells after treatment with the pro-apoptotic compounds **7c**, **7e**, **7g**, **7h**, **7k** and **7u**; suggesting that apoptosis may also occur through a caspase-independent pathway, a mechanism already observed in human pancreatic adenocarcinoma cell lines [23]. Therefore, in the pancreatic cancer cell line, MIA PaCa-2, the analysis of caspase-3/7 activity was not able to entirely predict the occurrence of apoptosis; accordingly, the compound **7l** significantly induced caspase activity without DNA fragmentation (see Table 2). This notion seems to be in line with data showing that, in the absence of apoptotic stimuli, tumour cells with high levels of the anti-apoptotic proteins, survivin and XIAP, such as MIA PaCa-2, displayed a low sensitivity to apoptosis induction by overexpression of caspase genes [24]. However, when the activity of caspase-3/7 was markedly increased in MIA PaCa-2 cells (see **7n** and **7t**; Table 3), such an effect was associated with a modest but significant induction of apoptotic cell death (Table 2).

4. Conclusions

Antitumour data from the present study demonstrates in vitro anticancer effects of new indole-based 3,5-substituted oxadiazoles, particularly against cancer cell lines derived from colon (COLO 320) and pancreatic (MiaPaCa-2) tissues. The new compounds were found to be both synthetically accessible in one key chemical step, and amenable to generation of a small library of compounds for structure–activity analysis. A number of compounds were found to have IC_{50} values in the low micromolar range (notably **7n** and **7u**), and corresponding tests on the H9c2 cardiac muscle cell line (characterised by a high abundance of mitochondria) revealed selectivity for cancer cell lines in most cases.

Through structure–activity relationship studies of indole-based 3,5-disubstituted oxadiazoles, we found in particular that certain compounds characterised by an unsubstituted indole nitrogen and a phenyl substituent at the R^1 position (**7g** and **7h**) were able to induce apoptotic cell death in both COLO 320 and MIA PaCa-2 cell lines, whereas the structurally-related compounds **7e** and **7k** induced apoptosis only in COLO 320 cells.

Some of the test compounds were found to be active in promoting apoptosis via activation of caspase-3/7, most notably compounds **7l** (R^1 = 4-Cl-Ph; R^2 = OMe), **7n** (R^1 = 4-Cl-Ph; R^2 = NO_2) and **7t** (R^1 = 4-MeO-Ph; R^2 = F) or through a caspase-independent pathway, depending on the cell type and compound examined. For example, cell necrosis was demonstrated for the compound **7u** (R^1 = 4-MeO-Ph; R^2 = Cl) and this type of cell death may be also taken into account to interpret the cytotoxic effects of other compounds. These results are relevant considering that development of resistant mechanisms for apoptosis confers high survival ability and low drug sensitivity of tumour cells.

Table 3
Induction of caspase-3/7 activity (fold change over control values) after treatment with new substituted indolyl-oxadiazoles in the sensitive cell lines.[a]

Compd.	Cell line	
	COLO 320	MIA PaCa-2
7c	1.5 ± 0.2	1.3 ± 0.2
7e	3.1 ± 0.1	1.2 ± 0.3
7g	3.6 ± 0.1	0.7 ± 0.1
7h	4.4 ± 0.6	1.2 ± 0.3
7j	1.3 ± 0.1	1.9 ± 0.4
7k	0.3 ± 0.1	1.2 ± 0.1
7l	4.5 ± 0.1	10.1 ± 1.7
7n	6.4 ± 0.2	23.9 ± 6.4
7s	1.1 ± 0.2	2.0 ± 0.4
7t	2.1 ± 0.3	5.0 ± 1.6
7u	4.2 ± 0.4	1.4 ± 0.6

[a] Determined by Apo-ONE Homogeneous Caspase-3/7 assay (100 μM for 24 h drug exposure); see Biological Experimental for details. Compounds were tested in duplicate, data expressed as mean ± SEM.

5. Experimental

5.1. Chemistry

Melting points were measured on a Griffin apparatus and are uncorrected. NMR spectra were recorded on a Bruker AVANCE 500 MHz instrument; coupling constants (*J* values) are in Hz. Merck silica gel 60 (40–60 μM) was used for column chromatography. All commercially available starting materials were used without further purification, and purchase/synthesis of the required amidoxime (**5a**–**d**) and indolyl ester (**6a**–**e**) starting materials was carried out according to well established literature methods as described in the chemistry results section. Compound **1** was synthesised as previously described [7]. The purity of tested compounds was found to be ≥95% as determined by combustion analysis (% C, H, N values with ±0.4% of theoretical; carried out in duplicate by Medac Ltd, U.K., www.medacltd.com) in the case of new compounds **7a**-**7u**, and accurate mass spectrometry (EPSRC National Mass Spectrometry Centre, Swansea, U.K.) in the case of the 3-aminooxadiazole **10**.

5.1.1. General method for the synthesis of 3-substituted-5-(5-substituted-1H-indol-2-yl)-1,2,4-oxadiazoles (*7a–f, 7h–o, 7q and 7s–u*)

To a stirred solution of sodium ethoxide in ethanol (2.6 mL of 21% solution in ethanol, 8 mmol) was added amidoxime (**5a**–**d**, 4 mmol). The mixture was stirred at room temperature for 30 min, then indole-2-carboxylate (**6a**–**e**, 4 mmol) was added and the mixture was heated under reflux for 2–5 h. The reaction mixture was left to cool to room temperature, then H_2O was added, and the precipitate formed was collected by filtration and recrystallised from ethanol to give pure indolyl-oxadiazole product in 8–48% yield.

*5.1.1.1. 5-(Indol-2-yl)-3-methyl-1,2,4-oxadiazole (**7a**).* Yield 11%; mp 121–122 °C. ^{1}H NMR ($CDCl_3$) δ 2.47 (3H, s, CH_3), 7.12 (1H, td, J = 7.5, 0.9 Hz, Ar-H), 7.28 (1H, td, J = 7.7, 1.1 Hz, Ar-H), 7.31 (1H, dd, J = 7.1, 0.9 Hz, Ar-H), 7.38 (1H, dd, J = 8.3, 0.9 Hz, Ar-H), 7.65 (1H, dd, J = 8.15, 0.80 Hz, Ar-H), 8.98 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 11.9 (CH_3), 108.19, 111.8, 121.3, 122.4, 125.7 (C3, C4, C5, C6, C7 of indole), 121.5, 127.8, 137.4 (C2, C3a, C7a of indole), 167.4, 169.8 (C3, C5 of oxadiazole). Anal. calcd for $C_{11}H_9N_3O$: C, 66.32; H, 4.55; N, 21.09. Found: C, 66.13; H, 4.54; N, 21.09.

*5.1.1.2. 5-(5-Methoxyindol-2-yl)-3-methyl-1,2,4-oxadiazole (**7b**).* Yield 13%; mp 127–130 °C. ^{1}H NMR ($CDCl_3$) δ 2.48 (3H, s, CH_3), 3.90 (3H, s, OCH_3), 7.02 (1H, dd, J = 8.8, 2.5 Hz, Ar-H), 7.10 (1H, s, Ar-H), 7.25 (1H, d, J = 2.9 Hz, Ar-H), 7.32 (1H, d, J = 8.8 Hz, Ar-H), 9.05 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 11.6 (CH_3), 55.5 (OCH_3), 102.4, 107.7, 112.7, 117.3

(C3, C4, C6, C7 of indole), 121.9, 128.3, 132.8, 155.1 (C2, C3a, C5, C7a of indole), 167.4, 169.7 (C3, C5 of oxadiazole). Anal. calcd for $C_{12}H_{11}N_3O_2$: C, 62.87; H, 4.84; N, 18.33. Found: C, 62.67; H, 4.87; N, 18.17.

*5.1.1.3. 5-(5-Fluoroindol-2-yl)-3-methyl-1,2,4-oxadiazole (**7c**).* Yield 15%; mp 148–149 °C. ^{1}H NMR (DMSO-d_6) δ 2.45 (3H, s, CH_3), 7.15–7.18 (1H, m, Ar-H), 7.34 (1H, s, Ar-H), 7.46–7.50 (2H, m, Ar-H), 12.5 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 11.1 (CH_3) 105.9, 106.8, 113.7, 113.9 (C3, C4, C6, C7 of indole), 122.8, 127.2, 134.8, 156.5, 158.4 (C2, C3a, C5, C7a of indole), 167.5, 169.2 (C3, C5 of oxadiazole). Anal. calcd for $C_{11}H_8FN_3O$: C, 60.83; H, 3.71; N 19.35. Found: C, 60.79; H, 3.61; N, 18.87.

*5.1.1.4. 5-(5-Chloroindol-2-yl)-3-methyl-1,2,4-oxadiazole (**7d**).* Yield 14%; mp 174–175 °C. ^{1}H NMR ($CDCl_3$) δ 2.50 (3H, s, CH_3), 7.28–7.31 (2H, m, Ar-H), 7.37 (1H, d, J = 8.8 Hz, Ar-H), 7.69 (1H, s, Ar-H), 9.13 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 11.6 (CH_3), 107.4, 112.9, 121.6, 126.2 (C3, C4, C6, C7 of indole), 121.7, 127.1, 128.8, 135.6 (C2, C3a, C5, C7a of indole), 167.5, 169.8 (C3, C5 of oxadiazole). Anal. calcd for $C_{11}H_8ClN_3O$: C, 56.54; H, 3.45; N, 17.98. Found: C, 56.28; H, 3.34; N, 17.72.

*5.1.1.5. 5-(Indol-2-yl)-3-phenyl-1,2,4-oxadiazole (**7e**).* Yield 42%. mp 168–169 °C. ^{1}H NMR (DMSO-d_6) δ 7.15 (1H, td, J = 7.6, 0.9 Hz, Ar-H), 7.33 (1H, td, J = 7.6, 1.0 Hz, Ar-H), 7.49 (1H, d, J = 1.1 Hz, Ar-H), 7.55 (1H, dd, J = 8.3, 0.9 Hz, Ar-H), 7.61–7.67 (3H, m, Ar-H), 7.74 (1H, d, J = 8.0 Hz, Ar-H), 8.11–8.13 (2H, m, Ar-H), 12.49 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 107.5, 112.6, 120.7, 122.0, 125.9 (C3, C4, C5, C6, C′7 of indole), 127.2, 129.3, 131.7 (C2, C3, C4, C5, C6 of phenyl), 121.0, 127.1, 138.2 (C2, C3a, C7a of indole), 126.1 (C1 of phenyl), 168.1, 170.2 (C3, C5 of oxadiazole). Anal. calcd for $C_{16}H_{11}N_3O$: C, 73.55; H, 4.24; N, 16.08. Found: C, 73.38; H, 4.07; N, 15.98.

*5.1.1.6. 5-(5-Methoxyindol-2-yl)-3-phenyl-1,2,4-oxadiazole (**7f**).* Yield 37%; mp 147–149 °C. ^{1}H NMR ($CDCl_3$) δ 3.89 (3H, s, OCH_3), 7.03 (1H, dd, J = 9.1, 2.5 Hz, Ar-H), 7.12 (1H, d, J = 2.4 Hz, Ar-H), 7.25 (1H, s, Ar-H), 7.35 (1H, m, Ar-H), 7.48–7.54 (3H, m, Ar-H), 8.15 (2H, m, Ar-H), 9.02 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 55.7 (OCH_3), 102.5, 107.8, 112.7, 117.4 (C3, C4, C6, C7 of indole), 127.5, 128.9, 131.3 (C2, C3, C4, C5, C6 of phenyl), 121.5, 128.4, 132.8, 155.1 (C2, C3a, C5, C7a of indole), 126.7 (C1 of phenyl), 168.6, 169.9 (C3, C5 of oxadiazole). Anal. calcd for $C_{17}H_{13}N_3O_2$: C, 70.09; H, 4.05; N, 14.42. Found: C, 69.90; H, 4.37; N, 14.37.

*5.1.1.7. 5-(5-Fluoroindol-2-yl)-3-phenyl-1,2,4-oxadiazole (**7h**).* Yield 36%; mp 169–170 °C. ^{1}H NMR (DMSO-d_6) δ 7.20 (1H, td, J = 9.3, 2.6 Hz, Ar-H), 7.45 (1H, s, Ar-H), 7.50 (1H, dd, J = 9.7, 2.5 Hz, Ar-H), 7.55 (1H, dd, J = 9.0, 4.6 Hz, Ar-H), 7.60–7.66 (3H, m, Ar-H), 8.11 (2H, m, Ar-H), 12.60 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 106.8, 108.1, 112.8, 114.8 (C3, C4, C6, C7 of indole), 127.6, 129.0, 131.5 (C2, C3, C4, C5, C6 of phenyl), 122.6, 127.3, 131.9, 156.5, 158.4 (C2, C3a, C5, C7a of indole), 126.0 (C1 of phenyl), 168.2, 169.9 (C3, C5 of oxadiazole). Anal. calcd for $C_{16}H_{10}FN_3O$: C, 68.81; H, 3.61; N, 15.05. Found: C, 68.73; H, 3.52; N, 14.79.

*5.1.1.8. 5-(5-Nitroindol-2-yl)-3-phenyl-1,2,4-oxadiazole (**7i**).* Yield 39%; mp 240–241 °C. ^{1}H NMR (DMSO-d_6) δ 7.67–7.73 (3H, m, Ar-H), 7.75 (1H, d, J = 9.1 Hz, Ar-H), 7.82 (1H, s, Ar-H), 8.17 (2H, m, Ar-H), 8.28 (1H, dd, J = 9.1, 2.4 Hz, Ar-H), 8.84 (1H, d, J = 2.3 Hz, Ar-H), 13.3 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 109.7, 112.1, 119.6, 120.9 (C3, C4, C6, C7 of indole), 127.6, 129.0, 133.4 (C2, C3, C4, C5, C6 of phenyl), 124.6, 125.9, 140.8, 141.8 84 (C2, C3a, C5, C7a of índole), 126.43 (C1 of phenyl), 168.3, 169.5 (C3, C5 of oxadiazole). Anal. calcd for $C_{16}H_{10}N_4O_3.0.67H_2O$: C, 60.30; H, 3.69; N, 17.58. Found: C, 60.09; H, 3.58; N, 17.93.

*5.1.1.9. 5-(5-Chloroindol-2-yl)-3-phenyl-1,2,4-oxadiazole (**7j**).* Yield 45%. mp 184–185 °C. ^{1}H NMR ($CDCl_3$) δ 7.32 (1H, dd, J = 8.8, 2.1 Hz, Ar-H), 7.38 (1H, dd, J = 2.1, 0.9 Hz, Ar-H), 7.41 (1H, d, J = 8.8 Hz, Ar-H), 7.50–7.56 (3H, m, Ar-H), 7.72 (1H, d, J = 2.0 Hz, Ar-H), 8.15 (2H, m, Ar-H), 9.15 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 107.5, 112.9, 121.6, 126.3 (C3, C4, C6, C7 of indole), 127.6, 129.0, 131.4 (C2, C3, C4, C5, C6 of phenyl), 122.8, 126.5, 127.1, 128.8, 135.7 (C2, C3a, C5, C7a of índole and C1 of phenyl), 168.7, 169.5 (C3, C5 of oxadiazole). Anal. calcd for $C_{16}H_{10}ClN_3O$: C, 64.98; H, 3.41; N, 14.21. Found: C, 65.05; H, 3.27; N, 14.13.

*5.1.1.10. 3-(4-Chlorophenyl)-5-(indol-2-yl)-1,2,4-oxadiazole (**7k**).* Yield 41%. mp 188–189 °C. ^{1}H NMR (DMSO-d_6) δ 7.15 (1H, t, J = 7.5 Hz, Ar-H), 7.33 (1H, t, J = 7.7 Hz, Ar-H), 7.34 (1H, d, J = 8.3 Hz, Ar-H), 7.49 (1H, s, Ar-H), 7.70 (1H, dd, J = 6.8, 1.9 Hz, Ar-H), 7.75 (1H, d, J = 8.1 Hz, Ar-H), 8.12 (2H, dd, J = 6.8, 1.9 Hz, Ar-H), 12.50 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 107.7, 112.6, 120.7, 121.9, 125.2 (C3, C4, C5, C6, C7 of indole), 128.9, 129.7 (C2, C3, C5, C6 of phenyl), 120.9, 127.2, 138.4 (C2, C3a, C7a of indole), 125.0 (C1 of phenyl), 136.4 (C4 of phenyl), 167.3, 170.4 (C3, C5 of oxadiazole). Anal. calcd for $C_{16}H_{10}ClN_3O$: C, 64.98; H, 3.41; N, 14.21. Found: C, 64.85; H, 3.41; N, 13.79.

*5.1.1.11. 3-(4-Chlorophenyl)-5-(5-methoxyindol-2-yl)-1,2,4-oxadiazole (**7l**).* Yield 35%; mp 184–185 °C. ^{1}H NMR (DMSO-d_6) δ 3.83 (3H, s, OCH_3), 7.00 (1H, dd, J = 8.9, 2.5 Hz, Ar-H), 7.18 (1H, d, J = 2.3 Hz, Ar-H), 7.38 (1H, s, Ar-H), 7.43 (1H, d, J = 8.9 Hz, Ar-H), 7.70 (2H, dd, J = 6.7, 1.8 Hz, Ar-H), 8.11 (2H, dd, J = 6.7, 1.8 Hz, Ar-H), 12.40 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 55.2 (OCH_3), 101.8, 107.2, 113.5, 116.8 (C3, C4, C6, C7 of indole), 128.9, 129.3 (C2, C3, C5, C6 of phenyl), 121.0, 127.5, 133.6, 154.3 (C2, C3a, C5, C7a of indole), 125.0 (C1 of phenyl), 136.3 (C4 of phenyl), 167.3, 170.3 (C3, C5 of oxadiazole). Anal. calcd for $C_{17}H_{12}ClN_3O_2$: C, 62.68; H, 3.71; N, 12.90. Found: C, 62.40; H, 3.58; N, 12.64.

*5.1.1.12. 3-(4-Chlorophenyl)-5-(5-fluoroindol-2-yl)-1,2,4-oxadiazole (**7m**).* Yield 15%; mp 225–226 °C. ^{1}H NMR (DMSO-d_6) δ 7.20 (1H, td, J = 9.3, 2.6 Hz, Ar-H), 7.47 (1H, s, Ar-H), 7.51 (1H, dd, J = 9.0, 2.5 Hz, Ar-H), 7.55 (1H, q, J = 4.6 Hz, Ar-H), 7.71 (2H, dd, J = 6.9, 1.9 Hz, Ar-H), 8.12 (2H, dd, J = 6.7, 1.9 Hz, Ar-H), 12.62 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 105.0, 107.5, 113.7, 114.8 (C3, C4, C6, C7 of indole), 127.2, 128.9 (C2, C3, C5, C6 of phenyl), 122.5, 127.2, 135.0, 156.5, 158.4 (C2, C3a, C5, C7a of indole), 124.9 (C1 of phenyl), 136.4 (C4 of phenyl), 167.3, 170.1 (C3, C5 of oxadiazole). Anal. calcd for $C_{16}H_9FClN_3O$: C, 61.26; H, 2.89; N, 13.39. Found: C, 61.01; H, 2.80; N, 13.17.

*5.1.1.13. 3-(4-Chlorophenyl)-5-(5-nitroindol-2-yl)-1,2,4-oxadiazole (**7n**).* Yield 27%; mp 257–259 °C. ^{1}H NMR ($CDCl_3$) δ 7.51 (2H, dd, J = 8.4, 1.8 Hz, Ar-H), 7.58 (1H, d, J = 9.1 Hz, Ar-H), 7.62 (1H, s, Ar-H), 8.10 (2H, dd, J = 8.1, 1.8 Hz, Ar-H), 8.28 (1H, dt, J = 9.1, 2.0 Hz, Ar-H), 8.74 (1H, s, Ar-H), 9.48 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 109.9, 113.3, 119.4, 119.8 (C3, C4, C6, C7 of indole), 129.0, 129.5 (C2, C3, C5, C6 of phenyl), 124.8, 136.5 (C1, C4 of phenyl), 126.4, 141.0, 141.8 (C2, C3a, C5, C7a of vndole), 167.5, 169.6 (C3, C5 of oxadiazole). Anal. calcd for $C_{16}H_9ClN_4O_3.H_2O$: C, 53.57; H, 3.09; N, 15.62. Found: C, 53.14; H, 3.40; N, 15.15.

*5.1.1.14. 5-(5-Chloroindol-2-yl)-3-(4-chlorophenyl)-1,2,4-oxadiazole (**7o**).* Yield 47%; mp 212–214 °C. ^{1}H NMR (DMSO-d_6) δ 7.33 (1H, dd, J = 8.8, 2.1 Hz, Ar-H), 7.46 (1H, s, Ar-H), 7.54 (1H, d, J = 8.8 Hz, Ar-H), 7.70 (2H, dd, J = 6.7, 2.0 Hz, Ar-H), 7.81 (1H, d, J = 1.9 Hz, Ar-H), 8.15 (2H, dd, J = 6.7, 2.0 Hz, Ar-H), 12.70 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 107.1, 114.2, 121.0, 125.2 (C3, C4, C6, C7 of indole), 128.9, 129.5 (C2, C3, C5, C6 of phenyl), 122.6, 125.2, 128.1, 136.4 (C2, C3a, C5, C7a of

indole), 124.9 (C1 of phenyl), 136.6 (C4 of phenyl), 167.3, 170.4 (C3, C5 of oxadiazole). Anal. calcd for $C_{16}H_9Cl_2N_3O$: C, 58.20; H, 2.75; N, 12.73. Found: C, 58.27; H, 2.59; N, 12.63.

*5.1.1.15. 5-(Indol-2-yl)-3-(4-methoxyphenyl)-1,2,4-oxadiazole (**7q**).* Yield 39%; mp 157–159 °C. ^{1}H NMR ($CDCl_3$) δ 3.90 (3H, s, OCH_3), 7.02 (2H, m, Ar-H), 7.20 (1H, td, J = 7.6, 0.9 Hz, Ar-H), 7.37 (1H, td, J = 7.7, 1.1 Hz, Ar-H), 7.44 (1H, dd, J = 2.1, 0.9 Hz, Ar-H) 7.49 (1H, dd, J = 8.3, 0.9 Hz, Ar-H), 7.74 (1H, dd, J = 8.1, 0.8 Hz, Ar-H), 8.12 (2H, m, Ar-H), 9.09 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 55.4 (OCH_3), 108.1, 111.8, 121.3, 122.4, 125.7 (C3, C4, C5, C6, C7 of indole), 114.3, 129.2 (C2, C3, C5, C6 of phenyl), 121.7, 127.9, 137.4 (C2, C3a, C7a of índole), 119.1, 162.1 (C1, C4 of phenyl), 168.3, 169.7 (C3, C5 of oxadiazole). Anal. calcd for $C_{17}H_{13}N_3O_2$: C, 70.09; H, 4.05; N, 14.42. Found: C, 69.81; H, 4.39; N. 14.30.

*5.1.1.16. 5-(5-Methoxyindol-2-yl)-3-(4-methoxyphenyl)-1,2,4-oxadiazole (**7s**).* Yield 27%; mp 174–176 °C. ^{1}H NMR (DMSO-d_6) δ 3.90 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 7.03 (1H, dd, J = 9.0, 2.5 Hz, Ar-H), 7.22 (1H, d, J = 2.4 Hz, Ar-H), 7.41 (1H, s, Ar-H), 7.48 (1H, d, J = 8.9 Hz, Ar-H), 7.24 (2H, d, J = 8.9 Hz, Ar-H), 8.10 (2H, d, J = 8.9 Hz, Ar-H), 12.35 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 55.3 (OCH_3), 55.4 (OCH_3), 101.9, 106.9, 113.4, 116.6 (C3, C4, C6, C7 of indole), 114.7, 128.8 (C2, C3, C5, C6 of phenyl), 121.3, 127.6, 133.5, 154.3 (C2, C3a, C5, C7a of indole), 118.4 (C1 of phenyl), 161.8 (C4 of phenyl), 167.4, 169.3 (C3, C5 of oxadiazole). Anal. calcd for $C_{18}H_{15}N_3O_3$: C, 67.28; H, 4.71; N, 13.08. Found: C, 67.14; H, 4.66; N, 12.93.

*5.1.1.17. 5-(5-Fluoroindol-2-yl)-3-(4-methoxyphenyl)-1,2,4-oxadiazole (**7t**).* Yield 20%; mp 190–191 °C. ^{1}H NMR ($CDCl_3$) δ 3.90 (3H, s, OCH_3), 7.02 (2H, dd, J = 6.9, 2.1 Hz, Ar-H), 7.13 (1H, td, J = 9.0, 2.5 Hz, Ar-H), 7.36 (1H, d, J = 2.5 Hz, Ar-H), 7.39 (1H, m, Ar-H), 8.42 (1H, dd, J = 9.0, 4.3 Hz, Ar-H), 8.09 (2H, dd, J = 6.9, 2.1 Hz, Ar-H), 9.1 (1H, s, NH). ^{13}C NMR ($CDCl_3$) δ 55.4 (OCH_3), 106.7, 107.9, 112.6, 114.8 (C3, C4, C6, C7 of indole), 114.4, 129.2 (C2, C3, C5, C6 of phenyl), 118.3, 161.8 (C1, C4 of phenyl), 122.8, 127.3, 134.9, 156.5 (C2, C3a, C5, C7a of índole), 167.8, 169.6 (C3, C5 of oxadiazole). Anal. calcd for $C_{17}H_{12}FN_3O_2$: C, 66.02; H, 3.91; N, 13.59. Found: C, 65.95; H, 3.84; N, 13.42.

*5.1.1.18. 5-(5-Chloroindol-2-yl)-3-(4-methoxyphenyl)-1,2,4-oxadiazole (**7u**).* Yield 46%; mp 221–222 °C. ^{1}H NMR (DMSO-d_6) δ 3.90 (3H, s, OCH_3), 7.03 (2H, dd, J = 6.5, 2.1 Hz, Ar-H), 7.31 (1H, dd, J = 8.8, 2.0 Hz, Ar-H), 7.37 (1H, d, J = 2.1 Hz, Ar-H), 7.42 (1H, d, J = 8.8 Hz, Ar-H), 7.72 (1H, d, J = 2.0 Hz, Ar-H), 8.08 (2H, dd, J = 6.9, 2.1 Hz, Ar-H), 12.65 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 55.4 (OCH_3), 107.4, 112.9, 121.6, 126.2 (C3, C4, C6, C7 of indole), 114.4, 129.2 (C2, C3, C5, C6 of phenyl), 123.0, 127.1, 128.8, 135.7 (C2, C3a, C5, C7a of indole), 119.0 (C1 of phenyl), 162.2 (C4 of phenyl), 168.4, 169.3 (C3, C5 of oxadiazole). Anal. calcd for $C_{17}H_{12}ClN_3O_2$: C, 62.68; H, 3.71; N, 12.90. Found: C, 62.40; H, 3.58; N, 12.64.

*5.1.2. Synthesis of 5-(5-hydroxyindol-2-yl)-3-phenyl-1,2,4-oxadiazole (**7g**)*

An ice-cold solution of 5-(methoxyindol-2-yl)-3-phenyl-1,2,4-oxadiazole **7f** (0.766 g, 2.63 mmol) was slowly added to a solution of boron tribromide (5.4 mL of 1 M in CH_2Cl_2, 5.4 mmol), followed by stirring at ambient temperature overnight. The resulting mixture was then poured onto ice (250 g) and the aqueous phase extracted with ethyl acetate (3 × 100 mL). The combined organic extracts were washed with water and brine (100 mL), dried (Na_2SO_4) and concentrated *in vacuo*. The resulting solid was washed with excess diethyl ether and dried under vacuum to give the required product **7g** as a yellow solid. Yield 85%; mp 205–207 °C. ^{1}H NMR (DMSO-d_6) δ 6.82 (1H, t, J = 7.6 Hz, Ar-H), 7.01 (1H, t, J = 8.1 Hz, Ar-H), 7.30 (1H, s, Ar-H), 7.43 (1H, d, J = 7.9 Hz, Ar-H), 7.60 (3H, m, Ar-H), 8.11 (2H, d, J = 7.8 Hz, Ar-H), 9.03 (1H, s, OH), 12.50 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 104.3, 106.5, 113.1, 116.6 (C3, C4, C6, C7 of indole), 127.1, 129.2, 131.6 (C2, C3, C4, C5, C6 of phenyl), 121.5, 128.4, 132.8, 155.1 (C2, C3a, C5, C7a of indole), 126.7 (C1 of phenyl), 168.6, 169.9 (C3, C5 of oxadiazole). Anal. calcd for $C_{16}H_{11}N_3O_2.H_2O$: C, 65.08; H, 4.44; N, 14.23. Found: C, 64.92; H, 4.36; N, 14.40.

*5.1.3. Synthesis of N-methylated indolyl-oxadiazoles (**7p** and **7r**)*

Sodium hydride (24.3 mg, 1 mmol) was added to a stirred solution of the 1*H*-indolyl-oxadiazole precursor **7o** or **7q** (1 mmol) in dry DMF (5 mL) under N_2 atmosphere at 5 °C. The reaction mixture was stirred at 5 °C for 30 min, then iodomethane (0.17 g, 1.2 mmol) was added and the mixture stirred at ambient temperature for 8 h. Water (50 mL) was added and the mixture extracted using ethyl acetate (3 × 50 mL). The organic extracts were dried ($MgSO_4$) and concentrated *in vacuo*. Recrystallisation of the residue from ethanol afforded the required product **7p** or **7r**.

*5.1.3.1. 3-(4-Chlorophenyl)-5-(5-chloro-1-methylindol-2-yl)-1,2,4-oxadiazole (**7p**).* Yield 83%; mp 182–184 °C. ^{1}H NMR ($CDCl_3$) δ 4.25 (3H, s, NCH_3), 7.36 (1H, dd, J = 8.9, 1.9 Hz, Ar-H), 7.40 (1H, d, J = 8.9 Hz, Ar-H), 7.45 (1H, s, Ar-H), 7.52 (2H, d, J = 6.7 Hz, Ar-H), 7.72 (1H, d, J = 1.8 Hz, Ar-H), 8.15 (2H, d, J = 6.7 Hz, Ar-H). ^{13}C NMR ($CDCl_3$) δ32.3(NCH_3), 108.5, 111.5, 121.4, 126.9 (C3, C4, C6, C7 of indole), 128.9, 129.2 (C2, C3, C5, C6 of phenyl), 122.6, 125.2, 128.1, 136.4 (C2, C3a, C5, C7a of indole), 124.9 (C1 of phenyl), 136.6 (C4 of phenyl), 167.8, 169.2 (C3, C5 of oxadiazole). Anal. calcd for $C_{17}H_{11}Cl_2N_3O$: C, 59.32; H, 3.22; N, 12.21. Found: C, 59.12; H, 2.97; N, 11.91.

*5.1.3.2. 3-(4-Methoxyphenyl)-5-(1-methylindol-2-yl)-1,2,4-oxadiazole (**7r**).* Yield 80%; mp 128–129 °C. ^{1}H NMR ($CDCl_3$) δ 3.90 (3H, s, OCH_3), 4.30 (3H, s, NCH_3), 7.02 (2H, dd, J = 6.9, 2.0 Hz, Ar-H), 7.19 (1H, td, J = 7.5, 0.9 Hz, Ar-H), 7.38 (1H, td, J = 7.5, 1.0 Hz, Ar-H), 7.44 (1H, d, J = 8.4 Hz, Ar-H) 7.49 (1H, s, Ar-H), 7.72 (1H, d, J = 8.1 Hz, Ar-H), 8.12 (2H, dd, J = 6.9, 2.0 Hz, Ar-H). ^{13}C NMR ($CDCl_3$) δ 32.0 (NCH_3), 55.2 (OCH_3), 109.0, 110.3, 120.9, 122.4, 125.1 (C3, C4, C5, C6, C7 of indole), 114.3, 129.2 (C2, C3, C5, C6 of phenyl), 122.4, 127.0, 139.8 (C2, C3a, C7a of índole), 118.9 (C1 of phenyl), 162.0 (C4 of phenyl), 168.2, 170.0 (C3, C5 of oxadiazole). Anal. calcd for $C_{18}H_{15}N_3O_2$: C, 70.81; H, 4.95; N, 13.76. Found: C, 70.76; H, 5.19; N, 13.34.

*5.1.4. Synthesis of 1H-indole-2-carbonyl cyanamide (**9**)*

Sodium hydride (0.49 g, 20 mmol) was added slowly to a solution of cyanamide (0.84 g, 20 mmol) in anhydrous DMF (20 mL) under nitrogen atmosphere at 0–5 °C. The mixture was stirred for 30 min at 5 °C, then a solution of índole-2-carbonyl chloride (1.80 g, 10 mmol) in anhydrous DMF (10 mL) was added, followed by stirring at ambient temperature for 1 h. Diethyl ether (200 mL) was added and the resulting precipitate collected by vacuum filtration. The filtrate was extracted with water (100 mL), and the aqueous extract acidified to pH 2 using HCl (aq) and extracted using ethyl acetate (3 × 100 mL). The combined organic layers were dried ($MgSO_4$) and concentrated *in vácuo* to afford the required product in 60% yield, which was used in the next step without further purification. ^{1}H NMR (DMSO-d_6) δ 7.10 (1H, td, J = 7.5, 0.9 Hz, Ar-H), 7.29 (1H, td, J = 7.6, 1.0 Hz, Ar-H), 7.35 (1H, d, J = 1.3 Hz, Ar-H), 7.46 (1H, d, J = 8.4 Hz, Ar-H), 7.71 (1H, d, J = 8.1 Hz, Ar-H), 12.0 (1H, s, NH).

*5.1.5. Synthesis of 3-amino-5-(indol-2-yl)-1,2,4-oxadiazole (**10**)*

A solution of indole-2-carbonyl cyanamide (10 mmol) in ethanol (20 mL) was added portionwise to a solution of hydroxylamine

hydrochloride (10 mmol) in pyridine (3 mL) with cooling (ice bath) for 15 min. The mixture was stirred at ambient temperature for 1 h, followed by standing for a further 16 h. 2 N NaOH (20 mL) was added to the reaction mixture to afford a precipitate, which was collected, washed with H_2O (50 mL), and dried *in vacuo*. Purification by column chromatography (ethyl acetate:hexane) afforded the required product. Yield 8%; mp 196–200 °C. ^{1}H NMR (DMSO-d_6) δ 6.40 (2H, s, NH_2), 7.10 (1H, t, J = 7.5 Hz, Ar-H), 7.24 (1H, s, Ar-H), 7.27 (1H, t, J = 7.7 Hz, Ar-H), 7.49 (1H, d, J = 8.3 Hz, Ar-H), 7.68 (1H, d, J = 7.5 Hz, Ar-H), 12.15 (1H, s, NH). ^{13}C NMR (DMSO-d_6) δ 106.1, 112.4, 120.4, 121.7, 124.5 (C3, C4, C5, C6, C7 of indole), 122.4, 127.0, 139.8 (C2, C3a, C7a of indole), 168.8, 170.2 (C3, C5 of oxadiazole). Acc. Mass ($C_{10}H_8N_4O$), found 200.0691, theoretical 200.0693.

5.2. Biology

5.2.1. Cell viability assay

The human cancer cell lines COLO 320 (colon), MIA PaCa-2 (pancreas), MCF-7 (breast; ER +ve) and Jurkat (leukemia) (American Type Culture Collection, Manassas, VA), were cultured in DMEM medium supplemented with L-glutamine (2 mM), 10% fetal bovine serum, 2.5% horse serum, 50 IU/mL penicillin and 50 μg/mL streptomycin (Sigma–Aldrich, Milano, Italy) at 37 °C in an atmosphere of 5% CO_2. Cell viability was measured using a method based on the cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenase activity (cell proliferation reagent WST-1; Roche, Mannheim, Germany). Cells (2×10^3/well) were seeded into 96-well microtiter plates and received compounds from 0.1 to 50 μM for 72 h. Following drug exposure, WST-1 was added and the absorbance was measured at 450 nm using a microplate reader. Inhibition of proliferation was assessed as the percentage reduction of absorbance of treated cells versus control cultures and the concentration of compounds that decreased cell viability by 50% (IC_{50}) was calculated by non-linear least squares curve fitting (GraphPad Software, San Diego, CA, USA). DMSO concentration in the culture medium never exceeded 0.2%.

Since cardiomyocytes have a high abundance of mitochondria, we performed additional in vitro experiments on the H9c2 cardiac muscle cell line (American Type Culture Collection) as a potential measure of side effects of the compounds. Cells were routinely grown in DMEM containing 10% fetal bovine serum and exposed to test compound concentrations equal to their IC_{50} mean values in the most responsive human cancer cell line (COLO 320). All other experimental conditions, such as cell number and time of exposure, were identical to those used in the anticancer screening experiments described above.

5.2.2. DNA fragmentation assay

Cells were treated at 100 μM for 24 h. Cells harvested by trypsinisation were combined with detached cells and apoptosis was assessed by the Cell Death Detection ELISA kit (Roche, Mannheim, Germany) based on the recognition of released nucleosomes after DNA internucleosomal fragmentation by a mouse monoclonal antibody directed against DNA and histones. The assay allowed the specific determination of mono- and oligonucleosomes in the cytoplasmic (apoptosis) or in the supernatant fraction (necrosis) of cell lysates.

5.2.3. Caspase activity assay

Enzyme activity was assessed by the Apo-ONE™ Homogeneous Caspase-3/7 assay (Promega, Madison, WI, USA). Cells were seeded at 7×10^3/well and treated with compounds at 100 μM for 24 h. Subsequently, the caspase-3/7 assay substrate was added and the fluorescence was measured by spectrofluorimeter at excitation and emission wavelengths of 485 and 530 nm, respectively. Values were expressed as ratio between fluorescent signals generated in cells treated with compounds and those produced in untreated cells (vehicle alone).

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