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Original Article

Synthesis of novel 1,8-acridinediones derivatives: Investigation of MDR reversibility on breast cancer cell lines T47D and tamoxifen-resistant T47D

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Abstract

Multi drug resistance (MDR) is a serious obstacle in the management of breast cancer. Therefore, overcoming MDR using novel anticancer agents is a top priority for medicinal chemists. It was found that dihydropyridines lacking calcium antagonistic activity (e.g acridinediones) possess MDR modifier potency. In this study, the capability of four novel acridine-1,8-diones derivatives 3a-d were evaluated as MDR reversing agents. In addition, the relationship between structural properties and biological effects of synthesized compounds was discussed. *In vitro* cytotoxicity of acridine-1,8-diones 3a-d derivatives in combination with doxorubicin (DOX) on T47D and tomoxifen-resistant T47D (TAMR-6) breast cancer cell lines were investigated using 3-(4,5-*dimethylthiazol*-2-yl)-2,5-*diphenyltetrazolium bromide* (MTT) test. Drug resistant index (DRI), which is equal to the ratio of IC_{50} in drug-resistant cells over IC_{50} in drug-sensitive cells, was calculated for each substance. Flowcytometry experiments were also implemented to distinguish cells undergoing apoptosis from those undergoing necrosis. The results from MTT and flowcytometry experiments indicated that 1 nM 3c derivative along with DOX significantly ($P<0.05$) increased the DOX cytotoxicity in T47D and TAMR-6 breast cancer cell lines. Synthesized compounds 3a and 3b also at concentrations of 1 nM with DOX significantly increased the cytotoxicity of DOX on T47D and TAMR-6 breast cancer cell lines. Meanwhile, 3d derivative with DOX did not exhibit good synergistic effect on cytotoxic activity of DOX, and slightly increased DOX cytotoxicity in both cell lines. Our results proposed that 3c may be an attractive lead compound for further development as a chemotherapeutic agent for MDR breast cancer therapy in combination with routine chemotherapeutic agents such as DOX.

Keywords: Acridinedione; Breast cancer; TAMR-6; T47D; MDR reversibility

INTRODUCTION

Multi drug resistance (MDR) is a serious obstacle in the management of breast cancer. Therefore, overcoming MDR using novel anticancer agents is a top priority for medicinal chemists. Among the dihydropyridine (DHP) analogoues, dexniguldipine and dibenzoyls show cytotoxic potency (1,2). It was found that DHPs lacking calcium antagonistic activity possess MDR modifier potency (3). Quantitative structure–activity relationship (QSAR) studies of DHP calcium channel antagonists propose that two acyl substituents at the 3 and 5 positions in the DHP ring might affect the activity of DHP calcium channel antagonists (4). As a matter of fact, the antagonist activity is optimized by ester substituent at the 3 and 5 positions and is decreased by their acetyl group substitutes (5).

The reverting activity of nifedipine derivative DHPs such as nimodipine has

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encouraged the search for exploring MDR reverters in this group of compounds.

The (R)-enantiomer of niguldipine and dexniguldipine which is fairly potent as MDR reverter, presents an affinity for the calcium channel some 40 times lower than that of its enantiomer (5). Dexniguldipine was verified to be an effective MDR modulator whose MDR reversing effect is associated with its ability to intensify intracellular concentration of rhodamine 123 (6). Furthermore, dexniguldipine was shown to inhibit dose-dependently photo affinity labeling of P-gp, by competing with azidopine for the same binding region of P-gp (7,8).

PAK-104P, a pyridine analogue, reverses paclitaxel and doxorubicin (DOX) resistance in resistant cell lines which over-expressed both P-gp and multidrug resistance-associated proteins (MRPs) (9). The aromatic structure of PAK-104P proves that MDR reversal ability is independent of calcium antagonism potency. More than that, the classical structure of DHP calcium antagonists can be considerably modified with reduction of their intrinsic cardiovascular activity, while conserving their good MDR reversibility.

Other studies emphasized that further reduction of calcium channel antagonism and improvement of MDR modulating characteristics have been obtained by substitution of the aryl group in the 4 position with different groups especially heterocyclic groups, as it has been accomplished with the series of nicardipine derivatives including NIK-250, N276-9 and N276-16 (10,11).

Previously the MDR reversibility of some acridones with different secondary amines was reported (12,13).

In this paper, for the first time, we investigated the MDR-reversal activities of tricyclic analogues of DHP (3,3,6,6-tetramethyl-9-aryl-octahydro-1,8-acridinones) against a breast cancer cell line and its tamoxifen resistant strain.

MATERIALS AND METHODS

Chemistry

Melting points were determined on electro thermal capillary apparatus, UK and are uncorrected. The IR spectra were obtained using a Perkin-Elmer model 1000, Japan. One H nuclear magnetic resonance (^{1}HNMR) was obtained on Bruker Ac-80 spectrophotometer, Germany and chemical shifts are in ppm relative to internal tetramethylsilane. C, H, and N analyses were within ± 0.4% of theoretical values. Title compounds (**3a-d)** were sensitive to light and all chemical procedures were shielded from light whenever present. Compounds were prepared as described previously (14). As an example, details for synthesis of **3a** are described here.

3,3,6,6-Tetramethyl-9-[1-(4-fluorobenzyl)-2-(methylthio)-5-imidazolyl]-2,3,4,5,6,7,9,10-octahydro-1,8-acridinedione [3a]

A mixture of ammonium acetate (0.32 g, 0.41 mmol), 2a (1 g, 0.41 mmol) and 5, 5-dimethyl-1,3-cyclohexanedione (1.18 g, 0.84 mmol) in methanol (15 ml) was protected from light and refluxed overnight. Then, the residue was poured in ice-water. The obtained precipitate was filtered to give 0.4 g of **3a** , m.p. 111.1 °C, yield 88.7%; IR (KBr): 1630 cm-1 (C=O); ^{1}HNMR (DMSO-d6): d 7.83-6.80 (m, 6H, arom, NH, H4-imidazole), 6.20 (s, H4-DHP), 5.00 (s, 2H, CH_2N), 2.80-1.80 (m, 11H, CH_2, CH_3S), 1.00 ppm (s, 12H, CH3).

AnalCalcd for $C_{28}H_{32}FN_3O_2S$: C, 68.13; H, 6.53; N, 8.51.Found: C, 68.09; H, 6.63; N, 8.48.

Cell culture

T47D and MCF-7 are two human hormone-dependent breast cancer cell lines commonly used as experimental models for *in vitro* and *in vivo* (tumor xenografts) breast cancer studies.

It must be noted that proteins involved in cell growth stimulation, anti-apoptosis mechanisms, and cancerogenesis are more strongly expressed in T47D than in MCF7. Due to this reason, we preferred T47D cell line to evaluate the *in vitro chemosensitizing capability* of synthesized compound in breast cancer chemotherapy (15,16).

The T47D, (NCBI C203, National Cell Bank of Iran, Pasteur Institute of Iran) was cultured in roswell park memorial institute (RPMI) 1640 medium (Gibco, England)

supplemented with 10% fetal bovine serum, L-glutamine 2 mM and penicillin/streptomycin 100 unit per ml (all from Sigma, Germany) at 37 °C in humidified incubator with 5% CO_2 atmosphere. After three days of incubation, the cells were detached using 0.25% Trypsin-0.05% EDTA solution (Boehringer, Germany), and then resuspended in RPMI 1640 medium containing 10% FBS (17).

Development of tamoxifen resistant T47D cell line

Resistance to tamoxifen was developed *in vitro* by bringing drug sensitive T47D cells to augmenting concentrations of tamoxifen.

Tamoxifen was dissolved in ethanol 96% and Phosphate buffer solution (PBS) at 1×10^{-3} M concentration as stock solution, light protected, stored at 4 °C, and used for preparing serial dilutions.

The final concentration of ethanol was never more than 0.05% in either blank or treated samples. Resistance was started against a concentration of 1×10^{-8} M of tamoxifen. Following three serial passage of cells at each concentration, viable cells were exposed to the next higher concentration of tamoxifen. At the end, the highest concentration in which cells were still grown rapidly was found to be 1×10^{-6} M of tamoxifen. The cells were grown successively in the medium containing tamoxifen for 3 months to obtain more stable tomoxifen-resistant T47D (T47D /TAMR-6) cells (18).

In vitro cytotoxicity

The T47D or T47D/TAMR-6 cells were seeded at the density of 5000 cells per well, in 96-well micro titer plates and incubated at 37 °C. Viability of the cells was assessed using the 3-(4,5-*dimethylthiazol*-2-yl)-2,5-*diphenyltetrazolium bromide* (MTT, Sigma, Germany) exclusive dye test. On the basis of preliminary studies 1 nM concentration of synthesized compounds was selected for *in vitro* cytotoxicity study (Data not shown).

The cytotoxicity of each synthesized analogues in conjunction with DOX on T47D and T47D/TAMR-6 cells was investigated after 24 h. During the course of these experiments, micro titer's wells that contained T47D or T47D/TAMR-6 cells and no additive, either DOX or synthesized analogues alone, were noted as negative controls, micro titer's wells that contained T47D or T47D/TAMR-6 cells and 2 µM concentration of DOX were noticed as positive control. To determine if the synthesized analogues show any cytotoxicity, test-included wells contained 1 nM of each of synthesized analogues. Each experiment was assayed in triplicate.

After 24 h incubation period, MTT was dissolved in PBS at a concentration of 5 mg/ml and then was added to each well at a concentration of 0.5 mg/ml. Subsequently, micro plate was further incubated under the same conditions for 3 h. The culture medium containing MTT solution was removed from the wells and 150 µl dimethyl sulfoxide (DMSO) (Merck, Germany) was added to each well and mixed thoroughly to dissolve the crystals. The plates were read at 545 nm in a micro plate reader (Dynatech, USA) to obtain the absorbance values (19,20).

Flow cytometry analysis

TheT47D or T47D/TAMR-6 cells were exposed to 1 nM of synthesized analogues in combination with 2 µM concentration of DOX for 24 h at 37 °C, and then detached and collected. The untreated and treated T47D or T47D/TAMR-6 cells were washed twice with citrate phosphate buffer, fixed with 0.5 ml ice-cold 70% ethanol and stored at 4 °C for 2 h.

Propidium iodide (PI) was then added to a final concentration of 50 µg/ml which can bind to double stranded DNA by intercalating between base pairs.

The suspension of the cells and PI was then mixed gently and incubated for 1 min in the dark at room temprature.

At final stage, the fluorescence of 100,000 fixed cells which stained with PI was analyzed on a FACScaliber (Bector Dickinson, USA) using FL-2 channel (21). The number of apoptotic cells was analyzed using the WinMDI 2.8 program (The Scripps Research Institute, San Diego, USA).

Since in the cells undergoe apoptosis, DNA is partially degraded, they lost low molecular weight DNA but the non-degraded DNA remained in the cell nucleus.

When apoptotic cells are stained with PI and analyzed with a flow cytometer, they exhibit a broad hypodiploid peak, while normal cells display narrow sharp peak of diploid DNA in the FL2 channels (Sub-G1).

Data analysis

The results were reported as means ± SD (n ≥3). Data were analysed by one-way analysis of variance (ANOVA). A probability value of less than 0.05 was considered significant.

RESULTS

Chemistry

1,8-acridinedione derivatives **3a-d** were prepared by the reaction of 5,5-dimethy l-1,3-cyclohexanedione (1) with aromatic aldehydes **2a-d** in the presence of ammonia in methanol (Fig. 1).

The purity of the compounds was confirmed through thin layer chromatography. The structure of the compounds was elucidated by IR, ^{1}HNMR and elemental analyses. All spectral data are in accordance with assigned structures. In IR spectra, N-H and C-O stretching bands were observed at spectra expected values. In the ^{1}HNMR spectra, methyl protons were seen at 0.90-1.00 ppm as separated singlets. Aromatic, methylene, methine and NH protons were seen at expected values.

In virto cytoxicity and flow cytometry experiments

Table 1 represents the cytotoxicities (IC_{50}, μM) of DOX and DOX in combination with synthesized compounds as MDR inhibitors.

As shown in Table 1, results from MTT experiments indicated that 1 nM **3c** along with DOX significantly *(P*<0.05) increased DOX cytotoxicity on T47D and TAMR-6 breast cancer cell lines (Drug resistant index, DRI=1.94 vs. 7.92 for DOX alone).

A flow cytometric analysis of PI stained cells was also performed to assess the impact of synthesized compounds on the generation of sub-diploid cells. Treatment of T47D or T47D/TAMR-6 cells with synthesized compound in combination with DOX increased the sub-diploid population (Table 2). Treatment with **3c** as the strongest compound in combination with DOX increased the sub-diploid population 16 and 21 fold for T47D and T47D/TAMR-6 respectively (Fig. 2).

Since, treatment with synthesized compounds (1 nM) alone neither caused cytotoxicity nor increased sub-diploid population for T47D and T47D/TAMR-6 cells, **3a-d** are qualified MDR reverser in the following order: **3c** >**3a** >**3b** >**3d**.

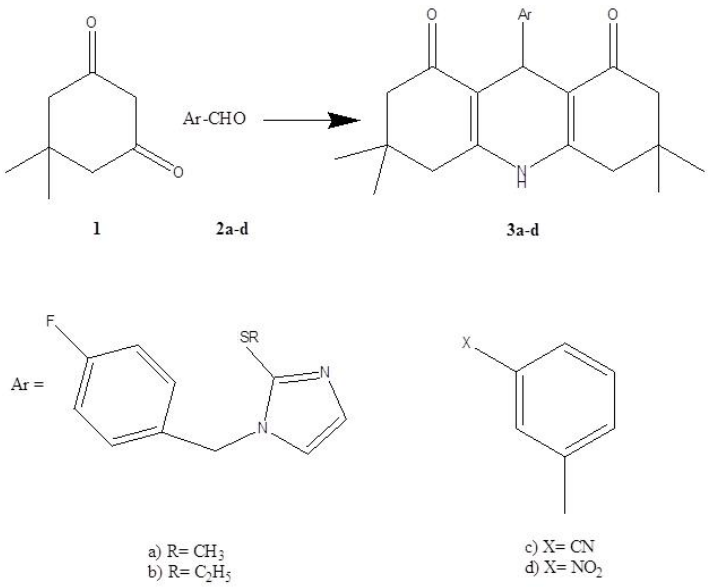


Fig. 1. Synthesis of compounds **3a-d**.

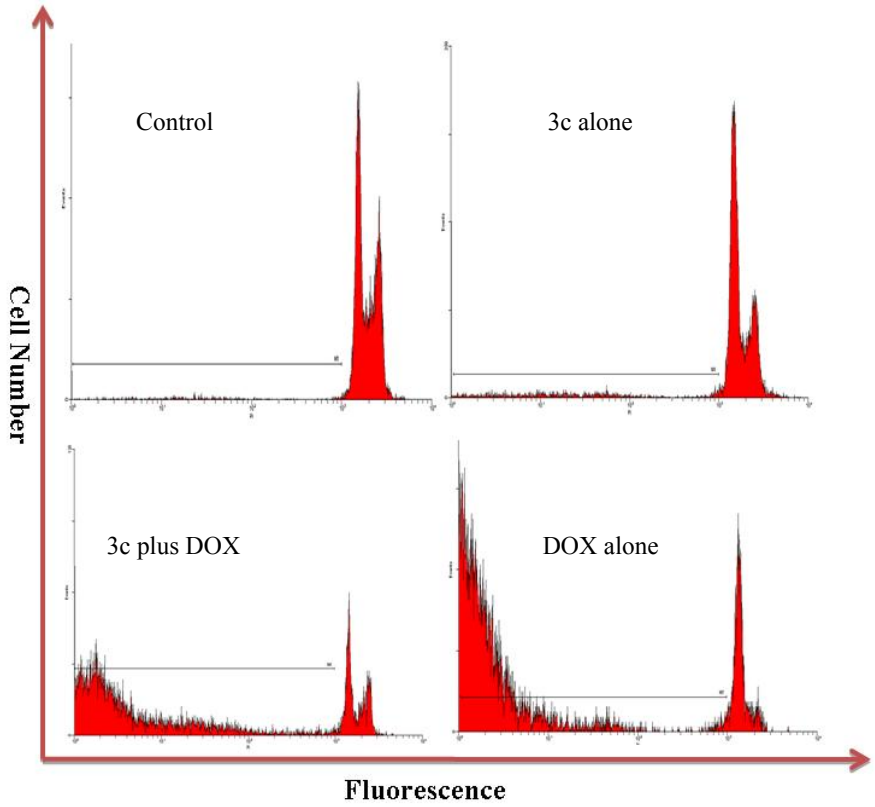


Fig. 2. Flow cytometry histograms of apoptosis assays by PI method in T47D corresponding drug resistance cell line **(**T47D/TMX**)** after 24 h. Cells were treated with 2 µM of DOX, 1nM of MDR inhibitor derivative **3c** plus 2 µM of DOX and 1 nM of MDR inhibitor derivative **3c** alone for 24 h. Sub-G1 peak as an indicative of apoptotic cells, was induced in DOX and DOX plus MDR inhibitors treated but not in control cells.

Table 1. IC_{50} (µM) of DOX and DOX and MDR Inhibitors in combination with DOX (MTT Assay).

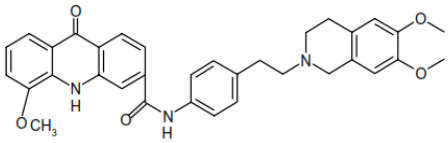
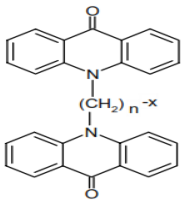
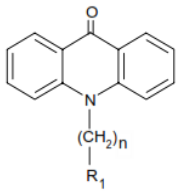
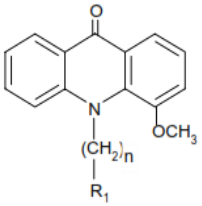
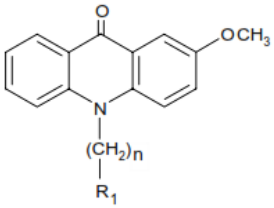
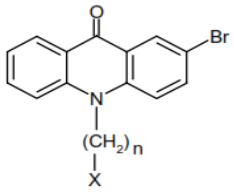
Compound	MDR Inhibitor	IC_{50} ± SEM (T47D/TAMR-6)	IC_{50} ± SEM (T47D)	DRI
DOX	None	2.06 ± 0.068	0.26 ± 0.045	7.92
3c	-CN (1 nM)	*0.33 ± 0.035	*0.17 ± 0.037	1.94
3a	SMT (1 nM)	*0.85 ± 0.015	0.20 ± 0.038	4.25
3d	$-NO_2$ (1 nM)	*1.38 ± 0.144	0.22 ± 0.079	6.27
3b	SET (1 nM)	*0.79 ± 0.022	0.22 ± 0.056	3.59

DRI is the abbreviation for drug resistant index, which is equal to the ratio of IC_{50} in TAMR-6 (tamoxifen-resistant T47D) over IC_{50} in drug-sensitive cells (breast cancer T47D cells). SEMs (standard errors of means) were derived from the IC_{50} values of three independent experiments. *represents the differences with DOX only treatment.

Table 2. Percent of apoptotic cells in T47D human breast cancer cell line and its corresponding drug resistance cell line T47D/TAMR-6 after 24 h of 2 µM of DOX, 1 nM of MDR inhibitors **3a-d** plus 2 µM of DOX, or 1 nM of MDR inhibitors alone.

Cell line	Control	MDR Inhibitor (1 nM) plus DOX (2 µM)				DOX (2 µM)	MDR Inhibitor (1 nM) alone			
		3c	3d	3b	3a		3c	3d	3b	3a
T47D	4.2 ± 0.21	89.3 ± 4.29	92.5 ± 6.89	78.8 ± 3.29	88.9 ± 4.57	89.8 ± 7.58	5.5 ± 0.35	3.1 ± 0.51	4.2 ± 0.27	3.7 ± 0.34
T47D/TAMR-6	3.9 ± 0.03	62.3 ± 8.26	38.5 ± 4 .97	53.6 ± 6.87	59.3 ± 3.45	33.6 ± 2.98	4.3 ± 0.94	4.1 ± 0.81	3.8 ± 0.57	4.3 ± 0.68

Table 3. Previously reported acridones derivatives as MDR reversal agents (*Chemosensitizer*).

No.	Structure	Substitution	Active against Pump	References
1		-----	ABC, BCRP	(12)
2		n=3 or 4	P-gp	(13)
3		n=3 or 4 R1=differed secondary amines	Not determined But enhanced uptake of vinblastine and verampil	(22)
4		n=3 or 4 R1=differed secondary amines	Not determined But enhanced uptake of vinblastine and verampil	(22)
5		n=3 or 4 R1=differed secondary amines	Not determined But enhanced uptake of vinblastine	(23)
6		n=3 or 4 X=differed secondary amines	Not determined But enhanced uptake of vinblastine	(24)

DISCUSSION

It is well known that the pivotal structural properties required for modulator binding to P-gp are two planar ring domains and a basic nitrogen atom (12). Previously it was proved that the structure of most active P-gp modulating compounds include highly lipophilic aromatic planar ring system substituted with a preferably cyclic, tertiary amino group. In this regard, acridones has a tricyclic hydrophobe planar ring which is very important for the reversal of MDR. Table 3 represents the previously reported acridine derivative with MDR reversibility. The MDR modulating activity of a series of acridione

derivatives which was reported earlier, prompted us to synthesize four novel 1,8-acridindione derivatives and to evaluate their MDR reversibility.

In this study 1,8-acridinedione scaffold was synthesized through introduction of carbonyl group to C1 and C8 positions at acridine rings. Then the fluoro benzyl imidazole, cyano or nitro benzyl substitution at C9 position of 1,8-acridinedione scaffold was prepared and consequently the MDR reversibility of 4 new derivatives (Fig. 1) was evaluated. Synthesized compound with substitution of fluoro benzyl ethylthio imidazole **3b** or fluoro benzyl methythiol imidazole **3a** at C9 position of acridinedione at concentration of 1 nM with DOX can increase cytotoxicity of DOX on T47D and tamoxifen-resistant T47D breast cancer cell lines (DRI=3.59 and 4.25 vs. 7.92 for DOX alone, respectively).

Obtained results illustrated that by changing the alkyl chain length of the substituent on position 2 in imidazole ring from methylthio (**3a**) to ethylthio (**3b**), compounds showed increasing cytotoxicity and MDR reversibility effect.

On the other hand, derivative with nitro benzyl substitution at C9 position of acridinedione (**3d**) with DOX did not exhibit significant synergistic effect on cytotoxic activity of DOX, and can slightly increase DOX cytotoxicity on T47D and T47D/TAMR-6 breast cancer cell line (DRI=6.27 vs. 7.92 for DOX alone, respectively).

While the compound **3c** with cyano benzyl substitution at C9 position of acridinedione (**3c**) exhibited significant anti proliferative activity in conjunction with DOX in T47D and also in T47D/TAMR-6 cell lines, the compound **3d** with substituent of nitro on benzene ring significantly enhanced cytotoxicity of DOX at higher concentrations, 100 and 10000 nM, (data not shown), on either T47D or T47D/TAMR-6 breast cancer cell lines.

Obtained results demonstrated that depends on the substitution of the benzene ring, the anti proliferative activity changes accordingly. When the cyano (weaker electron donating group) was substituted in the benzene ring, compound **3c** showed higher cytotoxic activity. In contrast, when the nitro (electron withdrawing group) was introduced to the benzene ring the anti proliferative activity of compound **3d** was reduced.

The preliminary *in vitro* MDR reversibility and anti-breast cancer activity test showed that **3c** in combination with DOX had significant growth inhibitory ($P<0.05$) effect against T47D or T47D/TAMR-6 breast cancer cell lines.

Structure–activity relationship studies revealed that weak electron donating substituent of benzene ring and also longer chain of alkyl substituent of fluoro benzyl imidazole played an important role in the anti-breast cancer activity and MDR reversibility *in vitro*. Such activity was proved to be associated with the induction of apoptosis by the flow cytometry analysis using propidium iodide staining. Our results proposed that **3c** may be an attractive lead compound for further development as a chemotherapeutic agent for MDR breast cancer therapy in combination with routine chemotherapeutic agents such as DOX.

CONCLUSION

In conclusion, we have presented an efficient protocol for synthesis of 1,8-acridinedione derivatives. Our goal in this study was to evaluate capability of 1,8-acridinedione derivatives to reverse MDR in breast cancer. All synthesized compounds were evaluated on T47D or T47D/TAMR-6 breast cancer cell lines for tumour cell cytotoxicity and MDR reversibility in combination with DOX. Obtained results demonstrated that among synthesized compounds, derivative with cyano benzyl substitution at C9 position of 1,8-acridinedione is the strongest MDR reverser compound. Overall, the results reported in this study provide a fruitful insight into the design of new series of compounds with MDR reversibility.

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