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Synthesis and Evaluation of Pyridinium-Hydrazone Derivatives as Potential Antitumoral Agents

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ABSTRACT

The hydrazones of 4-hydrazinylpyridinium bearing alkylphenyl groups on pyridinium nitrogen were synthesized and evaluated for their cytotoxic activity against MCF-7, PC3, U2OS and HEK293 cell lines by Wst1 cell proliferation assay.

Cytotoxic activity results indicated that **d** derivatives having butylene chain; **4** and **5** series having naphthalene and anthracene ring systems, showed high cytotoxic activity (IC_{50}= 3.27-

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8.54 μM) on cancer cells. **3d** (4-(2-(4-hydroxybenzylidene)hydrazinyl)-1-(4-phenylbutyl)pyridinium bromide) was the most cytotoxic compound with IC_{50} value of 3.27 μM against MCF-7. The most active derivatives (**1d**, **2d, 3d, 4** and **5** series) were selected to investigate for the effects on autophagy by analyzing the expression of autophagy marker proteins. The conversion of LC3-I to its lipidated form LC3-II is essential for autophagy and related to autophagosomes. According to our results, all tested compounds except for **3d**, induced lipidated form LC3-II accumulation. Then, the effects of the compounds on p62 protein level were also analyzed by the immunoblotting since the autophagy inhibition results in accumulation of p62.

Further molecular mechanistic studies including morphological analysis and live death assays indicated that all tested compounds (**1d**, **2d, 3d, 4** and **5** series) are potent antitumoral molecules and all except for **3d** have potential to inhibit autophagic flux.

KEYWORDS

pyridinium salts, hydrazone, anticancer activity, autophagy, p62

1 INTRODUCTION

Cancer is one of the causes of death affecting a significant amount of the population worldwide. The number of patients dealing with cancer increases every year. According to the International Agency for Research on Cancer reports, 14.1 million new cancer cases, 32.6 million people suffering from cancer (within 5 years of diagnosis) and 8.2 million cancer deaths were recorded in 2012 worldwide and it is estimated that deaths resulting from cancer will reach to 21.1 million by 2030.[1]

Although several chemotherapeutic agents have been used for the cancer treatment; serious side effects, high toxicity and the drug resistance limit their use in the treatment. Depending on elucidation of molecular mechanisms concerning cancer, new strategies have been developed for the treatment of this disease. Recently, researchers focus on discovering effective molecules targeting cell death pathways such as apoptosis, necrosis and autophagy.[2]

Apoptosis and autophagy are the main types of programmed cell death (PCD) related to cancer and its treatment.[3] Apoptosis is the best-characterized type of PCD and it has been targeted to prevent and treat cancer for decades. Exploring other types of PCDs creates new

opportunities for cancer treatment; especially when targeting apoptosis is not effective for cancer treatment in conditions seen in apoptosis-resistant cancer cells.[4] Autophagy is a physiological process that maintain cellular homeostasis by degrading and recycling organelles and proteins.[5] Autophagic defect may constitute metabolic stress, genetic damage susceptibility and later cancer promoting environment in cells. On the contrary, autophagy may increase stress tolerance and survival in cancer cells under adverse conditions. Thus, autophagy has a context-dependent role in cancer, and both induction and inhibition of autophagy may be the target of cancer therapy.[6, 7]

Hydrazones are a special group of the Schiff bases. Hydrazone derivatives have important biological activities and this functional group is found as a core structure in many chemotherapeutic agents having antimicrobial, antimalarial, antileishmanial, antiviral and anticancer effects.[8-10] Many researchers have reported hydrazones as promising anticancer agents.[11-18] According to the reports, these hydrazone compounds display anticancer activity via diverse molecular mechanisms.[l6-21] Among them, thiazole-hydrazone derivative (CPTH6) was found to have anticancer potency via apoptosis and autophagy.[22]

Researchers have also carried out studies for novel anticancer agents with quaternary nitrogen. These studies have revealed that the cationic charge might play a role not only for interacting with the target, but may also enhance water solubility, cell membrane permeability and cellular uptake.[23-26]

Many studies have shown that some of the antimicrobial compounds exhibit cytotoxic activity against cancer cells.[27-29] In our previous studies, oxime-ether and hydrazone derivatives with quaternary pyridinium core were found to have remarkable antimicrobial and antileishmanial activities.[30-32]

On the other hand, 1,4-dihydropyridine derivatives obtained from a new series pyridinium-hydrazones by using appropriate base[33] were evaluated as AChE inhibitory activity by our group.[34] In the light of the facts mentioned above, our main goal was to investigate anticancer profile of these pyridinium-hydrazone compounds with this study. We synthesized the pyridinium-hydrazone derivatives having an aromatic moiety connected to the pyridinium nitrogen via alkyl linkers of different lenght. At the opposite end of the molecule nonsubstituted-, *p*-hydroxy-, and *p*-nitrobenzaldehydes were selected as aldehyde function to form their hydrazones. Also, to investigate the effects of planar aromatic groups on the activity, 1-naphthaldehyde and 9-anthraldehyde derivatives were added to the series. Then, we planned to screen the cytotoxic activity on different cell lines.

Numerous studies identified that pyridinium or hydrazone containing molecules exhibited effects on autophagy. Compound VLX600 bearing pyridine-2-hydrazone moiety[35] and compound CCCP (carbonyl cyanide *m*-chlorophenylhydrazone)[36] were reported to have the effect on autophagy process. Besides, compounds LCL124 and C18-pyridinium ceramides having a long hydrophobic alkyl chain on quaternary pyridinium nitrogen were found to play a role on autophagy.[37, 38] On the other hand, chloroquine (CQ), lucanthone and acridine derivatives including planar ring system are the autophagic inhibitors under investigation as anticancer agents.[39-42] Due to these core structures' role on autophagy, as a further mechanistic study, we designed to test the effects on autophagy and ubiquitin proteasome system of the most active compounds.

2 METHODS AND MATERIALS

2.1 Chemistry

All melting points were determined using an Electrothermal IA9100 melting point apparatus (Electrothermal, Essex, U.K.) and are not corrected. The infrared (IR) spectra of the compounds were monitored by attenuated total reflectance (ATR) (PerkinElmer Spectrum 100 FT-IR, Waltham, MA, USA). ^{1}H- and ^{13}C-NMR spectra were recorded with a Varian AS 400 Mercury Plus NMR spectrometer (Varian, Palo Alto, CA, U.S.A.) operated at 400 and 100 MHz for ^{1}H and ^{13}C, respectively. Tetramethylsilane (TMS) was used as an internal standard and DMSO-d_6 was used as a solvent. Chemical shifts were measured in parts per million (δ). The *J* values were given in Hz. Abbreviations for data quoted are: s, singlet; d, doublet; t, triplet; quin, quintet; dd, doublet of doublets; m, multiplet; bs, broad singlet. All chemicals, reagents and solvents used for synthesis were high-grade commercial products and they were purchased from Sigma, Acros and Merck companies. LC/MS was recorded on a Thermo MSQ Plus (San Jose, CA, USA) mass spectrometer using ESI. Elemental analyses (C, H, N) were performed by Leco TruSpec Micro (Leco, St. Joseph, MI, USA). Analytical thin layer chromatography (TLC) was run on Merck silica gel plates (Kieselgel 60 F_{254}) with detection by UV light (254 nm).

2.2 General procedure for the synthesis of compounds 1a-5d

The title compounds, except for **3** series, were previously synthesized by our group.[34] They were prepared as shown in Scheme 1. In the first step, 4-chloropyridine (0.1 mol) and hydrazine monohydrate (0.2 mol) in 1-propanol (30 ml) was refluxed for 18 h. In the second step, 4-hydrazinylpyridine (0.033 mol) was condensed with 0.04 mol various aromatic aldehydes (benzaldehyde, *p*-nitrobenzaldehyde, *p*-hydroxybenzaldehyde, 1-naphthaldehyde, 9-anthraldehyde) for 6-18 h in 1-propanol to yield the corresponding hydrazone derivatives (**1-5**). Finally, the title compounds (**1a-5d**) were obtained by quaternization of the pyridine-hydrazone derivatives **(**0.01 mol) with 0.02 mol appropriate substituted alkyl halide (benzyl chloride, 2-phenethyl bromide, 3-phenylpropyl bromide, 4-phenylbutyl bromide) in ethanol under reflux for 1-16 h. The precipitate was filtered and washed with cool ethanol and crystallized from ethanol to give the final compounds (**1a-5d**). The spectral data of all compounds were reported for the first time with this study (supplementary data). Compounds **3a-3d** are original.

2.3 Biological activities

2.3.1 Cell culture

Three cancer cell lines namely PC3 (human prostate cancer), MCF-7 (human breast cancer), U2OS (human osteosarcoma) and one non-tumoral cell line HEK293 cells (human embryonic kidney cells) were included in the study. Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and maintained in a humidifier incubator at 37 °C supplied with 5% CO_2. All the reagents were purchased from Biological Industries (Israel).

2.3.2 Wst1 cell proliferation assay

PC3, MCF-7, U2OS and HEK293 cells were seeded in 96-well plates (5000 cells/well). All tested compounds were dissolved in DMSO. 24 hours later, cells were treated with the compounds from the library with the final concentrations of 1, 2, 5, 10, 25 µM. DMSO and doxorubicin were used as negative solvent control and positive cytotoxic control,

respectively. In all samples DMSO concentration was 0.2%. Following 48 hours incubation, cell viability was determined by the colorimetric WST-1 assay (Roche) as indicated in the protocol provided by manufacturer. Formazan dye formation was evaluated using a scanning multiplate spectrophotometer (Varioscan, Thermo) at 450 nm and a 620-nm reference filter. To determine the IC_{50} values, a sigmoid-dose response curve was fitted to the data using nonlinear regression in GraphPad Prism 5 software.

2.3.3 Immunoblotting

U2OS cells were treated with the IC_{50} concentrations of compounds or DMSO control for 24 h. Equal amounts of cell lysates were loaded to the SDS-PAGE gels. Following electrophoresis, proteins were transferred to the PVDF membrane (Millipore). After blocking with 5% nonfat milk in PBS-T buffer, membranes were blotted with primary antibody for 1 h at room temperature. After 3 times of washing procedure membranes were incubated with secondary antibody for 1 h at room temperature. Visualization of the results were done using Clarity ECL Solution (BioRad) and Fusion FX (Vilber Lorurmat). Actin was used as loading control. Anti-LC3A/B, anti-p62 and anti-K48 specific polyubiquitin antibodies were purchased from Cell Signaling, while anti-actin antibody was from Sigma.

2.3.4 DAPI staining

U2OS cells were inoculated on coverslips in 12-well plates for 24 h. Then the cells were treated with IC_{50} concentrations of the compounds for 24 h. To determine nuclear condensation by DAPI (Sigma Aldrich) staining, cells were fixed with 4% paraformaldehyde for 30 min at 4 °C and washed six times with ice cold PBS. Then coverslips were incubated with 0.5 μg/ml DAPI (4',6-diamidino-2-phenylindole) for 5 min in dark and examined under fluorescent microscope (Olympus IX71 fluorescent microscope).

2.3.5 Live Dead Cell staining

In order to distinguish between health live and dying apoptotic/necrotic cells Live-Dead Cell Staining Kit (Biovision) was utilized considering the protocol of manufacturer. U2OS cells were inoculated on coverslips in 12-well plates for 24 h. Then the cells were treated with IC_{50} concentrations of the compounds for 24 h. This kit includes Live-Dye™, a cell-permeable green fluorescent dye, to stain live cells and propidium iodide (PI), a cell non-permeable red fluorescent dye to stain dying cells. Following incubation with the Staining Solution, coverslip was inverted on a glass slide and cells were visualized by fluorescence microscopy (Olympus IX71 fluorescent microscope).

3 RESULTS AND DISCUSSION

3.1 Chemistry

In this study, twenty 4-(2-arylidinehydrazinyl)pyridinium derivatives were synthesized according to mainly reported method.[33] The synthetic route is given in Scheme 1.

Firstly, 4-hydrazinylpyridine was furnished by nucleophilic substitution of hydrazine with 4-chloropyridine. Secondly, 4-hydrazinylpyridine and corresponding aromatic aldehydes were reacted in 1-propanol to give hydrazone derivatives (**1-5**). Finally, the title compounds **1a-5d** were achieved by quaternization of intermediate compounds (**1-5)** with alkyl halides in ethanol under reflux condition. These final compounds are very sensitive to convert into the 1,4-dihyropyridines in the presence of appropriate base such as NaOH with a good yield.[34]

The structures of the compounds were confirmed by spectral (IR, ^{1}H NMR, ^{13}C NMR and ESI Mass) and elemental analyses. The purity levels of compounds were determined by elemental analyses (C, H, N) and the results were within ±0.4% of the calculated values. ^{1}H NMR spectra of the title compounds were consistent with expected resonance signals in terms of chemical shifts and integrations. All the ^{13}C NMR findings confirmed the structures proposed. The mass spectra of the title compounds were recorded in respect to the positive ion mode electrospray ionization (ESI+) technique. The M^{+} ions of title compounds compromised with the calculated molecular weights.

3.2 Biological evaluation

The cytotoxic activity of the title compounds was evaluated against human cancer cell lines including MCF-7, PC3, U2OS and one non-tumoral cell line, HEK293, by using Wst1 cell proliferation assay. Doxorubicin was used as the reference compound. IC_{50} values of the title compounds were listed in Table 1.

In our previous study, the antimicrobial activity results of the pyridinium-hydrazone derivatives indicated that the longer the side chain on pyridinium nitrogen, the more antimicrobial activity it possesses.[30] Since various studies have revealed that some of the antimicrobial compounds having toxicity on bacteria display cytotoxic activity against cancer cells[27-29], we evaluated the effect of the distance between the pyridinium nitrogen and the phenyl ring on the cytotoxic activity. According to the activity results, among the nonsubstituted and the substituted phenyl series **(1a-1d, 2a-2d, 3a-3d)**; generally, **d** derivatives (**1d**, **2d**, **3d**) having butylene chain displayed the highest cytotoxic activity whereas **a** derivatives (**1a**, **2a**, **3a**) having methylene chain exhibited poor cytotoxicity against the studied cell lines. Moreover, **d** derivatives have approximately two times more cytotoxicity than the **c** derivatives on especially U2OS and MCF-7 cell lines. Unlike phenyl series, all naphthalene (**4**) and anthracene (**5**) series (**4a-4d, 5a-5d**) exhibited high cytotoxic activity.

Many studies indicated that planar compounds exhibited significant cytotoxic activity.[43-45] Then, we evaluated the effect of planar ring on cytotoxic activity. According to the cytotoxic activity results, it is obvious that replacement of the phenyl moiety with naphthalene (**4**) and anthracene (**5**) ring systems remarkably improved the cytotoxic activity for the derivatives especially bearing benzyl side chain. All compounds (IC_{50}= 3.33-7.54 µM) of series **4** and **5** displayed comparable cytotoxic activity on cancer cells to the reference compound, doxorubicin (IC_{50}= 1.89-2.88 µM).

The cytotoxic activity results suggest that the distance between the pyridinium nitrogen and the phenyl ring is critical for the activity in the series **1**, **2**, **3,** and generally the planarity of the ring system connecting to the hydrazone moiety positively influences the cytotoxic activity. On the other hand, the substitutions at para position of the phenyl ring connected to the hydrazone moiety did not lead to significant change in the cytotoxic activity. Among the tested compounds, series **3** bearing hydroxyl substituent displayed higher cytotoxic activity on MCF-7 cell line and nitrosubstituted phenyl derivatives (series **2**) showed better selectivity on MCF-7, PC3, U2OS cell lines compared to non-tumoral HEK293 cells. Besides, **d**

derivatives exhibited higher cytotoxic activity against U2OS than the other cell lines. Among the tested compounds, derivatives with the highest cytotoxic activity (**1d**, **2d, 3d, 4** and **5** series) were selected to further investigate their mechanism of activity.

Autophagy, a catabolic pathway that eliminates long-lived proteins and organelles, is one of the important targeted mechanisms in the treatment of cancer disease. While autophagy maintains at basal level under normal nutrient conditions, it can be upregulated to continue cell survival under unexpected conditions such as starvation, hypoxic stress. Autophagy serves as tumor suppressive or tumor inducer depending on the tumor stage. Autophagy can suppress the development of cancer in the early stage, whereas it can contributes to cancer cell survival in the formed cancer.[46, 47]

Several proteins are required for the formation of the autophagosome. One of these proteins is the microtubule-associated light chain 3 (LC3). During the autophagy, LC3 is hydrolyzed to LC3-I and then the conjugation of LC3-I with phosphatidylethanolamine produce the lipidated form LC3-II.[48, 49] Thus, LC3 is widely used marker of autophagy. We firstly evaluated the autophagy activity by the conversion of LC3-I to LC3-II by immunoblot analysis in U2OS cell. DMSO was used as a positive control. Bafilomycin A1 was used as the standard autophagy inhibitor compound. The amount of LC3-II correlates with the number of autophagosomes. Immunoblot analysis showed that LC3-II was accumulated in these compounds except for **3d** (Figure 1A). Besides LC3 conversion, accumulation of p62 is also commonly used as a autophagy marker. p62 is a substrate of the autophagic process and degraded by autophagy. Accumulation of p62 is related to the inhibition of autophagy flux.[50, 51] Thus, we also determined the amount of p62 to investigate the role of the selected compounds in autophagy (Figure 1A). The immunoblot analysis data revealed that p62 levels increased as a result of treatment the cells with the compounds, again except for **3d** (Figure 1A and 1B). These results indicate that the compounds are presumably potent autophagic flux inhibitors. They may be effective in the formed cancer due to their inhibitory effect on autophagy.

It is known that there are two major protein degradation pathway in cell, namely autophagy and ubiquitin proteasome system. Since we found some significant effect on autophagy, we also tested the effect of compounds on the ubiquitin mediated degradation monitoring total K48-linked polyubiquitinated proteins. MG132 was used as the standard proteasome inhibitor. Our data suggests that only some of our compounds slightly caused accumulation of total K48-linked polyubiquitinated proteins (Figure 1C and 1D).

Next, we prompted to investigate the effect of selected compounds (**1d**, **2d**, **3d**, **4d** and **5d**) on the cell and nuclear morphology, using light and fluorescence microscopy, respectively. As seen in Figure 2A, **1d**, **2d**, **4d** and **5d** severely changed the morphology of cells. It is also noteworthy to mention that these compounds, except for **3d,** caused formation of intracytoplasmic vacuoles. It is known that cells undergoing apoptosis demonstrates nuclear condensation and DNA fragmentation. Thus, we also analyzed the effect of tested compounds for their nuclear staining using DAPI and fluorescent microscopy. While control cells show regular nuclear DAPI staining with uniform distribution, cells treated with tested compounds showed fragmented nuclei (Figure 2B). Next, we wanted to distinguish between live cells and apoptotic/necrotic cells on compounds treated U2OS cells and our data indicates that while there is no PI staining observed in DMSO treated cells, **1d**, **2d**, **3d**, **4d** and **5d** treated cells all have significant PI staining (Figure 2C).

CONCLUSIONS

In this study, we assessed the anticancer potencies of 20 pyridinium-hydrazone derivatives against three different types of cancer cell lines. Almost all compounds were able to display cytotoxic activity in micromolar range of concentration. According to cytotoxic activity results, it can be concluded that the distance between the pyridinium nitrogen and the phenyl ring is critical for the activity in the series **1**, **2**, **3**, and generally, the planarity of the ring system connecting to the hydrazone moiety positively influences the cytotoxic activity. In addition, further mechanistic study revealed that all the tested compounds except for **3d** exhibited inhibitory effect on autophagy and some of them slightly showed proteasome inhibitory activity. It can be suggested that compounds **1d**, **2d**, **4** series (**4a**, **4b**, **4c**, **4d**) and **5** series (**5a**, **5b**, **5c**, **5d**) are presumably potentially autophagic flux inhibitors.

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CONFLICT OF INTEREST

The authors report no conflict of interests.

SUPPORTING INFORMATION

Supporting Information can be found in the online version of this article:

Spectral data of final compounds

^{1}H NMR spectra of final compounds

^{13}C NMR spectra of final compounds

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A LIST OF CAPTIONS

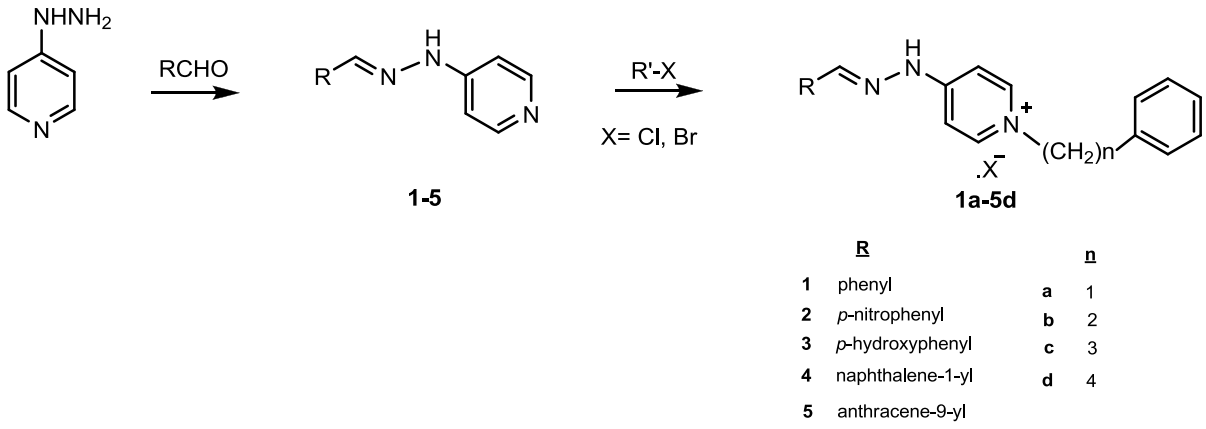
SCHEME 1 Synthesis of the title compounds

TABLE 1 Cytotoxic activities of the title compounds

FIGURE 1 A. U2OS cells were treated with compounds at IC_{50} doses for 24 h. At the end treatment cells were lysed and the autophagic marker proteins LC3 and p62 were analyzed by immunoblotting. The conversion of LC3-I and -II is shown. Actin was used as loading control. Bafilomycin (Baf) was used as standard autophagy inhibitor control. **B.** The accumulation of p62 was analyzed using the densitometric analyses of three independent assays using ImageJ software (http://imagej.nih.gov/ij/). The fold increase compared to DMSO treated control cell group was presented in the graph. **C.** U2OS cells were treated with compounds at IC_{50} doses. 24 hours later cells were harvested and analyzed via SDS-PAGE followed by immunoblotting. The total K48-linked ubiquitined protein levels were determined using a specific antibody against K48-linked ubiquitin chains. Mg132 was used as standard proteasome inhibitor. **D.** Densitometric analyses of K48-linked polyubiquitinated proteins were performed using ImageJ software (http://imagej.nih.gov/ij/). The data from three independent experiments was analyzed and the fold increase compared to DMSO treated control cell group was presented in the graph.

FIGURE 2 A. Live images of U2OS cells treated with compounds indicated compounds and DMSO control for 24 h and visualized under inverted microscope. **B.** DAPI staining of cells

treated with indicated compounds at IC_{50} concentrations for 24 h. Arrows indicates nuclear fragmentation. **C.** Live/dead cell staining of cells treated with indicated compounds at IC_{50} concentrations for 24 h. Green: Live-Dye™, Red: propidium iodide (PI).



	R		n
1	phenyl	a	1
2	*p*-nitrophenyl	b	2
3	*p*-hydroxyphenyl	c	3
4	naphthalene-1-yl	d	4
5	anthracene-9-yl		

SCHEME 1 Synthesis of the title compounds

TABLE 1 Cytotoxic activities of the title compounds

Compound	R	n	IC_{50} ± SD (μM)[a]			
			U2OS	MCF-7	PC3	HEK293
1a	phenyl	1	25>	25>	25>	25>
1b	phenyl	2	25>	25>	25>	20.97 ± 1.41
1c	phenyl	3	10.21 ± 0.09	9.07 ± 0.04	7.8 ± 0.10	10.79 ± 0.40
1d	phenyl	4	4.71 ± 0.05	5.75 ± 0.24	8.54 ± 0.30	7.98 ± 0.31
2a	*p*-nitrophenyl	1	19.33 ± 3.89	19.46 ± 1.05	18.60 ± 0.64	25>
2b	*p*-nitrophenyl	2	10.26 ± 0.19	8.39 ± 0.50	8.87 ± 0.07	22.50 ± 1.01
2c	*p*-nitrophenyl	3	12.67 ± 0.55	11.67 ± 0.47	8.84 ± 0.15	18.26 ± 0.90
2d	*p*-nitrophenyl	4	5.21 ± 0.25	5.59 ± 0.37	6.71 ± 0.16	9.60 ± 0.19
3a	*p*-hydroxyphenyl	1	25>	22.18 ± 2.98	25>	25>
3b	*p*-hydroxyphenyl	2	19.53 ± 1.24	4.67 ± 0.34	13.11 ± 2.31	25>
3c	*p*-hydroxyphenyl	3	10.41 ± 0.57	6.40 ± 0.52	9.95 ± 0.19	10.82 ± 0.08
3d	*p*-hydroxyphenyl	4	3.47 ± 0.73	3.27 ± 0.38	7.86 ± 0.37	8.05 ± 0.77
4a	naphthalene-1-yl	1	5.53 ± 0.23	7.54 ± 0.33	5.32 ± 0.50	8.21 ± 0.04
4b	naphthalene-1-yl	2	6.92 ± 0.30	5.83 ± 0.45	6.03 ± 0.25	7.72 ± 0.47
4c	naphthalene-1-yl	3	6.39 ± 0.34	6.11 ± 0.26	6.16 ± 0.29	7.50 ± 0.40
4d	naphthalene-1-yl	4	4.64 ± 0.05	6.44 ± 0.66	6.10 ± 0.23	8.92 ± 0.20
5a	anthracene-9-yl	1	3.73 ± 0.13	4.93 ± 0.06	3.61 ± 0.04	6.28 ± 0.24
5b	anthracene-9-yl	2	4.41 ± 0.06	6.84 ± 0.42	3.99 ± 0.10	6.99 ± 0.14
5c	anthracene-9-yl	3	3.65 ± 0.10	5.04 ± 0.12	4.09 ± 0.04	5.31 ± 0.18
5d	anthracene-9-yl	4	3.33 ± 0.10	3.57 ± 0.33	3.91 ± 0.11	4.57 ± 0.14
Doxorubicin	-	-	1.89 ± 0.28	1.99 ± 0.10	2.88 ± 0.19	1.10 ± 0.07

[a]Values represent mean ± SD of three independent experiments.