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Evaluation of Cytotoxicity and Apoptosis Inducing Effects of *N*-(5-mercapto-1,3,4-thiadiazol-2-yl)-2-phenylacetamide Derivatives as Caspase Enzymes Activators

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ABSTRACT

A new series of previously reported cytotoxic 1,3,4-thiadiazole analogs as potential anticancer agents were evaluated for induction of apoptosis. Namely, activation of caspases 3, 8 and 9 were assessed in Human Prostate Cancer Cell Line (PC-3) as well as Human Colorectal Adenocarcinoma Cell Line (HT-29). Cytotoxicity of intended compounds was evaluated in PC-3, HT-29 and Human Breast Adenocarcinoma Cell Line (MCF-7) using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) assay. Furthermore, activity of caspases 3, 8 and 9 were assessed in PC-3 and HT-29 cell lines and obtained results were compared to doxorubicin. Mitochondrial Membrane Potential (MMP) as well as capability of intracellular Reactive Oxygen Species (ROS) generation was also measured in these cell lines. A new series of recently reported cytotoxic 1,3,4-thiadiazole derivatives were studied mechanistically. Fortunately, the most of the investigated derivatives showed a significant increase in activity of caspases 3, 8 and 9, in PC-3 and HT-29 cell lines superior than doxorubicin. Only compound A (2-Cl) caused an increase in MMP in HT-29 cell line further than doxorubicin. None of the tested derivative did not tempted generation of reactive oxygen species in examined cell lines. Overall, the investigated derivatives could be introduced as potential anticancer lead compounds.

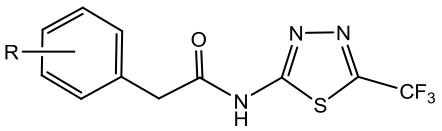
Keywords: 1,3,4-thiadiazole, Apoptosis, Caspase, Anticancer

INTRODUCTION

Apoptosis or programmed cell death is occurred in all organisms to control their cell numbers and to omit extra or damaged cells. The term 'apoptosis' was originally coined by Kerr et al., for explanation of a distinct morphological alterations in cell related to normal programmed cell death and certain pathological phenomenon *in vivo*. Cell shrinkage, loss of contact with neighboring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing and chromatin condensation are the main changes in an apoptosis process [1-5]. Cell lysis does not happen in an apoptosis process, but some parts of the cell bud off as apoptotic bodies that subsequently phagocytized by neighboring cells. Therefore, apoptosis is known as a very efficient process by which the body can remove a population of cells without the activation of an inflammatory response [6]. Inappropriate apoptosis induction could lead to the excessive cell death and is one of the main causes and origin of the degenerative disorders. In the other hand, inadequate apoptosis could result in over proliferation of cells and emergence of neoplasm. Defects in apoptosis signaling pathways could result in uncontrolled tumor cell growth as well as resistance to cancer treatment [2,7]. Hence, restoration of normal apoptosis or induction of apoptosis could lead to cancer cell death as well as increase the response to chemotherapeutical agents. In fact, it is known that the antineoplastic activity and efficacy of the most of anticancer drugs is correlated to their apoptosis inducing capability. The mechanism of apoptosis involves a cascade of initiator and effector caspases that are activated sequentially [2,8-12]. Caspases as cysteine proteolytic enzymes known are an enzyme family that precedes apoptosis. Activation of these protease enzymes which are normally present inside the cells in the form of inactive zymogens, lead to the cleavage of multiple protein substrates inside cells and result in irreversible apoptotic cell death [2]. Within the caspase family, caspase 3 has been identified as one of the key effector caspases that cleave multiple protein substrates in cells, and leading to irreversible cell death. Caspases 8 and 9 also have pivotal role in extrinsic and intrinsic pathway of apoptosis respectively. In addition, several clinically used cytotoxic agents, including paclitaxel, docetaxel and vinca alkaloids, are known to primarily act by inducing apoptosis in cancer cells [2]. Two major pathways are involved in the induction of apoptosis.

First one is called extrinsic pathway. This pathway starts by agents that activate a family of death receptors leading to the activation of the apoptotic cascade. The second pathway or intrinsic pathway begins by agents that stimulae the release of cytochrome C from mitochondria subsequently occurrence of apoptosis. Both of these pathways lead to the activation of caspases, a group of cysteine aspartyl proteases, which carry out the cleavage of both structural and functional elements of the cell [13]. Activation of caspases within the cell could lead to the induction of apoptosis.

During recent years there has been an intense attention towards the synthesis and biological evaluation of diverse derivatives of 1,3,4-thiadiazole compounds, many of which known to exert attractive pharmacological properties such as antimicrobial, antitubercular, antiinflammatory, anticonvulsants, antihypertensive, antioxidant, antifungal activity, anticancer and apoptosis inducers (Figure 1) [14-19]. Recently we reported the cytotoxic activity of some 1,3,4-thiadiazole derivatives (Figure 2) [20]. In the current project we embarked on the exploration of the exact mechanism of cytotoxicity of these compounds.



R: F, Cl, Br, OH, CH_3, CF_3, -OCH_3, NO_2

Figure 1: 1,3,4-thiadiazole based compounds as apoptosis inducers

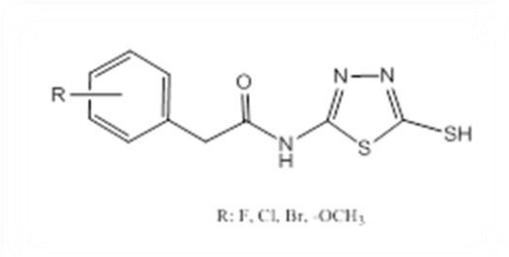


Figure 2: Recently reported cytotoxic 1,3,4-thiadiazole based compounds that likely mechanism were studied in the current project

MATERIALS AND METHODS

Reagents, cell culture material and kits

TritonX-100, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide (MTT) and Rhodamine 123 were obtained from Sigma (St Louis, MO, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCF-DA) was from Molecular Probes (Eugene, OR, USA. DMEM-F12 was purchased from Gibco (Gibco, Grand Island, NY, USA). Caspase-9, caspase-8 and caspase-3 assay kit were purchased from Calbiochem (San Diego, CA, USA).

Cell culture

Human Breast Adenocarcinoma Cell Line (MCF-7), Human Colorectal Adenocarcinoma Cell Line (HT-29) and SK-N-MC were obtained from Pasteur Institute (Tehran, Iran). Cells were cultured in 37°C, CO_2 incubator (5% CO_2) in Dulbecco's Modified Eagle's Medium (DMEM) with 5% v/v Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin and containing 10% heat-inactivated fetal bovine serum. 24 h after seeding cells were incubated overnight with different concentrations of the test compounds and then they were subjected to MTT assay for toxicity evaluation.

Cell viability (MTT) assay

A cytotoxic effect of derivatives was measured using the colorimetric assay in which live cells can reduce MTT to blue formazan crystals [21]. Cells were exposed to the compounds for 24 h and then they were incubated with MTT (0.5 mg/ml, Sigma-Aldrich) for 4.5 h at 37°C. The wells were aspirated and DMSO was added to dissolve blue formazan crystals. After 5 min the plate was read in a ELx808 microplate reader (Biotek) at 570 nm. Data were collected from 9 independent experiments. Some untreated cells underwent the above procedure and were chosen as controls. The following formula was used to determine the percentage of cell viability:

$$\text{Percentage of cell viability} = (\text{OD test}/\text{OD control}) \times 100$$

IC_{50} were, calculated by plotting the $\log_{10}$ of the percentage of viability versus drug concentration.

Detection of caspase activity

Caspase 3, 8 and 9 activities were assayed using the sigma colorimetric caspase kits [17]. The basis of all assays is the ability of the caspases to produce a chromophore from the enzyme substrates Ac-EVD-pNA (for caspase-3), Ac-IETD-pNA (for caspase-8), and Ac-LEHD-pNA (for caspase-9). Briefly cells (5×10^5) were harvested and lysed with 40 µl of the cell lysis buffer. After equalization for protein concentration, 10 µl of cell lysate was added to an equal amount of substrate reaction buffer containing caspase-3, 8, and 9 colorimetric substrates. After an incubation period of 2 h at 37°C the absorbance was measured with a micro-plate reader at 405 nm. The absorbance of the chromophore was compared with an un induced control and the fold change in caspase activity was determined after being corrected for baseline (protein and buffer without colorimetric substrate).

Measurement of mitochondrial membrane potential

The cells were cultured in the abovementioned conditions and then rhodamine 123 was added to the media at a final concentration of 40 µM. After 30 min of incubation, the cells were harvested and washed with Phosphate Buffered Saline (PBS). Cells were lysed by Triton-X 100 and the fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 520 nm using a micro-plate reader (BioTek, H1M, USA). During apoptosis mitochondrial cell membrane is depolarized and rhodamine is lost from the membrane hence intracellular fluorescence intensity is decreased [22].

Intracellular Reactive Oxygen Species (ROS) measurement

Intracellular oxidative stress was assessed by measuring ROS generation using the fluorescent dye DCF-DA [23]. Cells (3×10^5) were cultured and pretreated with the IC_{50} concentrations of each compound for 24 h. After incubation with 10 µM DCF-DA for 45 min, cells were washed with PBS and the intensity of the fluorescence was monitored using the excitation and emission wavelengths of 485 and 530 nm, respectively.

Statistical analysis

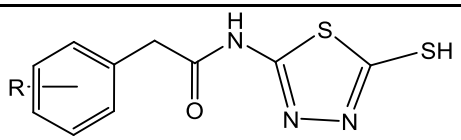
Data were analyzed using Student's t test and presented as mean ± standard deviation. All experiments performed at least three times independently. $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Cytotoxicity evaluation

Compounds A-E was, evaluated against three cancerous cell lines namely Prostate Carcinoma (PC-3), MCF-7 and HT-29. The obtained results were presented as IC_{50} µM in Table 1. Amongst utilized cell lines, HT-29 was the most sensitive one to tested compounds. Unfortunately, none of the tested derivatives demonstrated higher activity compared to doxorubicin towards applied cell lines. Compound B with meta positioning of the fluorine moiety rendered the highest potency against PC-3 cell line (IC_{50}=64.46). HT-29 cell line was so resistant to compound E with meta methoxy substituent. None of the tested compounds exerted acceptable cytotoxic activity against MCF-7 cell line. In fact, MCF-7 cell line was the most resistant cell line to the tested derivatives. As mentioned above, HT-29 cell line was the most sensitive cell line to tested compounds. Compound B with meta positioning of the fluorine atom displayed the highest cytotoxic potency against HT-29 cell line (IC_{50}=33.67 µM).

Table 1: Cytotoxicity results (IC_{50}, µM) of tested compounds A-E against cancerous cell lines (PC-3, MCF-7, HT-29)



Compounds	A	B	C	D	E	Doxorubicin
R	**2-Cl**	**3-F**	**4-F**	**2-OCH_3**	**3-OCH_3**	-
PC-3	71.44	64.46	77.18	75.81	> 80	4.76
MCF-7	> 80	> 80	> 80	> 80	> 80	1
HT-29	36.17	33.67	44.66	44.78	> 80	5.25

Caspase 3 activity

All compounds A-E were investigated for impacting on caspase 3 activities except compound E because of poor potency against all cell lines. Exploration of caspase 3, 8 and 9 activity was ignored in MCF-7 cell line because of the negative results in cytotoxic assay.

PC-3 cell line

All tested derivatives showed a significant increase in caspase 3 activity in comparison with doxorubicin as control drug in PC-3 cell line except for compound D (Figure 3). Compound D (2-OCH_3) also demonstrated an enhancement in caspase 3 activity but less than doxorubicin. Compound B (3-F) caused a remarkable enhancement in activity of caspase 3. The increase in caspase 3 activity was over 3 folds of control drug. Compounds A and C also exerted a robust increase in caspase 3 activity in PC3 cell line two folds of control drug.

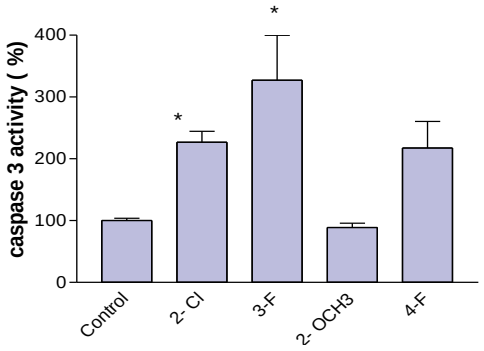


Figure 3: The effects of compounds A-E on the activity of caspase-3 in PC3 cell line compared to control drug (Doxorubicin)

HT-29 cell line

According to the Figure 4, all tested derivatives caused an increase in caspase 3 activity of HT-29 cell line. Amongst investigated compounds, only compound A with ortho-chlorine moiety possessed a superior potency in activation of caspase 3 compared to control drug. Although other tested derivatives promote the activation of caspase 3 activity in HT-29 cell line, none of them exerted higher potency than doxorubicin.

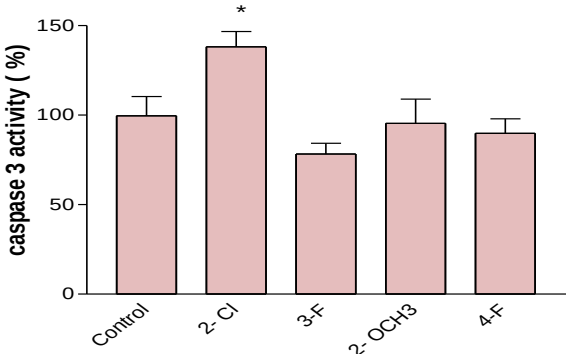


Figure 4: The effects of compounds A-E on the activity of caspase-3 in HT29 cell line compared to control drug (Doxorubicin)

Caspase 8 activity

PC-3 cell line

Compound B with meta fluorine moiety rendered the highest potency for increasing the caspase 8 activity (Figure 5). Compounds A (2-Cl) and C (4-F) also demonstrated higher capability for activation of caspase 8 compared to doxorubicin. Although compound D (ortho methoxy) enhanced the activation of caspase 8, this compound exhibited lower capability to activate caspase 8.

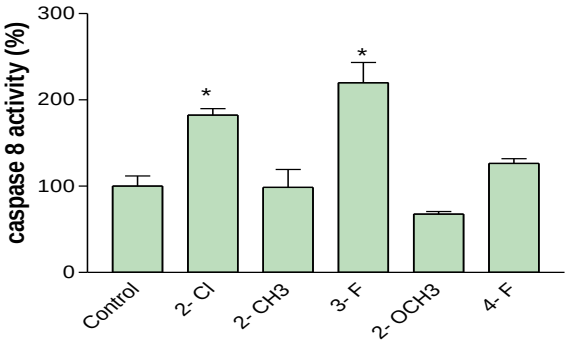


Figure 5: The effects of compounds A-E on the activity of caspase-8 in PC3 cell line compared to control drug (Doxorubicin)

HT-29 cell line

Two folds increase in activity of caspase 8 was observed for compound C (4-F) (Figure 6). Compound A with ortho positioning of chlorine atom also displayed higher potency for activation of caspase 8 in comparison with doxorubicin. Compound B (3-F) and D (2-OCH_3) possessed lower caspase activation than doxorubicin.

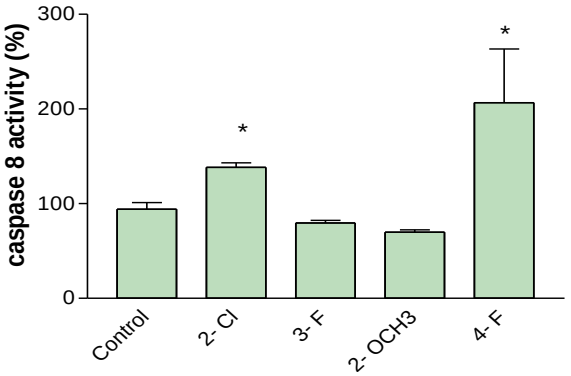


Figure 6: The effects of compounds A-E on the activity of caspase-8 in HT29 cell line compared to control drug (Doxorubicin)

Caspase 9

PC-3 cell line

According to the Figure 7, compounds A, B and C showed a better activity than doxorubicin for activation of caspase 9. Amongst these tree compounds, compound B (3-F) exerted the highest potency in this series. Whereas, compound C (4-F) the lowest potency for activation of caspase 9. Only compound D (2-OCH_3) caused weaker activation of caspase 9 than doxorubicin.

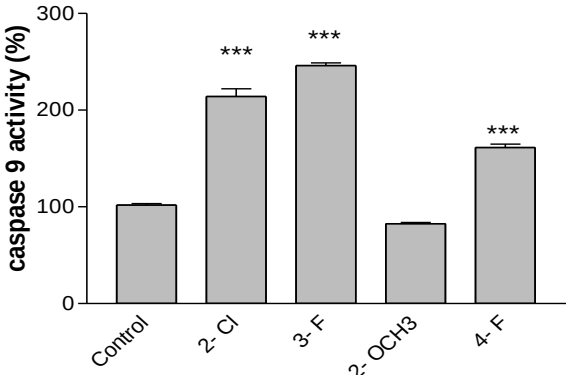


Figure 7: The effects of compounds A-E on the activity of caspase-9 in PC3 cell line compared to control drug (Doxorubicin)

HT-29 cell line

Compound A (2-Cl) induced the activation of caspase 9 in HT-29 cell line significantly. In fact, two folds of enhancement in caspase 9 activity were observed for compound A compared to doxorubicin (Figure 8). Compound D (2-OCH_3) was also more active than doxorubicin. Others, namely compounds B and C tempted favorable enhancement in caspase 9 activity with a trivial superior activity in comparison with doxorubicin.

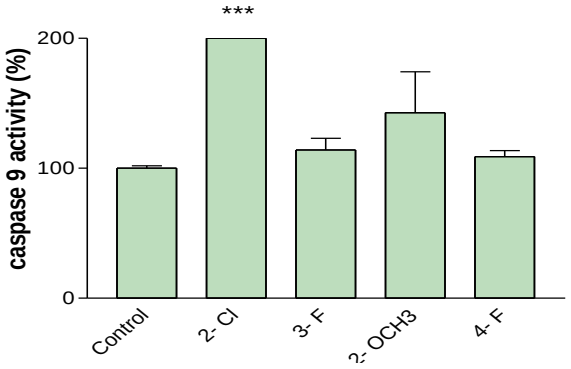


Figure 8: The effects of compounds A-E on the activity of caspase-9 in HT-29 cell line compared to control drug (Doxorubicin)

A new series of cytotoxic previously 1,3,4-thiadiazole derivatives were investigated as apoptosis inducers. Activity of caspases 3, 8 and 9 were measured after treatment of PC-3, HT-29 and MCF-7 cell lines. MCF-7 cell line was the most resistant cell line to the tested compounds. It is likely that tested 1,3,4-thiadiazole compounds have not enough capability for penetration to the MCF-7 cell line or do not have adequate potency for cytotoxic activity on this cell line. Compound B (3-F) was the most active derivative against PC-3 cell line. Whereas, compound E (3-OCH_3) the lowest cytotoxic potency towards PC3 cell line. It is probable replacement of the fluorine atom as electron withdrawing group at position meta of the phenyl residue with methoxy moiety as electron donating group is not a suitable electronic change for interaction with the corresponding receptor. Movement of the fluorine atom to the para position of the phenyl ring also was detrimental for cytotoxic activity and reduced the potency. Substitution of the methoxy moiety at position ortho of the phenyl residue caused an increase in cytotoxic activity. In fact, potentiation of the electronic charge of the phenyl residue enhanced the cytotoxic activity. Utilization of the chlorine substituent at position ortho of the phenyl residue was not so beneficial for activity because of the reducing and negative effect on the charge of the phenyl ring.

HT-29 cell line was the most sensitive cell line to the tested compounds. Approximately, a similar trend like PC-3 cell line was also in this cell line. Namely, compound B (3-F) was the most active compound against HT-29 cell line and compound E (3-OCH_3) was the weakest one. According to the Figures 3-8 compound A (2-Cl) demonstrated a robust activity towards activation of caspase 3, 8 and 9 in all cell lines. Generally, compound A, B and C exhibited high potency for activation of all types of caspases in PC-3 cell line. In the other words, these tree derivatives caused a so effective activation of caspase enzymes in PC-3 cell line superior than doxorubicin. Besides compound A (2-Cl) as potent caspase activator in HT-29 cell line, compound C (4-F) also activated the caspase 8 (Figure 6).

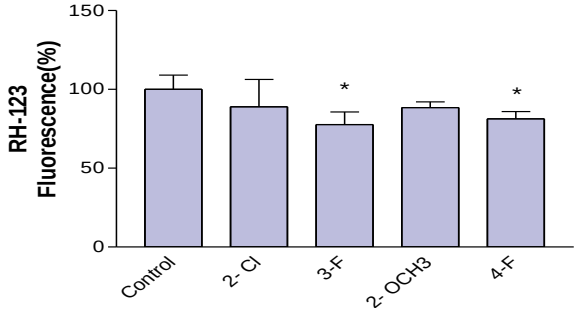


Figure 9: The effects of compounds A-E on the mitochondrial membrane potential of PC-3 cell line

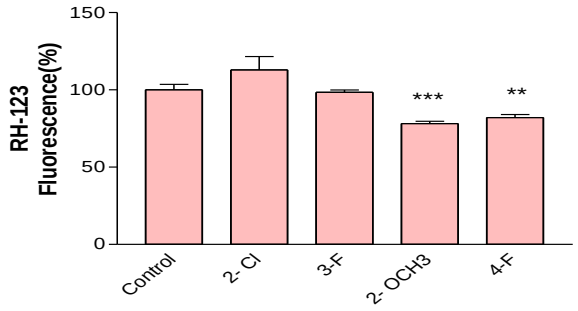


Figure 10: The effects of compounds A-E on the mitochondrial membrane potential of HT-29 cell line

CONCLUSION

Fortunately, the most of the investigated derivatives showed a significant increase in activity of caspases 3, 8 and 9, in PC-3 and HT-29 cell lines superior than doxorubicin. These compounds could be introduced as potent apoptosis inducers that exert their mechanism through intrinsic as well as extrinsic pathway of apoptosis. The tested compounds did not exhibited remarkable activity in MCF-7 cell line. Obtained results were compared to doxorubicin. Besides, Mitochondrial Membrane Potential (MMP) and intracellular ROS were also measured (Figures 9 and 10). Only compound A (2-Cl) caused an increase in MMP in HT-29 cell line further than doxorubicin. None of the tested derivative induced generation of reactive oxygen species in examined cell lines.

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