European Journal of Medicinal Chemistry 77 (2014) 323–333

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Induction of intrinsic apoptosis pathway in colon cancer HCT-116 cells by novel 2-substituted-5,6,7,8-tetrahydronaphthalene derivatives

Amira M. Gamal-Eldeen [a,*], Nehal A. Hamdy [b,*], Hatem A. Abdel-Aziz [b,c], Enas A. El-Hussieny [d], Issa M.I. Fakhr [b]

[a] Cancer Biology Laboratory, Center of Excellence for Advanced Sciences, National Research Center, Dokki, 12622 Cairo, Egypt
[b] Applied Organic Chemistry Department, National Research Centre, Dokki, Cairo, Egypt
[c] Department of Pharmaceutical Chemistry, College of Pharmacy, King Saud University, P.O. Box 2457, Riyadh 11451, Saudi Arabia
[d] Zoology Department, Faculty of Science, Ain Shams University, Cairo, Egypt

ARTICLE INFO

Article history:
Received 26 October 2010
Received in revised form
7 March 2014
Accepted 9 March 2014
Available online 12 March 2014

Keywords:
2-Acetyl tetralin
Enaminone
Pyranone
Pyrazolo[1,5-*a*]pyrimidine
1,2,3-Triazolo[1,5-*a*]pyrimidine
Pyrido[2,3-*d*]pyrimidine
Mitochondrial membrane potential
Apoptosis

ABSTRACT

2-Acetyl tetralin (**1**) reacted with *N*,*N*-dimethylformamide dimethylacetal (DMF-DMA) to afford the enaminone **3**. The reaction of **3** with piperidine and morpholine afforded the trans enaminone **5a**,**b**, respectively. Compound **3** was treated with primary aromatic amines to give secondary enaminones **6a**–**e**. The enaminone **3** reacted with acetylglycine and hippuric acid to yield pyranones **10a**, **b**, respectively. The reaction of enaminone **3** with 1,4-benzoquinone and 1,4-naphthoquinone gave benzofuranyl tetralin derivatives **14a**,**b**, respectively. Also, when **3** reacted with 5-amino-3-phenyl-1*H*-pyrazole **15a** and 5-amino-1,2,3-triazole **15b**, it afforded the new pyrazolo[1,5-*a*]pyrimidine **17a** and 1,2,3-triazolo[1,5-*a*]pyrimidine **17b**, respectively. While the reaction of **3** with pyrimidines **18a**, **b** resulted in the formation of pyrido[2,3-*d*]pyrimidine derivatives **20a**, **b**, respectively. Investigations of the cytotoxic effect of those compounds against different human cell lines indicated that some compounds showed high selective cytotoxicity against colon cancer HCT-116 cells. Some of these compounds led to DNA damaging and fragmentation that was associated with the induction of apoptosis *via* mitochondrial pathway. This pathway is initiated by the impairment of mitochondrial transmembrane potential ($\Delta\psi_m$) and in response to that the mitochondria released cytochrome c increased, that in turn activated caspase-9 and caspase-3 and induced apoptosis. Compounds **17b** and **20b** were promising anti-cancer agents that induced intrinsic apoptosis pathway in colon cancer cells.

1. Introduction

Apoptosis, programmed cell death, plays an important role in developmental processes by eliminating unwanted cells so as to maintain homeostasis in healthy tissue [1]. Perturbations in its regulation contribute to numerous pathological conditions, including cancer and autoimmune and degenerative diseases [2,3]. On the other hand, a wide variety of chemotherapeutic agents have been shown to cause the death of cancer cells by inducing apoptosis [4]. Two principal signal pathways have been established for the induction of apoptotic cell death, the extrinsic pathway (the death receptor pathway), which is triggered following the activation of cell-surface-expressed death receptors such as CD95 (also known as Fas receptor) and tumor necrosis factor receptor [5], followed by the activation of caspase-8, which activates the downstream effector caspases −3 and −7, and then −6. The intrinsic apoptotic pathway (the mitochondrion-mediated pathway) is initiated in response to a variety of stress signals [6], and a complex interplay of Bcl-2 proteins relays this signal to the mitochondrial outer membrane (OM) to initiate Bak and Bax activation, oligomerisation and OM damage. Breaching the mitochondrial OM releases apoptogenic factors, including cytochrome c and Smac, which activate a group of aspartate-specific proteases (caspases) [7]. Caspases, in turn, cleave several hundred cellular proteins to coordinate the destruction of the cell [8,9]. By contrast, the extrinsic apoptotic pathway can activate caspases without the participation of mitochondria [5].

In certain types of cells, effector caspase activation requires amplification of death-inducing signaling complex signals by engagement of the cell-intrinsic pathway. A critical step in the cell-intrinsic pathway is the activation of Bax, leading to dissipation of mitochondrial transmembrane potential ($\Delta\psi_m$) and cytochrome c

* Corresponding authors.
E-mail addresses: aeldeen7@yahoo.com (A.M. Gamal-Eldeen), drnehalhamdy63@hotmail.com (N.A. Hamdy).

http://dx.doi.org/10.1016/j.ejmech.2014.03.021
0223-5234/

release into the cytosol [10]. The compounds that induce apoptosis may be implemented by the cell-intrinsic pathway, which regularly begins with the disruption of $\Delta\psi_m$ and the release of apoptogenic factors such as cytochrome c from the intermembrane space into the cytosol [11]. These factors activate caspase-9, which in turn activates the executioner caspase-3. In this pathway, mitochondrion is the center of cell death control, and the mitochondrial membrane is the primary site of action by proapoptotic and anti-apoptotic factors [10].

The biological activities for pyrazolo[1,5-*a*]pyrimidines have stimulated chemists to develop the chemistry of this class of compounds [12], however, enaminones have been reported as useful precursors for the synthesis of pyrazolo[1,5-*a*]pyrimidines [13,14]. Enaminones are versatile reagents and their utility in heterocyclic synthesis has received a considerable attention [15]. On the other hand, a great deal of interest has been focused on the synthesis of tetralin derivatives due to their biological potentialities [16]. Moreover, pyridopyrimidine derivatives were reported as adenosine kinase inhibitors and potent anticancer drugs [17]. Similarly, the importance of pyran-2-one derivatives as building blocks in the field of synthetic and medicinal chemistry has been well established as a consequence of their interesting structural features and diverse pharmacological properties [18]. In addition, many benzofuran derivatives are reported as interesting pharmaceutical compounds [19–21]. They have potent pesticidal and insecticidal [22], antitumor [23], anthelmintic [24], nematocidal [24], antifungal [25], and anti-inflammatory [26] activities, and its nucleus is incorporated in various natural products [27].

All the previous findings and in continuation of our interest in the synthesis of a wide variety of heterocyclic systems for biological screening [28,29], we report here the potentiality of *E*-3-(*N*,*N*-dimethylamino)-1-(5,6,7,8-tetrahydronaphthalen-2-yl)prop-2-en-1-one (**3**) in the synthesis of the title compounds. We also evaluated their influence on the cancer cell death and of both of the extrinsic and intrinsic pathways of apoptosis.

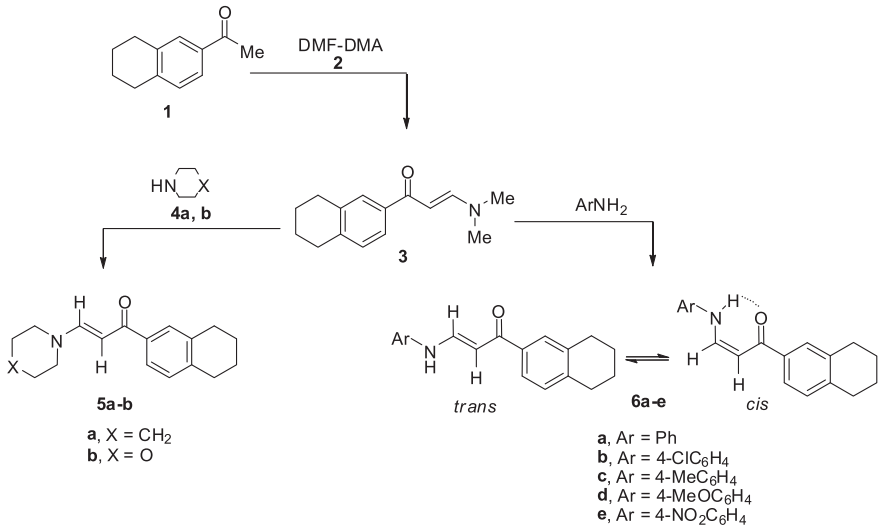
2. Results and discussion

Treatment of 2-acetyl tetralin (**1**) with *N*,*N*-dimethylformamide dimethylacetal (DMF-DMA) in refluxing dry toluene, afforded *E*-3-(*N*,*N*-dimethylamino)-1-(5,6,7,8-tetrahydronaphthalen-2-yl)prop-2-en-1-one (**3**) [28] (Scheme 1) and its structure was confirmed by elemental analysis and spectral data, however, the *E*-configuration of the latter enaminone **3** have been reported [28,30].

The reactivity of the enaminone **3** towards some nitrogen nucleophiles was investigated. Thus compound **3** was reacted with some secondary amines namely piperidine **4a** and morpholine **4b** in refluxing ethanol to yield the corresponding tertiary amines **5a**, **b** respectively. The IR spectra of the latter products showed carbonyl absorption bands at the region 1637, 1657 cm^{-1} respectively. Their ^1H NMR spectra are free of signals characteristic for the dimethylamine protons, in addition the value of the coupling constant ($J = 12.2$ Hz) for the olefinic protons indicates that the enaminones **5a**, **b** exist in the *trans* configuration.

The enaminone **3** reacted with primary aromatic amines namely aniline, 4-chloroaniline, 4-toludine, 4-anisidine and 4-nitroaniline to yield the corresponding secondary amines **6a**–**e**, respectively, through nucleophilic addition substitution reaction as a singlet product in each case (as examined by TLC). The stereochemistry of each compound (**6a**–**e**) was assigned, based on the ^1H NMR spectroscopy in DMSO-d_6. They were found to exist in the *E*/*Z* geometric conjugation forms. ^1H NMR spectra revealed two sets of doublet each belonging to the NH group of the *cis* and *trans* conformers were observed between δ 9.78–10.50 ppm and δ 11.95–12.20 ppm for a total of one proton. The up-field lines of –NH protons were assigned to *cis* conformer and downfield lines of the protons of the same group to *trans* conformer [31].The formation of the *Z*-form was proven by the coupling constant of the hydrogen atoms attached on the double bond ($J = 8.0$ Hz) and also by a distinct splitting of the hydrogen atom on the NH group ($J = 12.5$ Hz) fixed *via* an intramolecular hydrogen bond [32]. The *E*-form was confirmed by the coupling constant of the hydrogen atoms attached on the double bond ($J = 12.5$ Hz). In general, tertiary enaminones tend to adopt the *E* form, which are sterically less hindered while secondary enaminones exist predominantly, and in many cases completely, in the *Z* form in polar solvents. This allows for intramolecular hydrogen bonding. This observation is also in accordance with the results in the literature [33–35].

Reacting compound **3** with acetylglycine (**7a**) or hippuric acid (**7b**) in acetic anhydride afforded the 3-pyranyl tetrahydronaphthalene derivatives **10a**, **b**, respectively. These are assumed to be formed *via* initial cyclization of acetylglycine and hippuric acid into the oxazolones **8a**, **b** which then added to the activated double bond system of a molecule of compound **3** yielding compounds **9a**, **b** followed by further rearrangement to give **10a**, **b**, as shown in



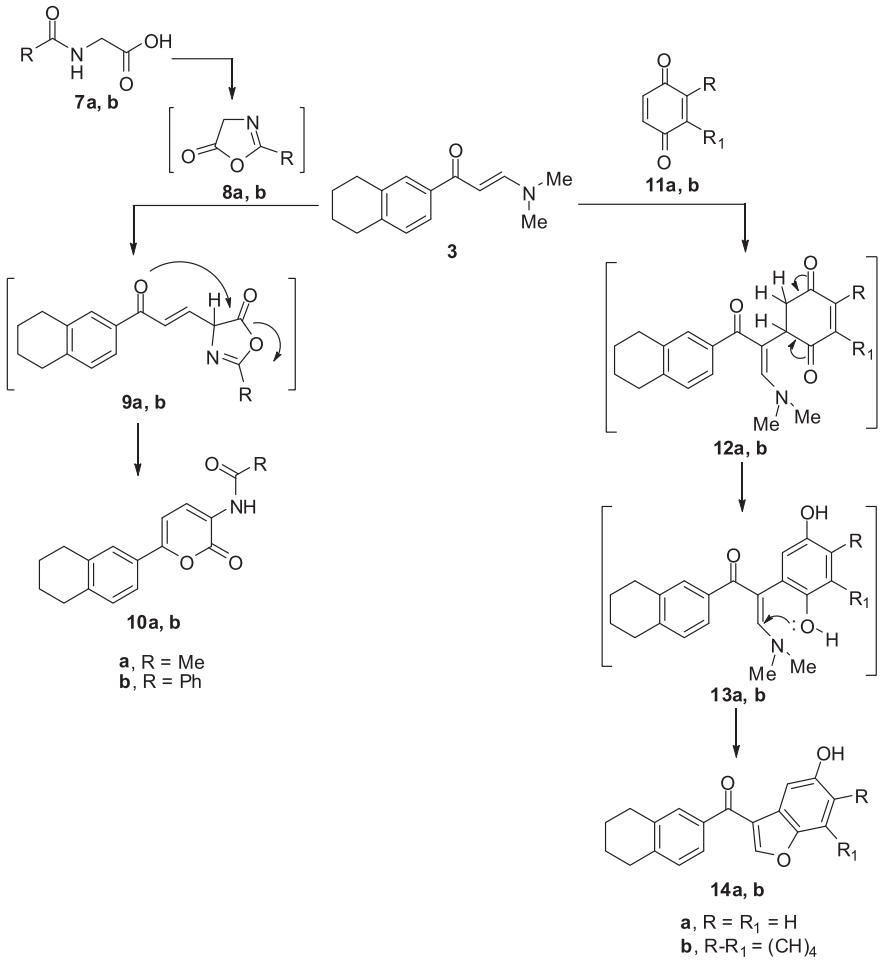
Scheme 1. Synthesis of enaminones **5a, b** and **6a-e**.

Scheme 2. This is similar to the well known Kepe pyranone synthesis [36a] that has been adopted by Elnagdi et al. [36b]. The structures of latter compounds were confirmed on the basis of the elemental analysis and spectral data. For example, their IR spectra revealed absorption bands at 3287 and 3391 cm^{-1}, respectively, due to NH function in addition to two absorption bands in the regions 1704–1705 cm^{-1} and 1677–1658 cm^{-1}, respectively due to two carbonyl function. Their ^1H NMR spectra showed the protons of pyran moiety in addition to D_2O exchangeable broad singlet due to NH function.

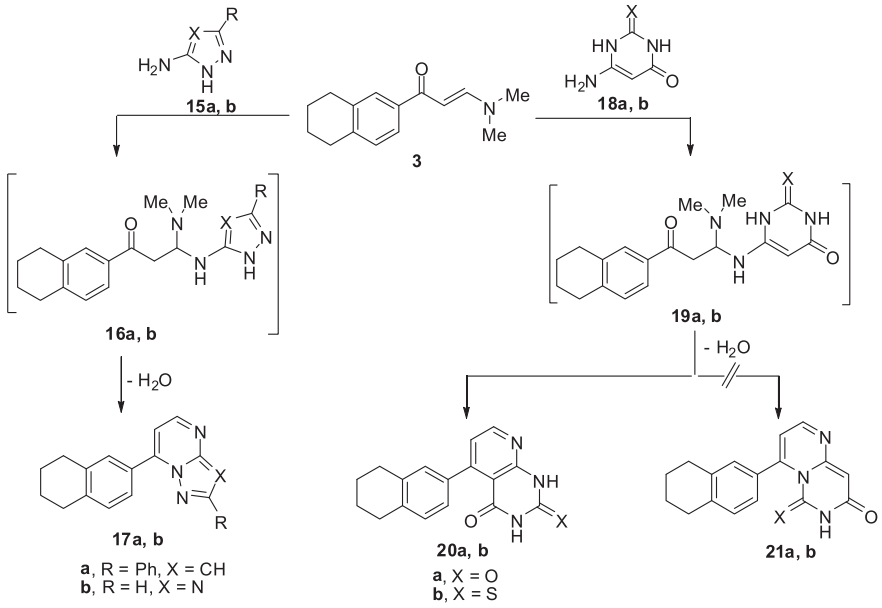
Compound **3** reacted with 1,4-benzoquinone (**11a**) and 1,4-naphthoquinone (**11b**) yielding the benzofuran **14a** and naphthofuran **14b**, respectively. It is believed that compound **3** firstly added to the quinone to yield the intermediate phenolic adduct that cyclized *via* loss of dimethylamine to yield final isolable products. Similar reaction sequence has been proposed to account for the formation of benzofuran and naphthofuran from reaction of enaminones with quinones [37]. Structures of **14a** and **14b** were supported by spectral data. For example, their IR spectra showed broad band of hydroxyl group at 3330–3241 cm^{-1} and it also revealed the carbonyl absorption band at 1619–1626 cm^{-1}, respectively. Their ^1H NMR spectrum showed a characteristic downfield signal at δ 8.54 and 8.69 ppm of C_1 proton of furan moiety and revealed D_2O exchangeable broad singlet at δ 9.40 and 10.21 ppm due to hydroxyl group, respectively.

The reaction of enaminone **3** with some heterocyclic amines as potential precursors for fused heterocyclic systems was also investigated. Thus when compound **3** was treated with 5-amino-3-phenyl-1*H*-pyrazole (**15a**) or 3-amino-1,2,4-triazole (**15b**) in refluxing ethanol, in the presence of a catalytic amount of piperidine furnished in each case a single product identified as pyrazolo[1,5-*a*]pyrimidine **17a** and 1,2,3-triazolo[1,5-*a*]pyrimidine **17b** derivatives, respectively (Scheme 3). The structures of the latter compounds were confirmed by elemental analysis and spectral data. For example, the IR spectrum of **17a**, **b** revealed no bands due to amino or carbonyl functions. Moreover, the ^1H NMR spectrum for **17a** revealed a singlet signal due to pyrazole proton at δ 5.31 ppm in addition to doublet signals at δ 8.12, 8.56 ppm due to pyrimidine protons. Also the ^1H NMR spectrum for **17b** revealed doublet signals at δ 8.23, 8.86 ppm due to pyrimidine protons, in addition to a singlet signal due to the triazole proton at δ 8.73 ppm. Although the endo cyclic nitrogen in compounds **15a**, **b** is the most nucleophilic site [38,39], it is sterically hindered site. Formation of compounds **17a**, **b** are therefore assumed to take place *via* an initial Michael addition of the exo-cyclic amino group in compounds **15a**, **b** to the activated double bond in compound **3** to give the acyclic non-isolable intermediate **16a**, **b** which undergo cyclization *via* loss of both dimethylamine and water molecules to produce the final isolable products **17a**, **b** as depicted in Scheme 3.

Enaminone **3** condensed readily with 6-aminopyrimidin-2,4(1*H*,3*H*)-dione (**18a**) or 6-amino-2-thioxo-2,3-dihydropyrimidin-4(1*H*)-one (**18b**) in boiling acetic acid to give pyrido[2,3-*d*]pyrimidine derivatives **20a**, **b** not the isomeric structures **21a**, **b** (Scheme 3). The ^1H NMR spectra of the resulting products **20a**, **b** displayed two doublet signals recognizable as arising from two CH of pyridine in the regions δ 7.89–8.29 and 7.86–8.26 ppm and two NH groups of



Scheme 2. Synthesis of pyranones **10a**, **b**, benzofuran **14a** and naphthofuran **14b**.



Scheme 3. Synthesis of pyrazolo[1,5-a]pyrimidine **17a**, 1,2,3-triazolo[1,5-a]pyrimidine **17b** and pyrido[2,3-d]pyrimidines **20a, b**.

pyramidie in the region δ 8.92–11.68 and 8.99–11.93 ppm, respectively. The formation of compounds **20a**, **b** can be explained on the basis of an initial Michael type addition of the amino group in aminopyrimidines **18a**, **b** to the double bond in enaminone **3** to afford the non-isolable intermediates **19a**, **b**, which underwent cyclization and aromatization *via* loss of both dimethylamine and water molecules producing the final isolable pyridopyrimidine derivatives **20a**, **b** and not **21a**, **b** on the basis of the spectral data of the isolated products, which in agreement with that recently reported [40].

Screening of the cytotoxic effect of the synthesized compounds against variable human cancer cell line (Hep-G2, HeLa, HCT-116 and MCF-7), using MTT assay, revealed that most of the tested compounds showed low cytotoxic effect against Hep-G2, HeLa, and MCF-7 cells as indicated by their high IC_{50} values (Table 1). On the other hand, some compounds showed high selective cytotoxicity against HCT-116 cells as indicated by their low IC_{50} values (<10 μg/ml) in the following order **20b** < **17b** < **6e** < **5b** < **6b** < **10a** < **14a** (Table 1). **20b** showed the highest potential cytotoxicity against HCT-116 cells as effective as that of paclitaxel as concluded from their relatively close IC_{50} values. According to these findings, we selected HCT-116 cell line to explore the cell death mechanism due to the treatment with the promising cytotoxic compounds: **5b**, **6b**, **6e**, **10a**, **14a**, **17b**, and **20b**.

To detect the type of cell death, the IC_{50} of each compound in HCT-116 cells were examined and the apoptosis and necrosis percentages were recorded using acridine orange/ethidium bromide staining and analysis under fluorescence microscope. As shown in Fig. 1A, all of the tested compounds led to an apoptosis-dependant cell death 77–96% of the total number of dead cell population, while the percentage of necrotic cells were only 0–23% of the total number of dead cell population. Comparing the untreated cells with paclitaxel-treated HCT-116 cells indicated that the later significantly induced the DNA fragmentation up to 44% ($P < 0.001$), as shown in Fig. 1B. Similarly the treatment of HCT-116 cells with different compounds resulted in a high, to variable extents, DNA fragmentation levels that were significantly different from control (P < 0.01–0.001), in the following order: **20b** > **17b** > **10a** < **5b** < **6b** < **6e** < **14a**.

Mitochondria are subcellular organelles that are essential in the regulation of cellular bioenergetics as a major source of ATP, which is produced through oxidative phosphorylation by the mitochondrial respiratory chain (RC) that localized in the inner membrane of the mitochondria and includes cytochrome c as an electron carrier [41] The electron transport generates an electrochemical proton gradient across the inner membrane, measured as $\Delta\psi_m$, which drives ATP synthesis by the ATP synthase [41]. Since loss of $\Delta\psi_m$ induces release of apoptogenic factors into cytoplasm and decrease of ATP generation, leading to cell death, in the present study, we have demonstrated that the preferential target of some compounds (**6e**, **10a**, **17b**, and **20b**) was the mitochondria and through inducing of mitochondrial dysfunctions. The mitochondrial dysfunction was assessed by measuring $\Delta\psi_m$ in HCT-116 cells. Interestingly, the ability to lower the $\Delta\psi_m$ was observed to be dominant with **17b** ($P < 0.001$) and **20b** ($P < 0.001$), which were more effective than the positive control; CCCP (Fig. 2A). Moreover, the results indicated **5b**, **6b** and **14a** did not impair $\Delta\psi_m$, which may suggest that these compounds induced apoptosis through death receptors pathway rather than mitochondrial mediated pathway.

Table 1
The cytotoxicity effect of different synthesized compounds of Schemes 1–3 as measured by MTT assay, after 48 h of incubation. The results are expressed as IC_{50}; μg/ml.

Compounds	(IC_{50}; μg/ml)			
	Hep-G2	HeLa	HCT-116	MCF-7
3	22.3	33.4	18.9	29.3
5a	39.1	35.6	35.4	41.1
5b	18.2	41.2	7.2	38.6
6a	19.9	30.6	41.6	32.2
6b	30.3	19.9	7.9	28.7
6c	42.7	28.7	42.1	43.6
6d	31.8	46.1	31.8	43.1
6e	27.4	48.4	6.8	28.5
10a	45.3	30.4	8.4	20.9
10b	29.3	38.3	47.7	27.5
14a	39.2	41.6	9.5	40.3
14b	31.7	40.5	36.2	48.7
17a	37.6	36.2	26.7	19.9
17b	15.9	21.8	2.1	48.2
20a	39.9	29.1	18.6	24.4
20b	27.0	25.3	0.49	18.2
Paclitaxel	0.59	0.47	0.38	0.61

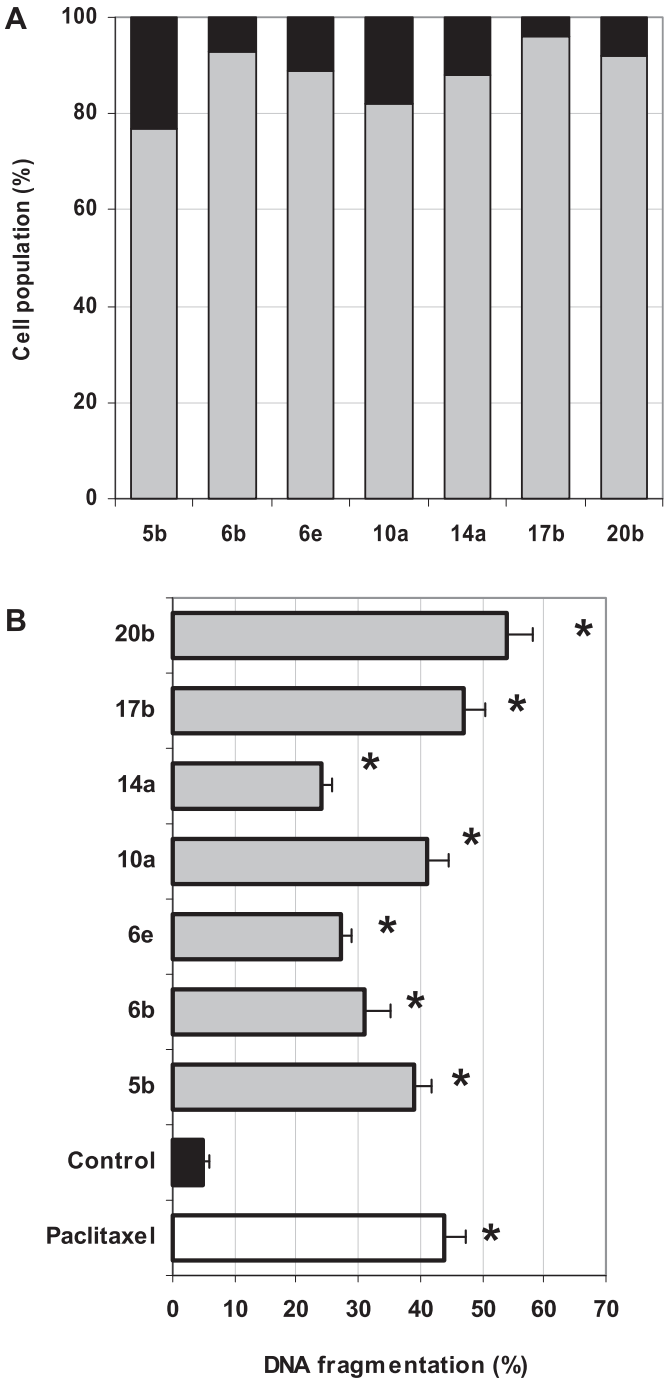


Fig. 1. A. The type of cell death was investigated in HCT-116 cells after the treatment with different compounds, using acridine orange/ethidium bromide staining to compare between the percentage of necrotic cells (black segment) and the apoptotic cells (gray segment). B. The effect of different compounds (gray bars) and paclitaxel (white bars) on the percentage of DNA fragmentation in colon cancer HCT-116 cells. The fragmented DNA was determined with the diphenylamine reaction. Values are means of three measurements (Mean ± SD), where (*) represent the $P < 0.05$ in comparison with control (black bars).

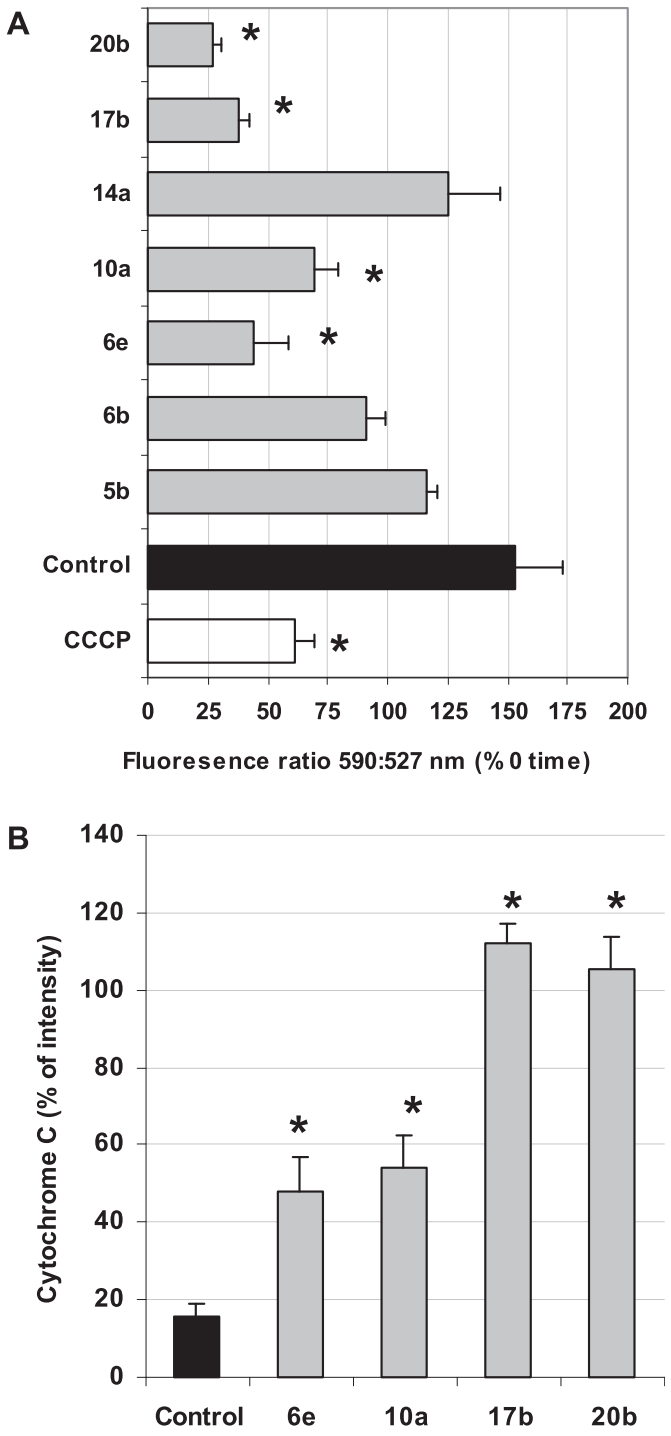


Fig. 2. A. The effect of different compounds (gray bars) on mitochondrial membrane potential ($\Delta\psi_m$) of colon cancer HCT-116 cells. CCCP was the positive control (white bars). Values are expressed as percentage of zero time readings and represent mean of three readings ± S.D. B. Dot blot analysis of the cytosolic content of cytochrome C in the compounds-treated HCT-116 cells. Data are expressed as the mean percentage of color intensity of dots of three replicates, where (*) represent the $P < 0.05$ in comparison with control (black bars).

Besides respiration, mitochondria also play important roles in the regulation of apoptosis. The mitochondrial pathway of apoptosis is initiated by the proapoptotic Bcl-2 family proteins, such as Bax and Bak, which form pores and induce mitochondrial outer membrane permeabilization [41]. This leads to a release of cytochrome c, loss of $\Delta\psi_m$, and activation of various caspases. These caspases cleave specific substrates within the cell to produce changes associated with apoptosis ($\Delta\psi_m$ is important for ATP production and mitochondrial protein transport) [42,43]. On the other hand, disruption of $\Delta\psi_m$ is also implicated in various apoptotic

phenomena [44] and mitochondrial dysfunction and dysregulation of apoptosis are implicated in many diseases such as cancer.

To investigate the effect of $\Delta\psi_m$ loss in the release of apoptogenic factors into cytoplasm we investigated the alteration in cytochrome C and caspases release after the treatment with **6e**, **10a**, **17b**, and **20b**. Cytochrome c, a component of the mitochondrial electron transfer chain that is present in the intermembrane space, is released into the cytosol during the early phases of apoptosis [45]. Therefore, we assayed the accumulation of mitochondrial cytochrome *c* release into the cytosol by immunoblotting of the cell lysate of compounds-treated HCT-116 cells. As shown in Fig. 2B, cytosol from untreated cells contained low cytochrome c content. In contrast, cytochrome c accumulated significantly in the cytosol of the cells after treated with **6e** and **10b** ($P < 0.01$) and highly accumulated after treated with **17b**, and **20b** ($P < 0.001$).

In many apoptotic systems, release of cytochrome *c* into the cytosol results in the activation of the executioner caspases of apoptosis. To determine whether caspases activation is involved in the compounds associated apoptosis, caspases were investigated in HCT-116 cells. The analysis revealed that there was a dramatic increase in the total caspases level after the treatment with **6e** and **10a** ($P < 0.05$), **17b** and **20b** ($P < 0.01$), as shown in Fig. 3. On the other hand, the investigation of the individual caspases indicated that the treatment of HCT-116 cells with **17b** and **20b** led to a significant increase ($P < 0.01$) in caspase-3 and -9 activities; whereas **17b** led to a significant enhancement ($P < 0.01$) in caspase-3 but not caspase-9, in addition to **10a** which led to significant enhancement ($P < 0.01$) in caspase-9 but not caspase-3, as shown in Fig. 3.

In the present work the mechanistic evaluation of the cytotoxic compounds against HCT-116 cells revealed that **6e**, **10a**, **17b** and **20b** are the most promising compounds as intrinsic apoptosis inducer agents. This activity may be due to the presence of the nitrophenyl group in **6e**, acetamide group in **10a**, triazolopyrimidine ring in **17b** and the pyridopyrimidine ring in **20b**. These active ring systems represent the characteristic difference of each compound from its corresponding family of compounds in Schemes 1–3 that may be responsible for their mechanistic role in apoptosis process.

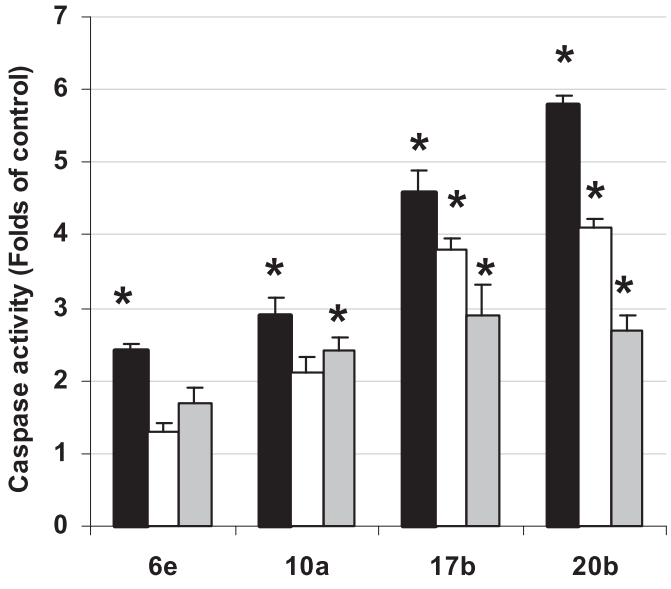


Fig. 3. The effect of different compounds on the level of total caspases (black bars), caspase-3 (white bars) and caspase-9 (gray bars) in the HCT-116 cells. Data are expressed as fold of control untreated cells activity, where (*) represent the $P < 0.05$ in comparison with control.

3. Conclusion

The important characteristic in any chemotherapeutic drugs is its ability to induce $\Delta\psi_m$ collapse and cytochrome c release, thereby induce the activation of the downstream caspases and apoptosis [46]. In the present work, some of the synthetic compounds showed high selective cytotoxicity against colon cancer HCT-116 cells among all the tested human cancer cell lines. The treatment of cells HCT-116 with **6e**, **10a**, **17b**, and **20b** resulted in DNA damaging and fragmentation that was associated with the induction of apoptosis *via* mitochondrial pathway. This pathway is initiated by **6e**, **10a**, **17b**, and **20b** treatment through the impairment of $\Delta\psi_m$ and in response to that besides the DNA damage and the mitochondria released cytochrome c that may in turn activated caspase-9 through autoproteolysis. The treatment with **17b** and **20b** resulted in activation of caspase-9 proteolyses activated caspase-3, which activates other downstream caspases causing the apoptotic phenotype. Compounds **17b** and **20b** are promising anti-cancer agents that induced intrinsic apoptosis pathway in colon cancer cells. These findings required further in vitro and in vivo investigations for confirmation.

4. Experimental

4.1. Chemistry

Melting points were measured with a Gallenkamp apparatus and are uncorrected. IR spectra were recorded on Shimadzu FT-IR 8101 PC infrared spectrophotometer. The ^{1}H NMR spectra were recorded on a Varian Mercury VX-300 NMR spectrometer. ^{1}H NMR spectra were run at 300 MHz and ^{13}C NMR spectra were run at 75.46 MHz in deuterated dimethylsulfoxide (DMSO-d_6). Chemical shifts are quoted in δ (ppm) and were related to that of the solvents. Mass spectra were measured on a GCMS-QP1000 EX spectrometer at 70 e.V. Elemental analyses was carried out at the Microanalytical center of Cairo University. 1-(5,6,7,8-Tetrahydronaphthalen-2-yl)ethanone (**1**) [47] was prepared by the reported method and 5-amino-3-phenyl-1H-pyrazole (**15a**), 3-amino-1,2,4-triazole (**17b**), Glycine derivatives **7a**-**7b** and pyrimidine derivatives **18a**–**18b** were used as obtained commercially.

4.1.1. *E*-3-(*N*,*N*-dimethylamino)-1-(5,6,7,8-tetrahydronaphthalen-2-yl) prop-2-en-1-one (**3**) [28]

To a mixture of 1-(5,6,7,8-tetrahydronaphthalen-2-yl)ethanone (**1**) [47] (1.74 g, 10 mmol) in dry toluene (50 mL), dimethylformamide-dimethylacetal (DMF-DMA) (1.34 g, 10 mmol) was added and the mixture was refluxed for 5 h. The solvent was evaporated and the residual reddish brown viscous liquid was taken in ether. The resulting yellow crystals were collected by filtration, washed thoroughly with ether, dried and finally recrystallized from EtOH to afford compound **3** [28] as yellow crystals in 58% yield, mp 70–72 °C; IR (KBr) (ν, cm^{-1}): 1634 (C=O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.71 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 2.72 (m, 4H, aliphatic 2CH$_2$ of tetrahydronaphthalene), 2.87 (s, 3H, CH$_3$), 3.10 (s, 3H, CH$_3$), 5.76 (d, 1H, J = 12.2 Hz, –CO–CH=), 7.05–7.58 (m, 3H Ar–H), 7.65 (d, 1H, J = 12.2 Hz, =CH–N–); ^{13}C NMR (DMSO-d_6): δ (ppm) 20.12, 20.13 (2CH$_3$), 22.48, 22.60, 28.70, 28.75 (4CH$_2$), 90.93 (CO–CH=), 124.25, 127.67, 128.44, 136.17, 137.52, 139.62 (Aromatic-C), 153.63 (=CH–N–), 185.70 (C=O); MS *m*/*z* (%): 229 (M$^+$, 92.4), 212 (99.0), 159 (43.5), 98 (100), 70 (74.5). Analysis (Calc/found %): for C$_{15}$H$_{19}$NO (229.32): C, 78.56/78.24; H, 8.35/8.30; N, 6.11/6.28.

4.1.2. General procedure reaction of the enaminone 3 with piperidine and morpholine

A mixture of *E*-3-(*N*,*N*-dimethylamino)-1-(5,6,7,8-tetrahydronaphthalen-2-yl)prop-2-en-1-one (**3**) (1.15 g, 5 mmol) and the

appropriate secondary amine piperidine **4a** or morpholine **4b** (3 mL) in ethanol (30 mL) was refluxed for 2 h, then left to cool. The solid so formed, in each case, was collected by filtration and recrystallized from ethanol to afford **5a** and **5b**, respectively.

*4.1.2.1. E-3-(piperidin-1-yl)-1-(5,6,7,8-tetrahydronaphthalen-2-yl) prop-2-en-1-one (**5a**).* Yield (64%); mp 134–136 °C; IR (KBr) (ν, cm^{-1}): 1637 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.56 (m, 6H, $3CH_2$ of piperidine), 1.71 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.73 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.39 (m, 4H, $(CH_2)_2$ = N– of piperidine), 5.94 (d, 1H, J = 12.2 Hz, –CO–CH =), 7.07–7.58 (m, 3H Ar–H), 7.60 (d, 1H, J = 12.2 Hz, ═CH–N–); MS *m*/*z* (%): 269 (M^+, 100%), 252 (96.2), 186 (65.8), 159 (80.2), 138 (73.3), 110 (98.6), 84 (99.8). Analysis (Calc/found %): for $C_{18}H_{23}NO$ (269.39): C, 80.26/80.08; H, 8.61/8.74; N, 5.20/5.03.

*4.1.2.2. E-3-morpholino-1-(5,6,7,8-tetrahydronaphthalen-2-yl)prop-2-en-1-one (**5b**).* Yield (65%); mp 115–117 °C; IR (KBr) (ν, cm^{-1}): 1657 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.72 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.74 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.00 (m, 4H, $(CH_2)_2$ = N– of morpholine), 3.92 (m, 4H, $-CH_2-O-CH_2-$ of morpholine), 5.70 (d, 1H, J = 12.2 Hz, –CO–CH =), 7.05–7.58 (m, 3H, Ar–H), 7.7 (d, 1H, J = 12.2 Hz, ═CH–N–); MS *m*/*z* (%): 271 (M^+, 23.3), 174 (32.7), 159 (100), 131 (58.5), 91 (48.6). Analysis (Calc/found %): for $C_{17}H_{21}NO_2$ (271.36): C, 75.25/75.25; H, 7.80/7.80; N, 5.16/5.16.

4.1.3. General procedure for the reaction of enaminone 3 with primary aromatic amines

To a mixture of *enaminone derivative* **3** (1.15 g, 5 mmol) in acetic acid (20 mL), the appropriate primary aromatic amine namely (aniline, 4-chloroaniline, 4-toludine, 4-anisidine and 4-nitroaniline) (5 mmol) was added and the reaction mixture was stirred for 3 h. The precipitated product was collected by filtration, washed with ethanol and dried. Crystallization from ethanol afforded the corresponding derivatives **6a**–**e**, respectively.

*4.1.3.1. 3-Phenylamino-1-(5,6,7,8-tetrahydronaphthalen-2-yl)prop-2-en-1-one (**6a**).* Yield (71%); mp 160–162 °C, the ratio of *cis/trans*: 60/40; IR (KBr) (ν, cm^{-1}): 3226 (NH), 1660 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.75 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 6.07 and 6.41 (d, 1H, J = 8.0 and 12.5 Hz, –CO–CH = , *cis and trans* conformers), 7.12–7.96 (m, 9H, Ar–H + ═CH–N–, *cis* and *trans* conformers), 9.90 and 11.99 (d, 1H, J = 12.0 Hz, NH, D_2O exchangeable, *cis* and *trans* conformers); MS *m*/*z* (%): 277 (M^+, 23.3), 276 (100), 159 (85.0), 146 (42.2), 91 (55.4). Analysis (Calc/found %): for $C_{19}H_{19}NO$ (277.37): C, 82.28/82.15; H, 6.90/6.83; N, 5.05/4.88.

*4.1.3.2. 3-[(4-Chlorophenyl)amino]-1-(5,6,7,8-tetrahydronaphthalen-2-yl)prop-2-en-1-one (**6b**).* Yield (75%); mp 142–144 °C, the ratio of *cis/trans*: 57/43; IR (KBr) (ν, cm^{-1}): 3350 (NH), 1634 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.75 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.77 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 6.09 and 6.43 (d, 1H, J = 8.0 and 12.5 Hz, –CO–CH = , *cis and trans* conformers), 7.14–7.90 (m, 8H, Ar–H + ═CH–N–), 10.0 and 11.95 (d, 1H, J = 12.5 Hz, NH, D_2O exchangeable, *cis* and *trans* conformers); MS *m*/*z* (%): 312 (M^++1, 73.8), 311 (M^+, 100), 201 (84.7), 159 (37.8), 91 (23.3). Analysis (Calc/found %): for $C_{19}H_{18}ClNO$ (311.81): C, 73.19/73.00; H, 11.37/11.23; N, 4.49/4.62.

*4.1.3.3. 3-[(4-Tolyl)amino]-1-(5,6,7,8-tetrahydronaphthalen-2-yl) prop-2-en-1-one (**6c**).* Yield (68%); mp 134–136 °C, the ratio of *cis/trans*: 62/38; IR (KBr) (ν, cm^{-1}): 3258 (NH), 1628 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.74 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.25 (s, 3H, CH_3), 2.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 6.08 and 6.44 (d, 1H, J = 8.0 and 12.5 Hz, –CO–CH═, *cis and trans* conformers), 7.13–7.95 (m, 8H, Ar–H + ═CH–N–, *cis* and *trans* conformers), 9.98 and 11.99 (d, 1H, J = 12.5 Hz, NH, D_2O exchangeable, *cis* and *trans* conformers); MS *m*/*z* (%): 292 (M^+ + 1, 25.3), 291 (M^+, 92.6), 290 (100.0), 160 (60.5), 91 (45.8). Analysis (Calc/found %): for $C_{20}H_{21}NO$ (291.40): C, 82.44/82.17; H, 7.26/7.40; N, 4.81/4.92.

*4.1.3.4. 3-[(4-Methoxyphenyl)amino]-1-(5,6,7,8-tetrahydronaphthalen-2-yl) prop-2-en-1-one (**6d**).* Yield (70%); mp 122–124 °C, the ratio of *cis/trans*: 69/31; IR (KBr) (ν, cm^{-1}): 3385 (NH), 1617 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.74 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.75 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 3.76 (s, 3H, CH_3), 6.00 and 6.34 (d, 1H, J = 8.0 and 12.5 Hz, –CO–CH═, *cis and trans* conformers), 6.92–7.24 (m, 8H, Ar–H + ═CH–N–, *cis* and *trans* conformers), 9.78 and 12.05 (d, 1H, J = 12.5 Hz, NH, D_2O exchangeable, cis and *trans* conformers); MS *m*/*z* (%): 308 (M^+ + 1, 15.8), 307 (M^+, 5.3), 306 (100.0), 159 (96.8), 85 (99.8). Analysis (Calc/found %): for $C_{20}H_{21}NO_2$ (307.40): C, 78.15/77.96; H, 6.89/7.83; N, 4.56/4.44.

*4.1.3.5. 3-[(4-Nitrophenyl)amino]-1-(5,6,7,8-tetrahydronaphthalen-2-yl)prop-2-en-1-one (**6e**).* Yield (78%); mp 195–197 °C, the ratio of *cis/trans*: 41/59; IR (KBr) (ν, cm^{-1}): 3442 (NH), 1645 (C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.77 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 5.75 and 6.61 (d, 1H, J = 8.0 and 12.5 Hz, –CO–CH═, *cis and trans* conformers), 6.92–7.58 (m, 8H, Ar–H and ═CH–N–, *cis* and *trans* conformers), 10.50 and 12.20 (d, 1H, J = 12.0 Hz, NH, D_2O exchangeable, *cis* and *trans* conformers); ^{13}C NMR (DMSO-d_6): δ (ppm) 22.48, 22.51, 28.75, 28.85 ($4CH_2$), 96.38 and 101.70 (–CO–CH = , *cis* and *trans* conformers), 124.50, 125.79, 128.09, 129.07, 135.48, 136.14, 140.84, 141.35, 141.62, 141.70, 141.80, 143.25 (Aromatic-C), 146.18, 147.240 (═CH–N–, *cis* and *trans* conformers), 187.500, 190,320 (C═O, *cis* and *trans* conformers); MS *m*/*z* (%): 323 (M^+ + 1, 34.6), 322 (M^+, 100.0), 159 (93.7), 91 (76.4). Analysis (Calc/found %): for $C_{19}H_{18}N_2O_3$ (322.37): C, 70.79/70.58; H, 5.63/5.54; N, 8.69/8.61.

*4.1.4. General procedure for the reaction of enaminone 3 with glycine derivatives **7a** and **7b***

A solution of the enaminone **3** (2.85 g, 10 mmol), acetylglycine (**7a**) or hippuric acid (**7b**) (1.7 g, 10 mmol) in acetic anhydride (30 mL) was refluxed for 3 h. The solid product obtained upon cooling was filtered off and recrystallized from DMF/H_2O to afford the pyran derivative **10a** and **10b**, respectively.

4.1.4.1. N-[2-Oxo-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-2H-pyran-3-yl]acetamide (10a). Yield (58%); mp 200–202 °C; IR (KBr) (ν, cm^{-1}): 3287 (NH), 1704, 1677 (2C═O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.33 (s, 3H, CH_3), 2.77 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 7.0–7.12 (m, 3H, Ar–H), 7.50 (d, 1H, J = 8.0 Hz, pyran), 8.20 (d, 1H, J = 8.0 Hz, pyran), 9.36 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): δ (ppm) 20.34 (CH_3), 22.41, 22.47, 28.57, 28.63 ($4CH_2$), 121.630, 123.71, 124.53, 124.84, 128.15, 128.63, 137.94, 139.03, 153.04, 158.15 (Aromatic-C), 164.89, 168.49 (2C═O); MS *m*/*z* (%): 384 (M^+ + 1, 24.1), 283 (M^+, 100.0), 159 (56.7), 91 (35.7). Analysis (Calc/found %): for $C_{17}H_{17}NO_3$ (283.33): C, 72.07/72.32; H, 6.05/6.14; N, 4.94/5.11.

*4.1.4.2. N-[2-Oxo-6-(5,6,7,8-tetrahydronaphthalen-2-yl)-2H-pyran-3-yl]benzamide (**10b**).* Yield (70%); mp 218–220 °C; IR (KBr) (ν,

cm^{-1}): 3391 (NH), 1705, 1658 (2C=O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.75 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.77 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 7.08–7.54 (m, 8H, Ar–H), 7.93 (d, 1H, $J = 7.8$ Hz, pyran), 8.20 (d, 1H, $J = 7.8$ Hz, pyran), 9.55 (s, 1H, NH, D_2O exchangeable); MS m/z (%): 346 ($M^+ + 1$, 30.6), 345 (M^+, 62.3), 212 (46.2), 105 (100.0), 77 (68.4). Analysis (Calc/found %): for $C_{22}H_{19}NO_3$ (345.40): C, 76.50/76.53; H, 5.54/5.77; N, 4.06/3.92.

4.1.5. General procedure for the reaction of enaminone 3 with p-benzoquinone and 1,4-naphthoquinone

To a stirred solution of the enaminone **3** (2.29 g, 10 mmol) in glacial acetic acid (50 mL), *p*-benzoquinone or 1,4-naphthoquinone (10 mmol) was added. Stirring was continued overnight at room temperature. The solid product was filtered off and recrystallized from EtOH/DMF to afford the corresponding derivatives **14a** and **14b**, respectively.

4.1.5.1. (5-Hydroxy-1-benzofuran-3-yl)(5,6,7,8-tetrahydronaphthalen-2-yl)methanone (14a). Yield (70%); mp 206–208 °C; IR (KBr) (ν, cm^{-1}): 3330 (OH), 1619 (C=O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.80 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 6.84–7.57 (m, 6H, Ar–H), 8.54 (s, 1H, furan), 9.40 (s, 1H, OH, D_2O exchangeable); MS m/z (%): 293 ($M^+ + 1$, 43.3), 292 (M^+, 100.0), 161 (80.4), 105 (50.1), 77 (39.5). Analysis (Calc/found %): for $C_{19}H_{16}O_3$ (292.34): C, 78.06/77.89; H, 5.52/5.48.

*4.1.5.2. (5-Hydroxynaphtho[1,2-b]furan-3-yl)(5,6,7,8-tetrahydronaphthalen-2-yl)methanone (**14b**).* Yield (55%); mp 268–270 °C; IR (KBr) (ν, cm^{-1}): 3241 (OH), 1626 (C=O); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.77 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.81 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 7.26–8.25 (m, 8H, Ar–H), 8.69 (s, 1H, furan), 10.21 (s, 1H, OH, D_2O exchangeable); MS m/z (%): 343 ($M^+ + 1$, 25.7), 342 (M^+, 100.0), 211 (71.6), 131 (80.8), 77 (78.2). Analysis (Calc/found %): for $C_{23}H_{18}O_3$ (342.40): C, 80.68/80.54; H, 5.30/5.22.

4.1.6. General procedure for the reaction of enaminone 3 with heterocyclic amines **15a**, **b**

A mixture of enaminone **3** (0.57 g, 2 mmol) and the appropriate amine **15a** or **15b** (10 mmol) in ethanol (25 mL), in the presence of catalytic amount of piperidine, was refluxed for 6 h. the reaction mixture left to cool, the solid product was filtered off, washed with ethanol, dried and finally recrystallized from DMF/H_2O to afford the corresponding derivatives **17a** and **17b**, respectively.

*4.1.6.1. 2-Phenyl-7-(5,6,7,8-tetrahydronaphthalen-2-yl)pyrazolo[1,5-a] pyrimidine (**17a**).* Yield (63%); mp 180–182 °C; IR (KBr) (ν, cm^{-1}): 1606 (C=N); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.77 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 5.31 (s, 1H, pyrazole), 7.17–8.03 (m, 8H, Ar–H), 8.12 (d, 1H, $J = 4.5$ Hz, pyrimidine), 8.56 (d, 1H, $J = 4.5$ Hz, pyrimidine); MS m/z (%): 325 (M^+, 24.6), 324 (100.0), 128 (15.3), 77 (66.8). Analysis (Calc/found %): for $C_{22}H_{19}N_3$ (325.42): C, 81.20/81.40; H, 5.89/5.97; N, 12.91/13.12.

*4.1.6.2. 7-(5,6,7,8-Tetrahydronaphthalen-2-yl)[1,2,4]triazolo[1,5-a] pyrimidine (**17b**).* Yield (60%); mp 130–132 °C; IR (KBr) (ν, cm^{-1}): 1608 (C=N); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.76 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.77 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 7.06–7.84 (m, 3H, Ar–H), 8.23 (d, 1H, $J = 4.5$ Hz, pyrimidine), 8.73 (s, 1H, triazole), 8.86 (d, 1H, $J = 4.5$ Hz, pyrimidine); ^{13}C NMR (DMSO-d_6): δ (ppm) 22.31, 22.41, 28.74, 28.80 ($4CH_2$), 109.07, 126.54, 129.10, 129.88, 137.05, 141.12, 147.35, 154.77, 155.39, 155.73, 168.41 (Aromatic-C); MS m/z (%): 250 (M^+, 100), 249 (93.36), 222 (18.73). Analysis (Calc/found %): for $C_{15}H_{14}N_4$ (250.31): C, 71.98/71.80; H, 5.64/5.68; N, 22.38/22.42.

4.1.7. General procedure for the reaction of enaminone 3 with pyrimidine derivatives (**18a**, **b**)

A mixture of enaminone **3** (0.57 g, 2 mmol) and the appropriate pyrimidine derivative **18a** or **18b** (10 mmol) in glacial acetic acid (25 mL) was refluxed for 6 h, the solid product was filtered off, washed with ethanol, dried and finally recrystallized from DMF/H_2O to afford the corresponding fused compounds **20a** and **20b**, respectively.

*4.1.7.1. 5-(5,6,7,8-Tetrahydronaphthalen-2-yl)pyrido[2,3-d]pyrimidine-2,4(1H,3H)-dione (**20a**).* Yield (64%); mp 251–253 °C; IR (KBr) (ν, cm^{-1}): 3171, 3050 (2NH), 1705, 1675 (2C=O), 1605 (C=N); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.73 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.74 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 7.14–7.79 (m, 3H, Ar–H), 7.89 (d, 1H, $J = 4.5$ Hz, pyridine), 8.29 (d, 1H, $J = 4.5$ Hz, pyridine), 8.92 (br. s, 1H, NH, D_2O exchangeable), 11.68 (br, s, 1H, NH, D_2O exchangeable); MS m/z (%): 294 ($M^+ + 1$, 34.8), 293 (M^+, 100.0), 131 (34.8), 77 (87.3). Analysis (Calc/found %): for $C_{17}H_{15}N_3O_2$ (293.33): C, 69.61/69.43; H, 5.15/5.32; N, 14.33/14.25.

*4.1.7.2. 5-(5,6,7,8-Tetrahydronaphthalen-2-yl)-2-thioxo-2,3-dihydropyrido[2,3-d]pyrimidin-4(1H)-one (**20b**).* Yield (68%); mp 288–290 °C; IR (KBr) (ν, cm^{-1}): 3385, 3064 (2NH), 1679 (C=O), 1607 (C=N); ^{1}H NMR (DMSO-d_6): δ (ppm) 1.74 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 2.75 (m, 4H, aliphatic $2CH_2$ of tetrahydronaphthalene), 7.17–7.84 (m, 3H, Ar–H), 7.86 (d, 1H, $J = 4.5$ Hz, pyridine), 8.26 (d, 1H, $J = 4.5$ Hz, pyridine), 8.99 (br. s, 1H, NH, D_2O exchangeable), 11.93 (br, s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6): δ (ppm) 22.39, 22.49, 28.63, 28.75 ($4CH_2$), 109.96, 116.23, 124.27, 127.73, 129.21, 133.75, 136.73, 136.98, 139.79, 151.24, 159.34, (Aromatic-C), 160.88 (C=O), 175.81 (C=S). MS m/z (%): 310 ($M^+ + 1$, 14.5), 309 (M^+, 58.9), 308 (100.0), 128 (14.7). Analysis (Calc/found %): for $C_{17}H_{15}N_3OS$ (309.39): C, 66.00/59.84; H, 4.89/5.03; N, 13.58/13.44; S, 10.36/10.14.

4.2. Cell culture

A panel of human cell lines was used in investigating the anticancer activity including: Hepatocellular carcinoma (Hep-G2), cervical carcinoma (HeLa), colon carcinoma (HCT-116), histiocytic lymphoma and breast adenocarcinoma (MCF-7) (ATCC, VA, USA). HCT-116 cells were grown in Mc Coy's medium, while all cells were routinely cultured in DMEM (Dulbeco's Modified Eagle's Medium) at 37 °C in humidified air containing 5% CO_2. All media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G sodium, 2 mM L-glutamine 100 units/ml, streptomycin sulfate, and 250 ng/ml amphotericin B. Monolayer cells were harvested by trypsin/EDTA treatment. Cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark), and all chemicals were from Sigma (USA). The tested compounds were diluted before assays and then tested for endotoxin using Pyrogent® Ultra gel clot assay that indicated endotoxin free of the samples. All experiments were repeated four times, unless mentioned, and the data was represented as (mean ± S.D.).

4.3. Cytotoxicity assay

The cytotoxic influence of the compounds on the growth of different human cancer cell lines was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

(MTT) assay [48]. Cells (5×10^4 cells/well) were incubated for 48 h with various concentrations of the compounds at 37 °C in a FBS-free medium, before submitted to MTT assay. The absorbance was measured with microplate reader (FluoStarOptima, BMG) at 570 nm. The relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. The data are expressed as the mean percentage of viable cells as compared to untreated cells. The relative cell viability was expressed as the mean percentage of viable cells as compared to the respective untreated cells (control). The half maximal growth inhibitory concentration (IC_{50}) value was calculated from the line equation of the dose–dependent curve of each compound. The results were compared with the cytotoxic activity of paclitaxel, a known anticancer drug.

4.4. Apoptosis and necrosis staining

The type of cell death was investigated in compounds-treated and untreated cells using acridine orange/ethidium bromide staining [49,50]. In brief, a mixture of 100 μg/ml acridine orange and 100 μg/ml ethidium bromide was prepared in PBS. The cell uptake of the stain was monitored under a fluorescence microscope, and the apoptotic, necrotic, and viable cells were counted. The early apoptotic cells had yellow chromatin in nuclei that were highly condensed or fragmented. Apoptotic cells also exhibited membrane blebbing. The late apoptotic cells had orange chromatin with nuclei that were highly condensed and fragmented. The necrotic cells had bright orange chromatin in round nuclei. Only cells with yellow, condensed, or fragmented nuclei were counted as apoptotic cells in a blinded, nonbiased manner.

4.5. DNA fragmentation

DNA fragmentation was essentially assayed as reported previously [51]. Briefly, compounds-treated and untreated cells pellet was re-suspended in 250 μl 10 mM Tris, 1 mM EDTA, pH 8.0 (TE-buffer), and incubated with an additional volume lysis buffer (5 mM Tris, 20 mM EDTA, pH 8.0, 0.5% Triton X-100) for 30 min at 48 °C. After lysis, the intact chromatin (pellet) was separated from DNA fragments (supernatant) by centrifugation. Pellets were re-suspended in TE-buffer and samples were precipitated by 0% trichloroacetic acid at 48 °C. The sample pellets were added to 5% trichloroacetic acid and boiled. DNA contents were quantified using the diphenylamine reagent [52,53]. The percentage of DNA fragmented was calculated as the ratio of the DNA content in the supernatant to the amount in the pellet [54].

4.6. Mitochondrial transmembrane potential ($\Delta\psi_m$)

5,59,6,69-Tetrachloro-1,19,3,39-tetraethybenzimidazol carbocyanine iodide (JC-1, Molecular Probes) is a lipophilic, cationic dye that enters mitochondria in proportion to the membrane potential and forms J-aggregates at the high intramitochondrial concentrations induced by higher $\Delta\psi_m$ values [55]. Measurement of mitochondrial transmembrane potential ($\Delta\psi_m$) was carried out by JC-1 [55,56]. In 96 well plates, cells were seeded and then preloaded with 10 μg/ml JC-1 dissolved in HBSS for 30 min, 37 °C. The JC-1 loaded cells were incubated with and without compounds at 37 °C for 2 h period. JC-1 exist as a monomer (emission 527 nm) at low $\Delta\psi_m$ but forms J-aggregates (emission 590 nm) at high $\Delta\psi_m$, which can be assessed by JC-1 by monitoring fluorescence emission ratios at (emission 590:527 nm). The ratio of red (590 nm) to green (527 nm) gives an index of the $\Delta\psi_m$: the higher the $\Delta\psi_m$ the greater proportion of JC-1 aggregates in the mitochondria, the greater the intensity of the red light signal and so with active mitochondria we see a relatively high ratio of 590/527 nm. Fluorescence values were monitored in plates at zero time and after 2h by microplate fluorescence reader (FluoStarOptima, BMG). Values are expressed as percent of zero time reading. Carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μM) was used as a positive control.

4.7. Preparation of cell lysates

After harvesting of Compounds-treated and untreated HCT-116 cells, they were washed and centrifuged for 10 min at 1000× g. The cell pellet was lysed in 0.5 mL of ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, and 10 mg/ml leupeptin (pH 7.4). The lysates were passed through a 21-gauge needle to break up cell aggregates. After centrifugation at 14,000× g for 15 min at 4 °C, the supernatants (total cell lysates) were submitted to measure cytochrome C and caspases. The cellular total protein content of the lysates was measured by bicinchoninic acid (BCA) assay [57] and bovine serum albumin (BSA), as a standard.

4.8. Immunoblotting of cytochrome C

Cytochrome C was assessed in cell lysate of from compounds -treated and -untreated HCT-116 cells. Briefly, 20 μg of isolated soluble proteins were applied at dot blotting set using nitrocellulose membranes. The change in cytochrome c protein was detected by dot immunoblotting using Cytochrome C Releasing Apoptosis Assay kit (#K257, BioVision, CA, USA). After being washed, bound antibody was detected using rabbit anti-goat antibody linked to horseradish peroxidase (Dako), and bound complexes were detected using O-phenylenediamine dihydrochloride (OPD) (Amersham-Pharmacia). The percentage of the enhanced color intensity was shown as fold induction after normalization of dot intensity with actin control. The dot photographing and analysis was performed using gel documentation system (Biometra, Goettingen, Germany).

4.9. Evaluation of caspases activity

The cell lysates of the compounds-treated and untreated HCT-116 cells were submitted to different kits to measure the level of total caspases, caspase-3, and caspase-9, according to the manufacturer instructions. Red Multi-Caspase Staining Kit (# PK-CA577-K190), PromoKine, Heidelberg, Germany was used for analysis of total caspases in a black microtiter plate with fluorescence plate reader at Ex. = 540 nm and Em. = 570 nm. The assay utilizes the caspase family inhibitor VAD-FMK conjugated to sulfo-rhodamine (Red-VAD-FMK) as the fluorescent *in situ* marker. Caspase-3 Colorimetric Detection Kit (# 907–013), Stressgen biotechnologies, Canada, was used to measure caspase-3 at 405 nm. One unit of Caspase-3 activity is defined as the amount of enzyme needed to convert one picomole of substrate per minute at 30 °C (U/ml). Caspase-9 Colorimetric Assay Kit (# PK-CA577-K119), PromoKine Heidelberg, Germany, was used to evaluated Caspase-9 activity. The method is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate LEHD-*p*NA. The *p*NA light emission can be quantified at 405 nm. In the assay, a constant substrate concentration (200 μM final concentration) was added to the assay reaction. A comparison of the fluorescence or absorbance readings of the treated and untreated-cells allows determination of the fold increase in the total caspases, caspase-3, and caspase-9 activities.

4.10. Statistical analysis

The Student's unpaired *t*-test was used to detect the statistical significance, where a *p* value less than 0.05 was considered significant.

Acknowledgments

This work was supported by the Center of Excellence for Advanced Sciences and Applied Organic Chemistry Department, National Research Center, Dokki, Cairo, Egypt.

References

[1] J.K. Brunelle, A. Letai, Journal of Cell Science 122 (2009) 437–441.
[2] J.M. Adams, S. Cory, Current Opinion in Immunology 19 (2007) 488–496.
[3] P. Meier, K.H. Vousden, Molecular Cell 28 (2007) 746–754.
[4] S.H. Kaufmann, W.C. Earnshaw. Experimental Cell Research 256(20004) 2–49.
[5] G. Dewson, R.M. Kluck, Journal of Cell Science 122 (2009) 2801–2808.
[6] S.N. Willis, J.M. Adams, Current Opinion In Cell Biology 17 (2005) 617–625.
[7] R.J. Youle, A. Strasser, Nature Reviews Molecular Cell Biology 9 (2008) 47–59.
[8] M.M. Dix, G.M. Simon, B.F. Cravatt, Cell 134 (2008) 679–691.
[9] A.U. Luthi, S.J. Martin, Cell Death Differ 14 (2007) 641–650.
[10] Q.Z. Zhang, J.Y. Wu, Experimental Biology and Medicine 232 (2007) 52–57.
[11] Y. Zhang, Z. Liu, T. Ng, Z. Chen, W. Qiao, F. Liu, Biochimie 99 (2014) 28–37.
[12] (a) S. Youssef, Monatshefte für Chemie 128 (1997) 493–503;
(b) Z.E. Kandeel, A.M. Farag, A.M. Negm, A.K. Khalafalla, M.A. Raslan, M.H. Elnagdi, Journal of Chemical Research (S) (1994) 416–422 (M) (1994) 2332–2337.
[13] K.M. Dawood, A.M. Farag, Z.E. Kandeel, Journal of Chemical Research (S) (1998) 208–215.
[14] T.M. Abu-Elmaati, Acta Chimica Slovenica 49 (2002) 721–732.
[15] (a) E. Bejan, H.A. Haddou, J.C. Daran, G.G.A. Balavoine, Synthesis (1996) 1012–1017;
(b) F. Al-Omran, M.M. Abdel-Khalik, A. Abuel Khair, M.H. Elnagdi, Synthesis (1997) 91–99;
(c) K.M. Al-Zayd, E.A. Hafez, Journal of Chemical Research (S) (1999) 360–368 (M) (1999) 1621–1627;
(d) B. Al-Saleh, N.A. Al-Awedi, H. Al-Kandari, M.M. Abdel-Khalik, M.H. Elnagdi, Journal of Chemical Research (S) (2000) 16–20 (M) (2000) 201–210;
(e) K.M. Al-Zayd, E.A. Hafez, Journal of Chemical Research (S) (1999) 360–367 (M) (1999) 1621–1629;
(f) K.M. Dawood, A.M. Farag, Z.E. Kandeel, Journal of Chemical Research (S) (1999) 88–93 (M) (1999) 537–544;
(g) A. Winter, N. Risch, Synlett 1 (2003) 1959–1964.
[16] (a) A.K. Dutta, X. Fei, M.E.A. Reith, Bioorganic & Medicinal Chemistry Letters 12 (2002) 619–622;
(b) M.H. Poiker, R. Chen, K.A. Conway, D.H.S. Lee, C. Luo, R.E. Boyd, S.O. Nortey, T.M. Ross, M.K. Scott, A.B. Reitz, Bioorganic & Medicinal Chemistry 10 (2002) 3565–3569;
(c) P. Taddei, A. Torreggiani, G. Fini, Biopolymers 67 (2002) 289–293;
(d) H. Hatori, B. Sato, I. Sato, T. Shibata, Y. Tsurumi, K. Sakamoto, S. Takase, H. Veda, M. Hino, T. Fujii, The Journal of Antibiotics 57 (2004) 264–270;
(e) J.H. Song, A.J. Slot, R.W.J. Ryan, G.M. Ross, Neuropharmacology 46 (2004) 984–993;
(f) H. Zhao, Z. Xin, J.R. Patel, L.T.J. Nelson, B. Liu, B.G. Szczepankiewicz, V.G. Schaefer, H.D. Falls, W. Kaszubska, C.A. Collins, H.L. Sham, G. Liu, Bioorganic & Medicinal Chemistry Letters 15 (2005) 1825–1828;
(g) K. Hattori, A. Tanaka, O. Okitsu, S. Tabuchi, K. Taniguchi, M. Nishio, S. Koyama, M. Higaki, J. Seki, K. Sakane, Bioorganic & Medicinal Chemistry Letters 15 (2005) 3091–3095.
[17] L. Cordeu, E. Cubedo, E. Bandres, A. Rebollo, X. Saenz, H. Chozas, M.V. Dominguez, M. Echeverria, B. Medivil, C. Sanmartin, J.A. Palop, M. Font, J.G. foncillas, Bioorganic & Medicinal Chemistry 15 (2007) 1659–1669.
[18] (a) J.D. Hepworth, in: A.R. Katritzky, C.W. Rees (Eds.), Comprehensive Heterocyclic Chemistry: Pyrans and Fussed Pyrans: (iii) Synthesis and Applications, vol. 3, Pergamon Press, Oxford, 1984, pp. 737–743;
(b) J.D. Hepworth, C.D. Gabbutt, B.M. Heron, in: A.R. Katritzky, C.W. Rees, E.F.V. Scriven (Eds.), Comprehensive Heterocyclic Chemistry II: Pyrans and Their Benzo Derivatives: Synthesis, vol. 5, Pergamon Press, Oxford, 1996, pp. 351–358;
(c) G.R. Geen, J.M. Evans, A.K. Vong, in: A.R. Katritzky, C.W. Rees, E.F.V. Scriven (Eds.), Comprehensive Heterocyclic Chemistry II: Pyrans and Their Benzo Derivatives: Applications, vol. 5, Pergamon Press, Oxford, 1996, pp. 469–504;
(d) K. Afarinkia, M.V. Vinader, in: E.J. Thomas (Ed.), Science of Synthesis, vol. 14, Thieme, Stuttgart, 2003, pp. 275–281.
[19] T.K. Vinh, M. Ahmadi, L.O.P. Delgado, F.S. Prerez, M.H. Walters, J.H. Smith, J.P. Nicholls, C. Simons, Bioorganic & Medicinal Chemistry Letters 14 (1999) 2105–2108.
[20] P.G. Wyatt, M.J. Allen, J. Chilcott, C.J. Gardner, D.G. Livermore, J.E. Mordaunt, F. Nerozzi, M. Patel, M.J. Perren, G.G. Weingarten, S. Shabbir, P.M. Woollard, P. Zhou, Bioorganic & Medicinal Chemistry Letters 12 (2002) 1405–1411.
[21] F. Yoneda, T. Moto, M. Sakae, H. Ohde, B. Knoll, I. Miklya, J. Knoll, Bioorganic & Medicinal Chemistry 9 (2001) 1197–1212.
[22] J.A. Findlay, S. Buthetezi, G. Li, M. Seveck, J.D. Miller, Journal of Natural Products 60 (1997) 1214–1215.
[23] I. Hayakawa, R. Shioya, T. Agatsuma, H. Furukawa, Y. Sugano, Bioorganic & Medicinal Chemistry Letters 14 (2004) 3411–3414.
[24] M. Masubuchi, H. Ebiike, K. Kawasaki, S. Sogabe, K. Morikami, Y. Shiratori, S. Tsujii, T. Fujii, K. Sakata, M. Hayase, H. Shindoh, Y. Aoki, T. Ohtsuka, N. Shimma, Bioorganic & Medicinal Chemistry 11 (2003) 4463–4478.
[25] S.S. Sangapure, R. Basawaraj, Indian Journal of Pharmaceutical Sciences 66 (2004) 221–225.
[26] (a) K.M. Dawood, H. Abdel-Gawad, E.A. Rageb, M. Ellithey, H.A. Mohamed, B. Hegazi, Archives of Pharmacal Research Life Sciences 339 (2006) 133–140;
(b) K.M. Dawood, H. Abdel-Gawad, M. Ellithey, H.A. Mohamed, B. Hegazi, Bioorganic & Medicinal Chemistry 14 (2006) 3672–3680.
[27] (a) A.J. Walker, K. Rossen, A.R. Reamer, P.R. Volante, J.P. Reider, Tetrahedron Letters 27 (1999) 4917–4920;
(b) M.P. Donate, R. Dasilva, J.V.G. Da Silva, C. Aleman, Synthetic Communications 31 (2001) 151–154.
[28] (a) N.A. Hamdy, W.M. El-Senousy, I.M.I. Fakhr, Journal of Heterocyclic Chemistry 50 (2013) 337–343;
(b) N.A. Hamdy, W.M. El-Senousy, Acta Poloniae Pharmaceutica. Drug Research 70 (2013) 99–110.
[29] (a) N.A. Hamdy, H.A. Abdel-Aziz, G.M. Kamal, I.M.I. Fakhr, Acta Poloniae Pharmaceutica. Drug Research 70 (2013) 469–480;
(b) H.A. Abdel-Aziz, N.A. Hamdy, A.M. Gamal-Eldeen, I.M.I. Fakhr, Zeitschrift fur Naturforschung C 66c (2011) 7–16;
(c) A.M. Farag, K.M. Dawood, H.A. Abdel-Aziz, N.A. Hamdy, I.M.I. Fakhr, Journal of Heterocyclic Chemistry 48 (2011) 355–360;
(d) N.A. Hamdy, A.M. Gamal-Eldeen, H.A. Abdel-Aziz, I.M.I. Fakhr, European Journal of Medicinal Chemistry 45 (2010) 463–470;
(e) N.A. Hamdy, A.M. Gamal-Eldeen, European Journal of Medicinal Chemistry 44 (2010) 4547–4556;
(f) H.A. Abdel-Aziz, A.M. Gamal-Eldeen, N.A. Hamdy, I.M.I. Fakhr, Archiv der Pharmazie 342 (2009) 230–237;
(g) H.A. Abdel-Aziz, N.A. Hamdy, A.M. Farag, I.M.I. Fakhr, Journal of Heterocyclic Chemistry 45 (2008) 1033–1037.
[30] S. Almazroa, M.H. Elnagdi, A.M. Salah El-Din, Journal of Heterocyclic Chemistry 41 (2004) 267–275.
[31] N. Demirbas, S.A. Karaoglu, A. Demirbas, K. Sancak, European Journal of Medicinal Chemistry 39 (2004) 793–804.
[32] D. Potin, F. Dumas, J. d'Angelo, Journal of the American Chemical Society 112 (1990) 2483–2493.
[33] L. Kozerski, W. Von Philipsborn, Organic Magnetic Resonance 17 (1981) 301–308.
[34] J. Dabrowski, K. Kamienska-Trela, Journal of the American Chemical Society 98 (1976) 2826–2834.
[35] L. Kozerski, K. Kamienska-Trela, L. Kania, W. Von Philipsborn, Helvetica Chimica Acta 66 (1983) 2113–2219.
[36] (a) V. Kepe, M. Kocevar, S. Polanc, Journal of Heterocyclic Chemistry 33 (1996) 1707–1716;
(b) F. Al-Omran, N.A. Al-Awedi, M.M. Abdel-Khalik, K. Kaul, A. Abuel-Khair, M.H. Elnagdi, Journal of Chemical Research (S) (1997) 84–91 (M) (1997) 601–610.
[37] F.M. Abel Aziz El-Taweel, M.H. Elnagdi, Journal of Heterocyclic Chemistry 38 (2001) 981–984.
[38] M.H. Elnagdi, F.A. Abdel All, R.M. Abdel Motaleb, F.F. Mahmoud, Collection of Czechoslovak Chemical Communications 54 (1989) 1082–1087.
[39] S.M. Sherief, A.M. Hussien, Monatshefte für Chemie 128 (1997) 687–692.
[40] H.M. Hassneen, T.A. Abdallah, Molecules 8 (2003) 333–341.
[41] H. Lu, X. Cao, Molecular Biology of the Cell 19 (2008) 1893–1902.
[42] N.N. Danial, S.J. Korsmeyer, Cell 116 (2004) 205–219.
[43] M.C. .Wei, W.X. Zong, E.H. Cheng, T. Lindsten, V. Panoutsakopoulou, A.J. Ross, K.A. Roth, G.R. MacGregor, C.B. Thompson, S.J. Korsmeyer, Science 292 (2001) 727–730.
[44] J.D. Ly, D.R. Grubb, A. Lawen, Apoptosis 8 (2003) .115–128.
[45] X. Liu, C.N. Kim, J. Yang, R. Jemmerson, X. Wang, Cell 86 (1996) 147–157.
[46] G.P. Amarante-Mendes, K.C. Naekyung, L. Liu, Y. Huang, C.L. Perkins, D.R. Green, K. Bhalla, Blood 91 (1998) 1700–1705.
[47] N.L. Allinger, E.S. Jones, Journal of Organic Chemistry 27 (1962) 70–78.
[48] M.B. Hansen, S.E. Nielsen, K. Berg, Journal of Immunological Methods 119 (1989) 203–210.
[49] J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, W. Strober, Current protocols in immunology, in: R. Coico (Ed.), Related Isolation Procedures and Functional Assays, vol. 1John Wiley & Sons, Inc, 1995, 3.17.1.
[50] D. Ribble, N.B. Goldstein, D.A. Norris, Y.G. Shellman, BMC Biotechnology 10 (2005) 5–12.
[51] U.K. Messmer, U.K. Reed, B. Brüne, Journal of Biological Chemistry 271 (1998) 20192–20197.
[52] K.A. Burton, Biochemical Journal 62 (1956) 315–323.
[53] U.K. MEÛMER, D.M. REIMER, B. BRUÈ NE, European Journal of Pharmacology 349 (1998) 333–343.

[54] U.K. Meûmer, G. Winkel, V.A. Briner, J. .Pfeilschifter, British Journal of Pharmacology 127 (1999) 1633–1640.
[55] J.F. Kidd, M.F. Pilkington, M.J. Schell, K.E. Fogarty, J.N. Skepper, C.W. Taylor, P. Thorn, Journal of Biological Chemistry 277 (2002) 6504–65010.
[56] J.S. Wadia, R.M. Chalmers-Redman, W.J. Ju, G.W. Carlile, J.L. Phillips, A.D. Fraser, W.G. Tatton, Journal of Neuroscience 18 (1998) 932–947.
[57] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Analytical Biochemistry 150 (1985) 76–85.