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Original article

New thiophene analogues of kenpaullone: synthesis and biological evaluation in breast cancer cells

Laurent Brault [a], Evelyne Migianu [a,b], Adrien Néguesque [a], Eric Battaglia [a], Denyse Bagrel [a,*], Gilbert Kirsch [a]

[a] *Laboratoire d'Ingénierie Moléculaire et Biochimie Pharmacologique, UFR SciFA, Université de Metz, Campus Bridoux, rue du Général Delestraint, 57070 Metz, France*

[b] *Present address: Laboratoire de Chimie Structurale et Spectroscopie Biomoléculaire, UMR CNRS 7033, 74, rue Marcel Cachin, 93017 Bobigny, France*

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Abstract

Thieno analogues of kenpaullone have been synthesized using an established method. We investigated the effect of five structural analogues of kenpaullone on vincristine sensitive and resistant MCF7 (human mammary adenocarcinoma) cells. One analogue, 8-Bromo-6,11-dihydro-thieno-[3′,2′:2,3]azepino[4,5-b]indol-5(4H)-one (**3a**), showed an antiproliferative activity in the drug sensitive cell line that led to cell accumulation in G2/M phase. In addition, repression of cdk1, a G2/M transition key regulator, as well as induction of p21 were observed at the mRNA level. Programmed cell death (apoptosis) was induced in early time treatments and was accompanied by p53 mRNA induction. The antiproliferative and proapoptotic properties of **3a** make this CDK inhibitor an interesting candidate for further investigations.

Keywords: Paullone analogues; Cytotoxicity; Cell cycle; Apoptosis; Breast cancer

1. Introduction

Over the last few years, comprehension of eukaryotic cell division mechanisms has largely improved. The progression of the cell cycle through its four distinct phases (G1, S, G2 and M) is dependent on the integration of extra- and intracellular signals among which cyclin-dependent kinases (CDKs) play a central role [1,2]. These enzymes are a family of serine/threonine kinases which become active when associated with regulatory proteins, their sequential activation ensuring the correct crossing of checkpoints separating each cell cycle phase. If numerous CDKs and cyclin-box-containing proteins have been isolated, only about 10 cyclin/CDKs complexes are identified as involved in the regulation of cell cycle progression [3]. While cyclin D is required for entering the cycle, the progression from G1 phase into and through S phase depends on the catalytically active complex cyclin E/CDK2 and cyclin A/CDK2. The entry into mitosis is under the control of the cyclin B/CDK1 complex [4]. Thus, CDKs and their direct partners play a key role in cell proliferation and they are also implicated in other physiological functions such as RNA transcription and processing, cell death and neuronal functions [5,6]. For these reasons, they are attractive targets in numerous diseases as different as cancer, neurodegenerative or cardiovascular disorders and viral infections. Therefore, multiple CDK pharmacological inhibitors have been identified to date, all being competitive inhibitors of these kinases, mostly by interacting with the ATP binding site [7]. They belong to various chemical classes, including flavones (flavopiridol), purine analogues (e.g. olomoucine, purvanalol, roscovitine), pyrimidine analogues, oxindoles and paullones [8]. Members of the latter class were identified as CDK inhibitors using the COMPARE analysis of a database of compounds screened in cancer cell lines [9]. Using this method, kenpaullone was identified as inhibiting ATP binding and, since then, numerous paullone derivatives have been synthesized, such as alsterpaullone and gwennpaullone [10–12].

Using an established synthesis procedure [13], we prepared four new thiophene derivatives of kenpaullone (**3b**–**3e**) (Fig. 1). We synthesized original compounds by replacing

* Corresponding author. Tel.: +33 3 87 37 84 04; fax: +33 3 87 37 84 23.
E-mail address: bagrel@sciences.univ-metz.fr (D. Bagrel).

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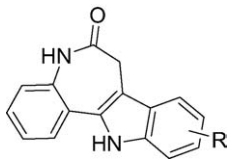


Fig. 1. General structure of paullones.

the aromatic cycle A by a variously substituted thiophene ring. The aim of this study was to characterize the antiproliferative action of these analogues of paullone in breast cancer cells, i.e. the adenocarcinoma cell line MCF7 and its counterpart resistant to vincristine, Vcr-R [14]. Cell cycle repartition as well as cell death mechanisms implicated were also evaluated for cells exposed to these compounds.

2. Chemistry

Having developed a method to access variously substituted 3-aminothiophenes [15], we used an established strategy to synthesize new analogs of paullones among them **3a** recently described by Kunick et al. [13]. The analogues **3** were prepared by applying the Fischer indole synthesis to 6,7-dihydro-4H-thieno[3,4-b]azepine-5,8 diones **2** (Table 1). The latter were prepared from the corresponding 3-amino-2-carbethoxy substituted thiophenes **1**. The synthesis of these thienoazepine-diones has been fully described earlier [15]. The Fischer indole synthesis was made by reacting the corresponding dione with the appropriate phenylhydrazine in acetic acid followed by cyclization at 70 °C for 1 h [13]. The yields of the isolated paullone analogues ranged from 9% to 49%.

3. Biological evaluation

Breast cancer cell lines used in this study were the wild-type MCF7, sensitive to miscellaneous drugs, and its counterpart Vcr-R, resistant to vincristine. Paullone analogues (compounds **3a** to **3e**) were screened by treating cells with 100 μM for 48 and 72 h, before using additional incubation times and concentrations for active molecules.

3.1. Antiproliferative activity

Cell viability was first evaluated in our screening conditions (vide supra). As shown in Fig. 2A, compound **3a** induced in MCF7 an important decrease of cell viability, the number of living cells being about 8% of control after 72 h of exposure. Except for analogue **3b** which displayed a slight effect (64% of viable cells as compared to control), the other compounds were inactive. In the resistant cell line Vcr-R, only compound **3a** showed a significant activity with 72% of living cells as compared to control. As referred to its activity toward MCF7 cells, **3a** was also tested at lower doses for 24–72 h-treatments (Fig. 2B). Our results indicate that this analogue exerts a dose- and time-dependent decrease of cell viability. Thus, a 25 μM concentration induced a significant effect for a 24 h-treatment. It lowered to 10 μM for a 72 h-exposure.

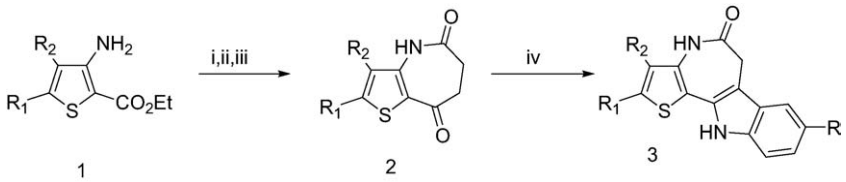
3.2. Cell cycle progression of MCF7 cells

Given the antiproliferative activity of these kenpaullone analogues, cell cycle distribution was assessed by flow cytometry. As shown in Fig. 3A, no significant modification was observed for the resistant cells whatever the compound. In contrast, a markedly change in MCF7 cell cycle repartition was induced only for compound **3a** for a 48 and 72 h-treatment (data not shown and Fig. 3A). Cell accumulation in G2/M phase, caused by **3a**, was accompanied by a diminution of cell proportion in G0/G1 phase. For this compound, the cell cycle blockage was observed as soon as 24 h. It persisted for longer treatments (Fig. 3B, C) and was dose-dependent for concentrations above 25 μM. This cell accumulation was accompanied by a dose-dependent repression of mRNA expression of cdk1 implicated in G2/M transition (Fig. 4). A dose-dependent induction was also observed for p21 mRNA, implicated in cell cycle blockage by inhibiting CDK activity but also in apoptosis induction.

3.3. Induction of apoptosis

As shown in Fig. 4, the level of p53 mRNA was increased after a 24 h-treatment by compound **3a**. p53 is well-known as

Table 1
Synthesis and structure of kenpaullone thieno analogues



1 2 3

Compound	R	R_1	R_2
3a	Br	H	H
3b	H	tBu	H
3c	Br	tBu	H
3d	H	Ph	CH_3
3e	Br	Ph	CH_3

(i) $ClCO(CH_2)_2CO_2CH_3$, K_2CO_3, toluene, reflux; (ii) KH, toluene,DMF,80 °C; (iii) H_2O, DMSO, 140 °C; (iv) $RPhNHNH_2$, AcOH,70 °C.

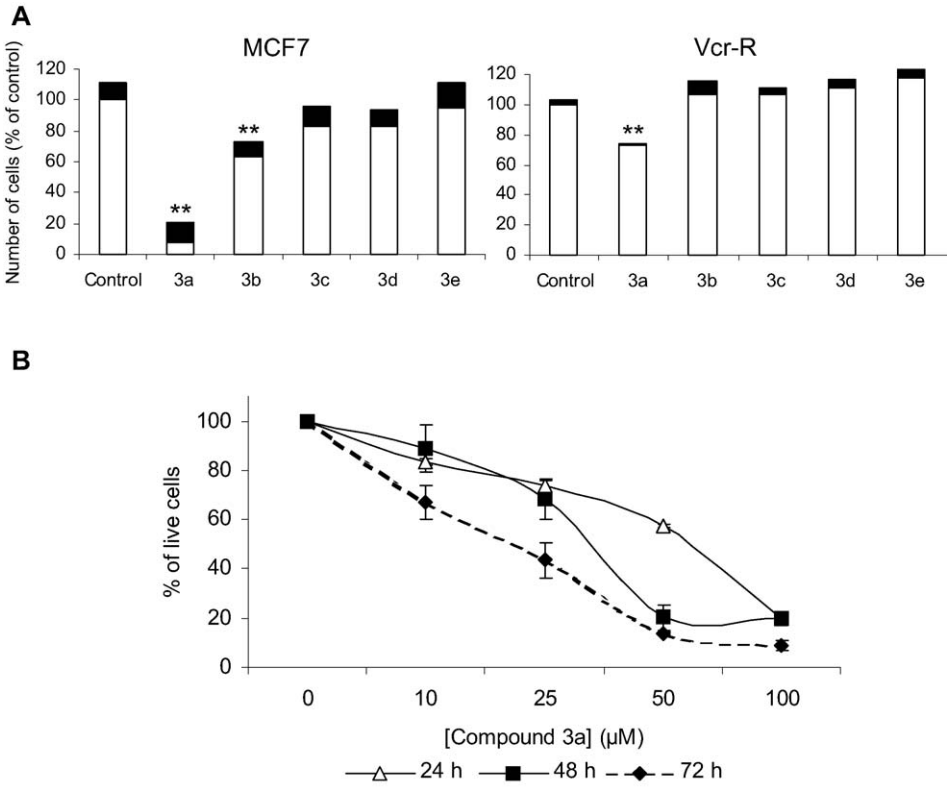


Fig. 2. Antiproliferative effect of thieno analogues of kenpaullone. (A) Cell viability (trypan-blue exclusion) of MCF7 and Vcr-R incubated with kenpaullone analogues at 100 μM for 72 h (□ live cells, ■ dead cells), ** significantly different from control $P < 0.01$. (B) Percent of live MCF7 cells after 24, 48 and 72 h treatment with compound **3a**. Each result represents the mean of three independent experiments. Results are significantly different from control ($P < 0.05$) except for 24 and 48 h-treatment at 10 μM.

a key regulator of apoptosis and is also implicated in positive regulation of the expression of p21.

Therefore, in order to determine if programmed cell death was induced together with the observed cell cycle arrest, Annexin-V binding was assessed by flow cytometry in both cell types. In our screening conditions, no significant cell death was induced for resistant cells, and was only detected in sensitive MCF7 cells following exposure to **3a** (Fig. 5A). It is worth noting that these results are consistent with those obtained with the cell viability test. Furthermore, cell death was significantly induced by a 24 h-treatment with 100 μM (Fig. 5B, C). For longer time treatments, a significant effect was observed with lower concentration (50 μM) of compound **3a** (Fig. 5C). However, apoptosis seems to be only slightly increased with this treatment (Fig. 5B). Thus, the proportion of early apoptotic cells, detected by flow cytometry, varied from 1.9 ± 0.6% for control to 4.8 ± 0.6% for a treatment with 100 μM of compound **3a**.

Given these results and taking into account the induction of the mRNA level of p53, we investigated if apoptosis is an early phenomenon by taking advantage of the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method. This assay revealed a significant induction of apoptosis for a 24 h-treatment at 50 μM with a large amount of necrotic cells which are in a large part in secondary necrosis (i.e. necrotic lysis of floating apoptotic bodies). When used at 100 μM compound **3a** led to an eightfold increase of the proportion of apoptotic cells (Fig. 6). Thus, **3a** is able to induce apoptosis and then apoptotic cells and bodies enter secondary necrosis in less than 24 h. The apparent discrepancy between TUNEL and Annexin-V results could be linked to the fact that the flow cytometric analysis reveals only early apoptosis, whereas the immunostaining technique allows to show late apoptosis. The only extensive study of apoptosis induction triggered by paullone derivatives was performed by Lahusen et al. [11]. They showed that, in Jurkat cells, the apoptosis induced by alsterpaullone was observed within early time treatment, which is in good agreement with our results. It is worth noting that cell death is not due to the induction of oxidative stress since treatments were not accompanied by a significant modification in reactive oxygen species level as assessed by the dichlorofluorescein-diacetate (DCF-DA) flow cytometric assay (data not shown).

4. Conclusions

Analogues of paullones occupy, as CDK inhibitors, an attractive position in the current generation of antitumor drugs. The ability to prepare variously substituted thiophenes permitted us to synthesize new analogues of paullone. These structural modifications allowed to get some informations about the structure–activity behavior of these compounds. Among them, **3a**, containing a bromo substitution (Table 1),

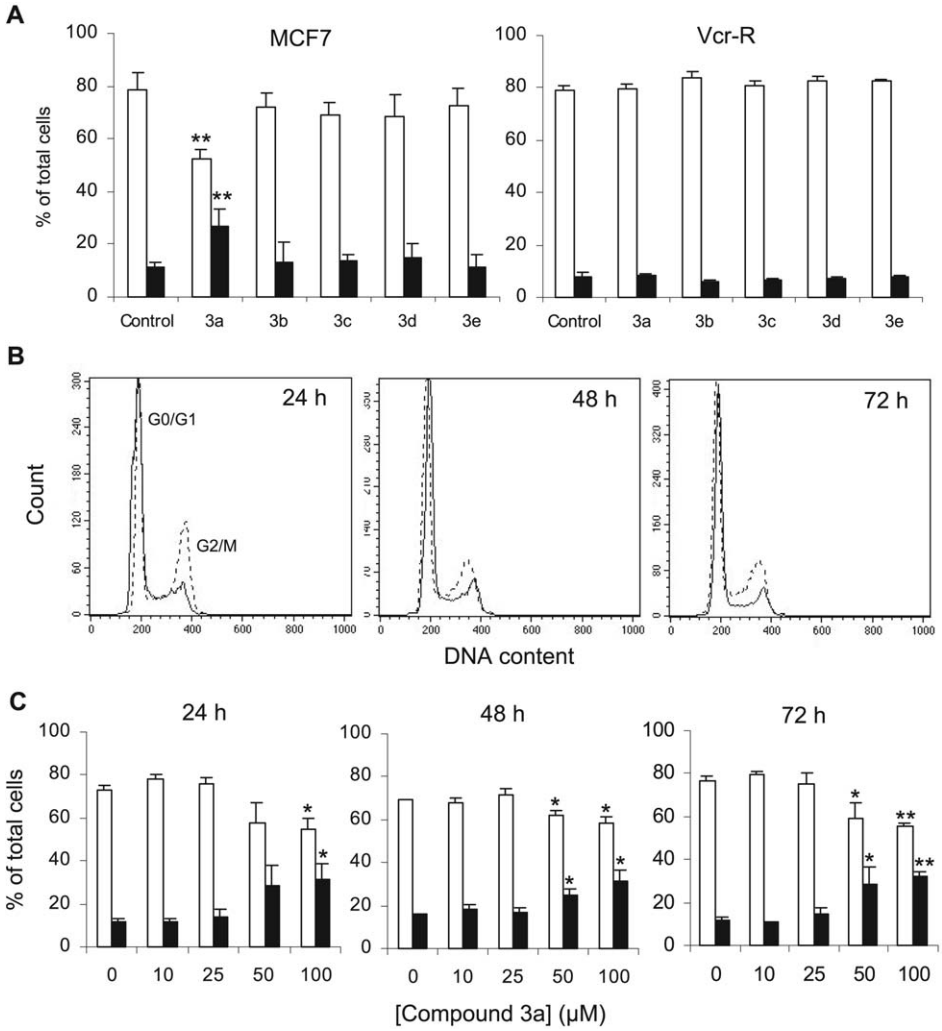


Fig. 3. Modulation of cell cycle progression. (A) MCF7 and Vcr-R cells were treated with paullone analogues (100 μM) for 72 h and analyzed by flow cytometry (□ G_0/G_1, ■ G_2/M). (B) DNA content of MCF7 cells exposed (dashed line) or not (full line) to compound **3a** (100 μM) for 24, 48 and 72 h. (C) Dose-dependent modulation of cell cycle distribution of MCF7 cells after 24–72 h incubation with compound **3a** (□ G0/G1, ■ G2/M). Each column and vertical bar represent mean ± S.E.M. ($n = 3$), * and ** significantly different from control $P < 0.05$ and $P < 0.01$, respectively.

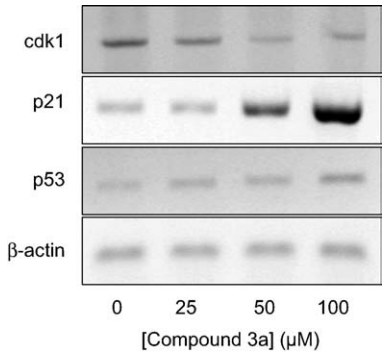


Fig. 4. RT-PCR analysis of the mRNA level of genes implicated in cell cycle and apoptosis regulation in MCF7 cells incubated with compound **3a**. Ethidium bromide stained gel showing amplicons corresponding to cdk1, p21, p53 and β-actin mRNA following exposure to compound **3a** at 0, 25, 50 and 100 μM for 24 h.

presents an interesting antiproliferative activity in MCF7 breast cancer cells. Furthermore, introduction of substitutions on the thiophene ring (**3c** and **3e**) was detrimental to the biological activity of this compound. The inhibitory effect of **3a** has been recently reported for purified CDK1 enzyme with an IC_{50} of 0.6 μM [16] which is in good agreement with our results on cultured cells. Our work was focused on the cellular effects of the thieno analogues of kenpaullone and we showed an antiproliferative activity of this compound characterized by the repression of the expression of ckd1 gene accompanied by a cell accumulation in G2/M phase. The decrease in mRNA level of cdk1 could be a direct consequence of inhibition of the activity of the kinase. Indeed, cdk1 expression is dependent on its own activity through a positive feedback loop. Compound **3a** was also able to increase p21 mRNA level which is implicated in cell cycle arrest, through inhibition of CDK activity especially CDK1, but also in apoptosis regulation. It also increased the expres-

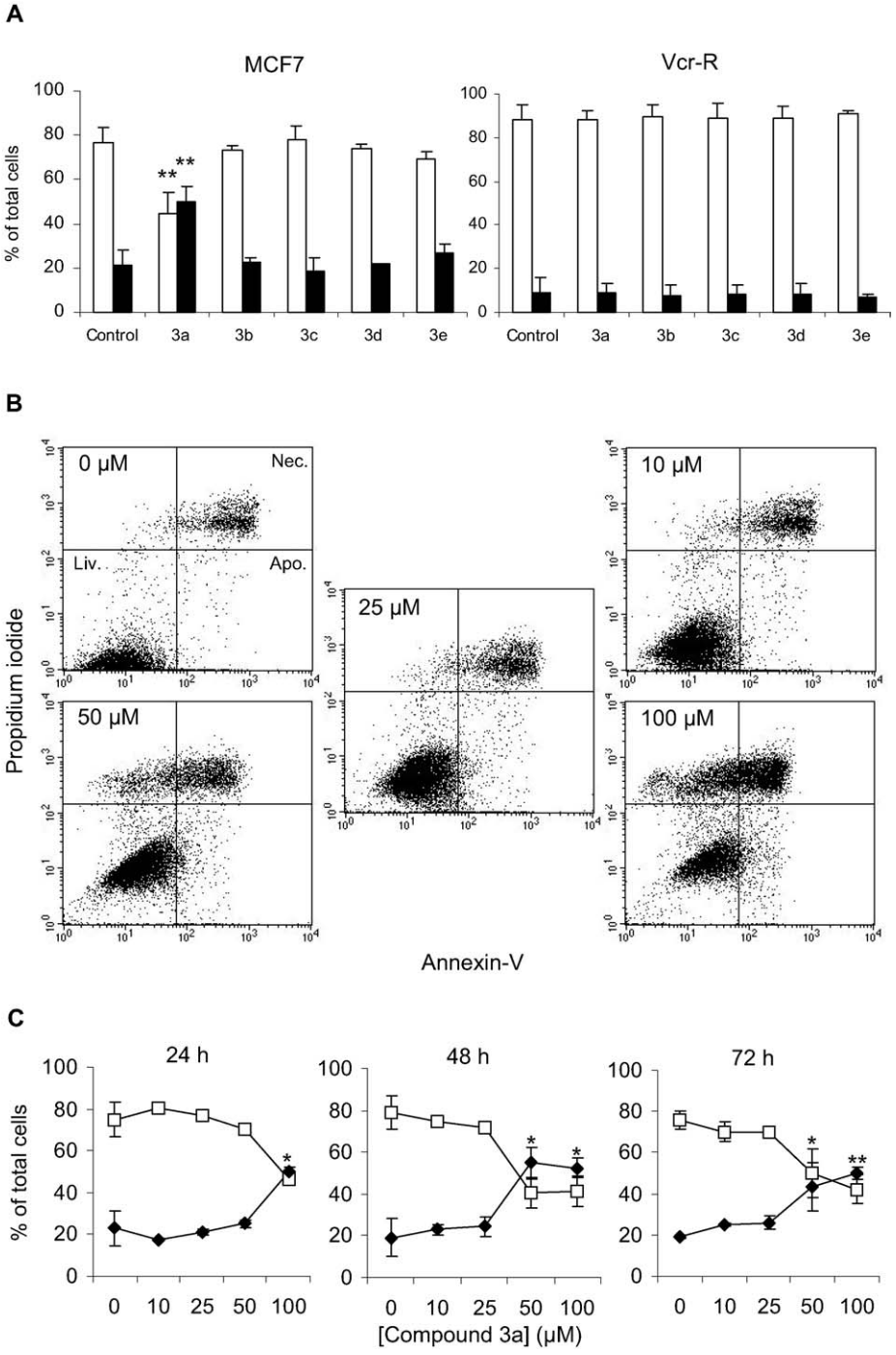


Fig. 5. Cell death pathway induced by the kenpaullone analogues. (A) Percent of dead cells of MCF7 and Vcr-R treated with the compounds (100 µM) for 48 h (□ live cells, ■ dead cells) assessed by Annexin-V staining. (B) Dose–response assessment of cell death upon exposure to compound **3a** during 24 h Liv.: live cells; Apo.: early apoptotic cells; Nec.: late apoptotic and necrotic cells. (C) Dose–response assessment of cell death for MCF7 cells exposed to compound **3a** for 24, 48 and 72 h (□ live cells, ♦ dead cells). Each column/symbol and vertical bar represent mean ± S.E.M. ($n = 3$), * and ** significantly different from control $P < 0.05$ and $P < 0.01$, respectively.

sion of p53, another well-known cycle regulator and apoptosis inducer. For MCF7 cells, this modulation of mRNAs levels went together with a rapidly induced apoptosis which occurred within 24 h as demonstrated by TUNEL analysis. While it displayed a very slight effect in vincristine resistant cells, the antiproliferative and proapoptotic properties of compound **3a** on MCF7 breast cancer cells highlight the potential interest of this molecule among the CDK inhibitors. Future studies of its pharmacological properties and in vivo activity would be of interest for therapeutic strategies against cancer.

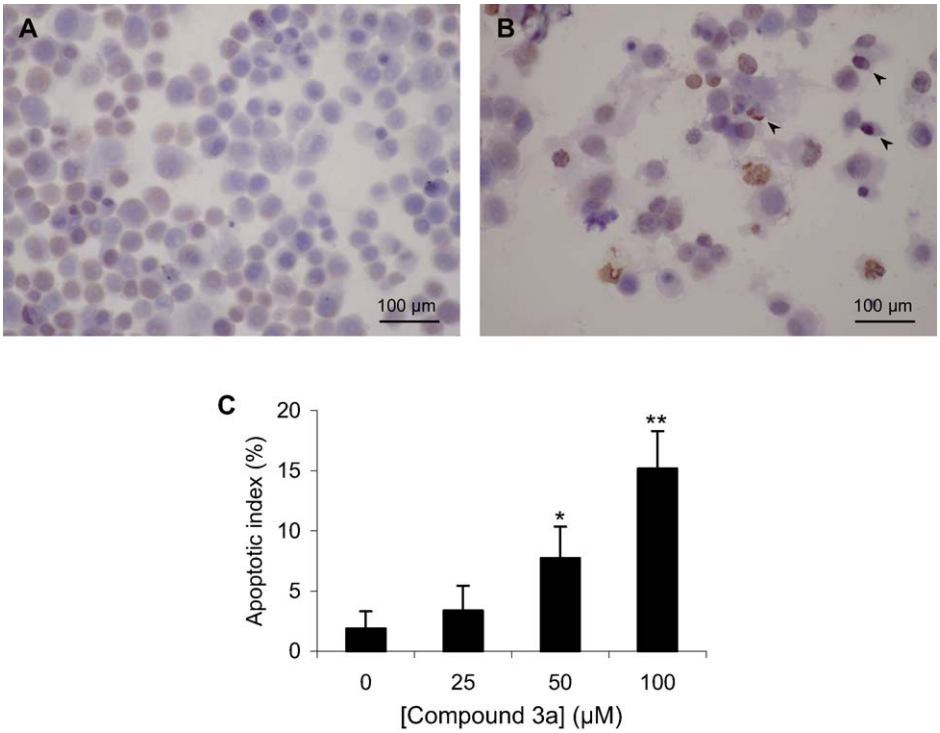


Fig. 6. TUNEL detection of apoptotic MCF7 cells. (A) Control cells. (B) 24 h-exposure to compound **3a** (100 µM). DAB labeling led to brown staining of apoptotic cell nuclei (examples: arrows). Harris' hematoxylin was used for nuclear counter-staining (blue). (C) Apoptotic indexes, evaluated as the percentage of apoptotic cells and bodies by reference to the total cellular population determined after a 24 h-treatment with compound **3a** at 0, 25, 50 and 100 µM. Columns and vertical bars represent mean ± S.E.M. for three independent experiments. A minimum of 600 cells were counted in each test. * and ** significantly different from control $P < 0.05$ and $P < 0.01$, respectively.

5. Experimental section

5.1. Chemistry

Thieno[b]azepinediones **1** were prepared according the procedure we described previously [15]. Phenylhydrazines **2** were purchased from Acros Organics (France). Melting points were determined on a Stuart SMP3 melting point apparatus and are uncorrected. Microanalysis (C, H, N) were within ± 0.4% of the theoretical values. ^{1}H-NMR spectra were recorded on a AC Bruker 250 MHz spectrometer in DMSO-d_6.

General procedure for the synthesis of thienoazepino-indol-5-ones **3** [13]. 0.12 ml (1.2 mmol) of adequate phenylhydrazine in 3 ml of acetic acid was added dropwise to 1 mmol of thieno[b]azepinedione **1** dissolved in 5 ml of acetic acid. The reaction mixture was stirred at 70 °C for 1 h and then cooled at 0 °C. Next, 0.1 ml of concentrated sulfuric acid was added and the reaction mixture was stirred again at 70 °C for 1 h. After incubation at room temperature, the mixture was poured into 20 ml of ice-cold 10% aqueous sodium acetate solution. The obtained precipitate was filtered, washed with water and dried. The crude product was purified by trituration in hot methanol and filtrated to give the expected thienoazepino-indol-5-one **3**.

8-Bromo-6,11-dihydro-thieno-[3′,2′:2,3]azepino[4,5-b]indol-5(4H)-one (**3a**) (9%). See Ref. [13].

2-tert-Butyl-6,11-dihydro-thieno-[3′,2′:2,3]azepino[4,5-b]indol-5(4H)-one (**3b**). Pale brown solid (34.5%); m.p. dec. > 300 °C; Anal. $C_{18}H_{18}N_2OS$ (C, H, N); ^{1}H-NMR (250 MHz, DMSO-d_6): 1.39 (s, 9H, $C(CH_3)_3$), 3.52 (s, 2H, CH_2), 6.73 (s, 1H, H_3), 7.03–7.16 (m, 2H, 2H indole), 7.36 (d, 1H, $J = 7.7$ Hz, H indole), 7.60 (d, 1H, $J = 7.7$ Hz, H indole), 10.23 (bs, 1H, NH, D_2O exchangeable H), 11.45 (bs, 1H, NH, D_2O exchangeable H). Anal. $C_{18}H_{18}N_2OS$ (C, H, N): C: 69.65, H: 5.84, N: 9.02; Found: C: 69.49, H: 6.03, N: 8.95.

8-Bromo-2-tert-butyl-6,11-dihydro-thieno-[3′,2′:2,3]azepino[4,5-b]indol-5(4H)-one (**3c**). Pale brown solid (14%); m.p. dec. > 300 °C; Anal. $C_{18}H_{17}BrN_2OS$ (C, H, N); ^{1}H-NMR (250 MHz, DMSO-d_6): 1.39 (s, 9H, $C(CH_3)_3$), 3.53 (s, 2H, CH_2), 6.73 (s, 1H, H_3), 7.22 (d, 1H, $J = 8.6$ Hz, H indole), 7.31 (d, 1H, $J = 8.6$ Hz, H indole), 7.84 (s, 1H, H indole), 10.28 (bs, 1H, NH, D_2O exchangeable H), 11.68 (bs, 1H, NH, D_2O exchangeable H). Anal. $C_{18}H_{17}BrN_2OS$ (C, H, N): C: 55.53, H: 4.40, N: 7.20; Found: C: 55.37, H: 4.59, N: 7.13.

3-Methyl-2-phenyl-6,11-dihydro-thieno-[3′,2′:2,3]azepino[4,5-b]indol-5(4H)-one (**3d**). Pale brown solid (49%); m.p. dec. > 300 °C; Anal. $C_{21}H_{16}N_2OS$ (C, H, N); ^{1}H-NMR (250 MHz, DMSO-d_6): 2.29 (s, 3H, CH_3), 3.54 (s, 2H, CH_2), 7.10–7.21 (m, 2H, 2H indole), 7.40–7.47 (m, 2H, 1H phenyl + 1H indole), 7.52–7.54 (m, 4H, 4H phenyl), 7.67 (d, 1H, $J = 7.9$ Hz, H indole), 9.91 (bs, 1H, NH, D_2O exchangeable H), 11.62 (bs, 1H, NH, D_2O exchangeable H). Anal. $C_{21}H_{16}N_2OS$ (C, H, N): C: 73.23, H: 4.68, N: 8.13; Found: C: 72.97, H: 4.61, N: 7.91.

8-Bromo-3-methyl-2-phenyl-6,11-dihydro-thieno-[3′,2′:2,3]azepino[4,5-b]indol-5(4H)-one (**3e**). Green solid (24%); m.p. dec. > 300 °C; Anal. $C_{21}H_{15}BrN_2OS$ (C, H, N); ^{1}H-NMR (250 MHz, DMSO-d_6): 2.28 (s, 3H, CH_3), 3.56 (s, 2H, CH_2), 7.28 (d, 1H, J = 8.5 Hz, H indole), 7.38 (d, 1H, J = 8.5 Hz, H indole), 7.43–7.48 (m, 1H, H phenyl), 7.52–7.55 (m, 4H, 4H phenyl), 7.92 (s, 1H, H indole), 9.95 (bs, 1H, NH, D_2O exchangeable H), 11.85 (bs, 1H, NH, D_2O exchangeable H). Anal. $C_{21}H_{15}BrN_2OS$ (C, H, N): C: 59.58, H: 3.57, N: 6.62; Found: C: 59.29, H: 3.50, N: 6.22.

5.2. Biology

5.2.1. Cell culture and viability test

Cells were grown in RPMI-1640 medium, containing 10% fetal calf serum, antibiotics and antimycotics [10^4 UI/ml penicillin G, 10 mg/ml streptomycin, 25 μg/ml amphotericin B] (Eurobio, France) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells in suspension in the culture medium were collected together with the adherent cells. Trypan-blue staining was used to test cellular viability: cells were suspended in 1% Trypan-blue in PBS (phosphate buffered saline), incubated for 5 min, and counted for viability using visible-light microscopy. Live cells remained white and light-refracting, while dead cells were blue-stained.

5.2.2. Cell cycle analysis

At the indicated times, total cells were trypsin-harvested, washed with PBS, fixed with 80% cold ethanol, stained with propidium iodide solution (50 μg/ml PI, 20 μg/ml of Rnase A, 0.1% Triton X-100) for 15 min, and analyzed by flow cytometry (FACScan, Becton-Dickinson, USA).

5.2.3. RNA isolation and RT-PCR

Total cellular RNA was isolated from cultured cells using the ExtractAll (Eurobio, France) according the manufacturer's instructions. The primer sequences for β-actin (an invariant housekeeping gene control), p21, p53 and cdk1 were as follows: β-actin (5′-ggacgacatggagaaaatctgg-3′, 5′-tggatagcaacgtacatggctg-3′), p21 (5′-aggaggcgccatgtcagaac-3′, 5′-aggactgcaggcttcctgtg-3′), p53 (5′-attctgggacagccaagtct-3′, 5′-ggagtcttccagtgtgatga-3′), cdk1 (5′-ggagttgtgtataagggtag-3′, 5′-gagtgttacctcatgtg-3′). Total RNA (5 μg) was transcribed using MMLV-reverse transcriptase according to the manufacturer's instructions and PCR was performed at an annealing temperature of 60 °C and 32 cycles.

5.2.4. Detection of apoptosis by Annexin-V and TUNEL staining

After exposure of the cells to the compounds, Annexin-V FITC staining was performed as instructed by the manufacturer (BD Pharmingen, USA). Briefly, the MCF7 cells were trypsinized, harvested by centrifugation together with suspension cells and resuspended in 100 μl of binding buffer. The cell suspension was incubated in the dark for 15 min with 5 μl of Annexin-V FITC and 5 μl of propidium iodide. Analysis was performed on a FACScan and Annexin-V FITC binding was detected in the FL-1 (green) and PI in the FL-2 (red). In some instances, parallels samples were assessed by the TUNEL in situ staining. TUNEL was performed according to an optimized protocol previously described [17]. Cells in suspension in the culture medium were collected together with the adherent cells, fixed and studied as cytospots. Peroxidase labeling was detected using diaminobenzidine (DAB) color reaction (brown). Samples were counterstained with Harris' hematoxylin, photographied and quantified using a Eclipse 80i microscope (Nikon, France) and the Lucia 4.8 software (Nikon, France).

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