Biochemical Pharmacology 77 (2009) 1401–1409

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Denbinobin inhibits nuclear factor-κB and induces apoptosis via reactive oxygen species generation in human leukemic cells

Gonzalo Sánchez-Duffhues [a], Marco A. Calzado [a], Amaya García de Vinuesa [a], Giovanni Appendino [b], Bernd L. Fiebich [c], Ulich Loock [d], Annette Lefarth-Risse [d], Karsten Krohn [d,1], Eduardo Muñoz [a,1,*]

[a] Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba, Córdoba, Spain
[b] Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche, Università del Piemonte Orientale, Novara, Italy
[c] Department of Psychiatry, University of Freiburg Medical School, Freiburg, Germany
[d] Department of Chemistry, University of Paderborn, Germany

ARTICLE INFO

Article history:
Received 17 December 2008
Received in revised form 11 January 2009
Accepted 14 January 2009

Keywords:
Denbinobin
1,4-Phenanthrenequinones
NF-κB
ROS
MAPKs
Apoptosis

ABSTRACT

Denbinobin, a 1,4-phenanthrenequinone firstly isolated from the stems of *Dendrobium moniliforme* (Shi-Hu in Chinese medicine), has been reported to exhibit anti-tumoral and anti-inflammatory activities through mechanism(s) not yet fully understood. Because of the critical role of the transcription factor NF-κB and of ROS-induced activation of stress regulated kinases in tumorigenesis, we have investigated the effect of denbinobin on these pathways. We found that denbinobin is a potent inhibitor of TNFα and PMA-induced NF-κB activation, and that it can block the phosphorylation and degradation of IκBα by inhibiting TAK1 activity, an event lying upstream of IKK activation. Moreover, treatment with denbinobin not only elicited apoptotic signalling, including mitochondrial membrane dysfunction, activation of caspases and cleavage of poly(ADP-ribose) polymerase, but also induced intracellular reactive oxygen species (ROS) generation and sustained activation of the mitogen-activated kinases (MAPKs) ERK1+2, p38 and JNK 1+2. The apoptotic effects of denbinobin could be prevented by pre-treatment with the intracellular ROS scavenger *N*-acetyl-L-cysteine, but not by pharmacological inhibition of MAPKs, suggesting that intracellular ROS generation underlies denbinobin-induced apoptosis, and that this effect takes place in an MAPKs-independent pathway. To define the structural elements critical for these activities, a series of phenanthrenequinones with different substituents in the phenanthrene- and/or in the quinone ring were prepared and assayed for NF-κB inhibition and ROS production. In this way, the major structure–activity relationships and the structural elements critical for the activity of denbinobin could be established.

1. Introduction

The phenanthrene system is relatively uncommon in natural products, and is usually generated by the oxidative coupling of stilbene precursors or by aromatization of diterpenoids. Some plant-derived phenanthrenequinones, and especially denbinobin, show interesting biological activities [1]. Denbinobin (5-hydroxy-3,7-dimethoxy-1,4-phenanthrenequinone) has been isolated from several orchidaceous plants, like *Dendrobium nobile* [2], *D. moniliforme* [3] and *Ephemerantha lonchophylla* [4] and from a variety of *Cannabis sativa* [5]. Interestingly, the stems of *D. moniliform* and *E. lonchophylla* known as Shi-Hu in Chinese medicine have been used for a long time to treat respiratory infections, as a tonic to nourish the stomach, and to reduce fever. Also, the total synthesis of denbinobin has been reported [6], and this compound has been shown to induce apoptosis in several cancer cell lines [7–9]. Furthermore, we have recently shown that denbinobin inhibits HIV-1 reactivation through an NF-κB-dependent pathway [5]. However, the molecular details involved in NF-κB inhibition and the induction of apoptosis are still unclear.

The imbalance between cell survival and death, a key feature of many neoplastic, degenerative, inflammatory and autoimmune diseases, may be caused by an aberrant turnover of reactive oxygen species (ROS), a process that, in turn, regulates the crosstalk between NF-κB and mitogen-activated protein kinases (MAPKs) [10,11]. NF-κB is a collective term used to describe members of the Rel family of transcription factor. The Rel family regulates

* Corresponding author at: Departamento de Biología Celular, Fisiología e Inmunología, Facultad de Medicina, Avda de Menendez Pidal s/n, 14004 Córdoba, Spain. Tel.: +34 957 218267; fax: +34 957 218229.
E-mail address: fi1muble@uco.es (E. Muñoz).
[1] These authors are equally senior co-authors.

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.01.004

transcription of large number of genes and is involved in the control of a variety of cellular processes, such as immune and inflammatory responses, development, cellular growth, apoptosis and HIV-1 gene expression [12]. NF-κB is an inducible transcription factor made up of homo- and heterodimers of p50, p65 (RelA), p52, relB and c-rel subunits that interact with a family of inhibitory IκB proteins, of which IκBα is the best characterised. In most cell types, these proteins sequester NF-κB in the cytoplasm by masking its nuclear localization sequence. Stimulation of cells with a variety of physiological or pathogenic stimuli leads to phosphorylation, ubiquitination, and the subsequent degradation of IκBα proteins [13]. Phosphorylation of IκBα at serines 32 and 36 is a key step involved in the activation of NF-κB complexes. This event is mediated by IκB kinases (IKKs), which are formed by a high-molecular weight complex (IKC) containing at least two kinase subunits (IKKα and IKKβ), and the associated modulatory protein NEMO/IKKγ [12,13]. Several upstream kinases, including TGFβ-activated kinase 1 (TAK1), have been described to regulate the activity of IKKs [12]. TAK1 is a ubiquitin-dependent kinase that is activated by innate immune stimuli including bacterial components and pro-inflammatory cytokines such as interleukin-1 and TNFα and plays an essential role in innate immune signalling by activating both IKKs and MAPK pathways [14–16]. Therefore, it has been proposed that inhibition of TAK1 activity may be effective in preventing inflammation and tissue destruction promoted by pro-inflammatory cytokines [17].

ROS metabolism has been proposed to play an important role in all types of programmed cell death: apoptosis, necrosis, and autophagy [11]. ROS are continuously produced during normal aerobic metabolism by the mitochondria electron chain and enhanced in response to many different physiological stimuli. In addition ROS are also produced by extra-mitochondrial cellular sources including endoplasmic reticulum, a lysosomal redox chain and plasma membrane Noxs [18]. It has been demonstrated that low- and intermediated levels of ROS can protect cell from apoptosis by activating anti-oxidant mechanisms, including activation of the NF-κB pathway, which in turn up regulates anti-apoptotic genes [19]. Conversely, high levels of intracellular ROS results in a breakdown of the transmembrane mitochondrial potential ($\Delta\Psi_m$), a common feature of early apoptosis that precedes DNA fragmentation regardless the cell type and the apoptotic stimuli [20]. The disrupture of $\Delta\Psi_m$ is mediated by mitochondrial megachannel opening (permeability transition, PT) [21], which leads to the release of a host of pro-apoptotic proteins (cytochrome *c*, Smac/Diablo) and the apoptotic inducing factor, which, in turn, mediates the activation of executor caspases [22]. Furthermore, high levels of ROS may inhibit NF-κB activation and induce a sustained c-Jun N-terminal kinase (JNK) activation that promotes the apoptotic pathway [10,23,24].

The MAPK signalling cascade was originally identified as an important pathway in the transduction of apoptotic signals initiated by stress or toxic stimuli [25]. Major participants in this kinase cascade are two members of the MAPKs, JNK and p38 MAP kinase, as well as their upstream kinases, such as MAP kinase kinases (MKKs). In contrast, p42/44 MAPKs (ERKs) are most frequently associated with pro-survival activity. Recent studies have demonstrated the essential role of the kinase cascade as well as its downstream mitochondrial-dependent reactive oxygen species production pathway during apoptosis induction by cellular stress [26–28]. Some chemopreventive compounds as well as antineoplastic drugs may induce apoptosis through modulation of the MAPK pathways [29,30].

Leukemia, currently one of the most threatening hematological malignant cancers, has been found to be very sensitive to anticancer chemotherapeutic reagents which either interfere with the cell cycle or cause apoptosis. Thus, the manipulation of apoptosis has the potential to provide novel strategies for cancer chemotherapy by selectively activating this process in malignant cells. We report here that denbinobin is endowed with a potent pro-oxidant activity, related to the presence of a quinone ring. As a result, denbinobin inhibits TAK1-dependent NF-κB activation and induces apoptosis on the human leukemia cell line Jurkat. The compound induces the collapse of $\Delta\Psi_m$ and the activation of caspases, suggesting that the main lethal apoptotic pathway induced by this phenanthrenequinone involves mitochondrial damage.

2. Material and methods

2.1. Isolation of denbinobin

The isolation of denbinobin from a chemotype of *C. sativa* (variety CARMA) cultivated at the I.S.I.C. (Rovigo, Italy) has been previously described [5]. Denbinobin was identified according to its physical and spectroscopic properties [3].

2.2. Synthesis of 1,4-phenanthrenequinones

The method applied in the synthesis of 1,4-phenanthrenequinones [1–20] and tri- and tetramethoxyphenanthrenes [21,22] used in this study has been previously described [6] (Supplementary information). The synthesis of 1,4-diacetoxy-7,8-dimethoxyphenanthrene [6] is also described in supplementary material.

2.3. Cell lines and reagents

Jurkat cells were grown at 37 °C and 5% CO_2 in supplemented RPMI 1640 medium containing 10% heat-inactivated FCS, 2 mM glutamine and antibiotics (Lonza, Basel, Switzerland). The 5.1 clone line is a Jurkat derived clone stably transfected with a plasmid containing the luciferase gene driven by the HIV-LTR promoter and was maintained in complete medium supplemented with G418 (200 μg/ml) (Sigma Co, St. Louis, MO, USA). The 293T cell line was obtained from the ATCC (Manassas, VA, USA) and cultured in complete DMEM (Lonza). The antibodies anti-IκBα (sc-371), anti-NF-κB-p65 (sc-8008), anti-p-ERK1+2 (sc-7383, Tyr 204), the rabbit polyclonal anti-IKKγ (sc-8330), and the goat polyclonal against p-IKKα/β (sc-21661, Ser 176) were purchased from Santa Cruz Biotechnology (San Diego, CA, USA). The antibodies against anti-phospho-p65 (Ser 536), anti-phospho-IκBα (5A5, Ser 32/36), anti-JNK (9252), anti-p-JNK 1+2 (9255, Thr 183/Tyr 185) and anti-phospho p38 (9211, Thr 180/Tyr 182) were from Cell Signalling (Danvers, MA, USA). The mAb anti-tubulin and anti-ERK1+2 (M5670) were purchased from Sigma. The rabbit polyclonal α-caspase-3 was from Dako Diagnostic (Copenhagen, DK), the antisera α-caspase-7, α-caspase-8 and the antibody anti-PARP were from Chemicon (Harrow, UK). Anti-c-myc, anti-HA and anti-GFP epitopes were purchased from Roche (Manheim, Germany). The p38 kinase inhibitor SB203085 was purchased from Sigma–Aldrich, the JNK inhibitor SP600125 was from Calbiochem (Darmstadt, Germany) and the ERK inhibitor PD98059 was purchased from Alexis (Lausanne, Switzerland). [γ-^{32}P]ATP (3000 Ci/mmol) was from MP Biomedicals (Irvine, CA, USA). All other reagents were from Sigma.

2.4. Plasmids, transient transfections and luciferase assays

The KBF-Luc contains three copies of the MHC enhancer κB site upstream of the conalbumin promoter, followed by the luciferase gene. The expression vectors for IKKα and IKKβ and the IKKα and β tagged GFP vectors have been described elsewhere [31]. The expression vector TRAF-2, TRAF-6, TAK1-HA, TAB1-myc were obtained from Dr. M.L. Schmitz (Giessen, Germany). To study the

NF-κB transcriptional activities the Jurkat cells were transfected with the KBF-Luc plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. 24 h post-transfection, cells were pre-treated with denbinobin for 30 min, and treated or not with PMA for 6 h. For the kinase overexpression experiments, 293T cells (5×10^5 cells/well) were transiently transfected with the expression vectors for TAK1/TAB1, TRAF-2, TRAF-6, IKKα and IKKβ, and the KBF-Luc plasmids. After 24 h, cells were treated with indicated concentrations of denbinobin for 6 h and luciferase activity measured. To determine NF-κB-dependent transcription of the HIV-LTR-luc 5.1 cells were pre-incubated for 30 min with denbinobin and analogues as indicated, followed by stimulation with TNFα (2 ng/ml) for 6 h (supplementary Tables I and II). For luciferase detection the cells were washed twice in PBS and lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol during 15 m at RT in a horizontal shaker. Then the lysates were spun down and the supernatant was used to measure luciferase activity using an Autolumat LB 9510 (Berthold) following the instructions of the luciferase assay kit (Promega Co, Madison, WI, USA). Results are represented as RLU or fold induction over untreated control. Results represent mean ± S.D. of four different experiments.

2.5. Isolation of nuclear extracts and mobility shift assays

Jurkat cells (10^6/ml) were pretreated with denbinobin at the indicated doses for 1 h and then were stimulated or not with PMA (50 ng/ml) for 2 h. Cells were then washed twice with cold PBS and proteins from nuclear extracts isolated as previously described [5]. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA). For the electrophoretic mobility shift assay (EMSA), double stranded oligonucleotide containing the consensus site for NF-κB, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ or the consensus site for SP-1, 5′-ATT CGA TCG GGG CGG GGC GAG C-3′ (Promega) were end-labeled with [γ-^{32}P]ATP. The binding reaction mixture contained 5 μg of nuclear extracts, 0.5 μg poly(dI-dC) (Amersham Biosciences Inc.), 20 mM Hepes pH 7, 70 mM NaCl, 2 mM DTT, 0.01% NP-40, 100 μg/ml BSA, 4% Ficoll, and 100,000 cpm of end-labeled DNA fragments in a total volume of 20 μl. After 30 min incubation at 4 °C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris-base, 89 mM boric acid and 2 mM EDTA. Gels were pre-electrophoresed for 30 min at 225 V and then for 2 h after loading the samples. These gels were dried and exposed to X-ray film at −80 °C.

2.6. Western blot

Jurkat cells (10^6 cells/ml) were stimulated with the indicated compounds. Cells were then washed with PBS and proteins extracted in 50 μl of lysis buffer (20 mM Hepes pH 8.0, 10 mM KCl, 0.15 mM EGTA, 0.15 mM EDTA, 0.5 mM Na_3VO_4, 5 mM NaF, 1 mM DTT, leupeptin 1 μg/ml, pepstatin 0.5 μg/ml, aprotinin 0.5 μg/ml, and 1 mM PMSF) containing 0.5% NP-40. Protein concentration was determined by the Bradford assay (Bio-Rad) and 30 μg of proteins were boiled in Laemmli buffer and electrophoresed in 10% SDS/polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; 4 °C) for 1 h. Blots were blocked in TBS solution containing 0.1% Tween 20 and 5% non-fat dry milk overnight at 4 °C, and immunodetection of specific proteins was carried out with primary antibodies using an ECL system (GE Healthcare, Piscataway, NJ, USA).

2.7. IKK kinase assay

Cells were lysed in NP-40 lysis buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM PMSF, 10 mM NaF, 0.5 mM sodium vanadate, leupeptine (10 μg/ml), aprotinin (10 μg/ml), 1% (v/v) NP-40 and 10% (v/v) glycerol) for 15 min at 4 °C, and after centrifugation for 10 min at 13,000 rpm, the supernatant was incubated with non-specific IgG and 25 μl protein A/G sepharose (preclearing) and incubated overnight on a spinning wheel. After centrifugation, the supernatants were incubated with 2 μg of anti-IKKγ antibody and 25 μl protein A/G sepharose and incubated for 2–4 h on a spinning wheel at 4 °C. The precipitate was washed three times in cold lysis buffer and three times in cold kinase buffer (20 mM Hepes/KOH pH 7.4, 25 mM β-glycerophosphate, 2 mM DTT, 20 mM $MgCl_2$). The kinase assay was performed in a final volume of 20 μl kinase buffer containing 40 μM ATP and 2 μg of the purified substrate protein GST-IκBα(1–54). After incubation for 20 min at 30 °C, the reaction was stopped by the addition of 5× SDS loading buffer. After separation by SDS-PAGE the proteins were transferred to nitrocellulose membranes and IκBα phosphorylation was detected by Western blot using the antibody anti-phospho-IκBα 5A5.

2.8. Analysis of cell cycle analysis and determination of cytotoxicity

The percentage of cells undergoing chromatinolysis (subdiploid cells) was determined by ethanol fixation (70%, for 24 h at 4 °C). Then, the cells were washed twice with PBS containing 4% glucose and subjected to RNA digestion (RNAse-A, 50 U/ml) and PI (20 μg/ml) staining in PBS for 1 h at RT, and analyzed by cytofluorimetry. With this method, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells and the subsequent staining allows the determination of the percentage of subdiploid cells (sub-G_0/G_1 fraction). For cytotoxicity assays Jurkat cells were seeded in 96-well plates in complete medium and treated with increasing doses of denbinobin for the indicated times. Samples were then diluted with 300 μl of PBS and incubated 1 min at RT in the presence of PI (10 μg/ml). After incubation, cells were immediately analyzed by flow cytometry in an EPICS XL flow cytometer (Coulter, Hialeah, FL).

2.9. Cytofluorimetric analysis of mitochondrial transmembrane potential and ROS generation

To evaluate the mitochondrial transmembrane potential ($\Delta\Psi_m$) and the superoxide anion generation (ROS), treated or untreated cells (5×10^5/ml) were incubated in PBS with $DiOC_6(3)$ (green fluorescence) (20 nM) (Molecular Probes, Eugene, OR, USA) and dihydroethidine (HE) (red fluorescence after oxidation) (2 μM) (Sigma) for 20 min at 37 °C, followed by analysis on an Epics XL flow cytometer.

3. Results

3.1. Differential effects of denbinobin on NF-κB activation and MAPKs signalling pathways

We have recently found that denbinobin inhibits TNFα-induced HIV-1 reactivation by targeting activation of the NF-κB canonical pathway, but the molecular details of this activity remained elusive [5]. To further confirm the anti-NF-κB activity of denbinobin, Jurkat cells were transiently transfected with a luciferase reporter construct under the control of a minimal promoter containing three binding sites for NF-κB. Activation by PMA increased the luciferase gene expression driven by this artificial promoter in Jurkat cells, and we found that denbinobin could effectively inhibit this promoter activity in a dose-dependent manner (Fig. 1A). In addition, using electrophoretic shift assays, we confirmed the inhibitory effect of denbinobin on PMA-induced NF-κB binding to DNA (Fig. 1B). Kinetic experiments demonstrate that denbinobin also inhibited PMA-induced IκBα phosphorylation and degradation as well as p65

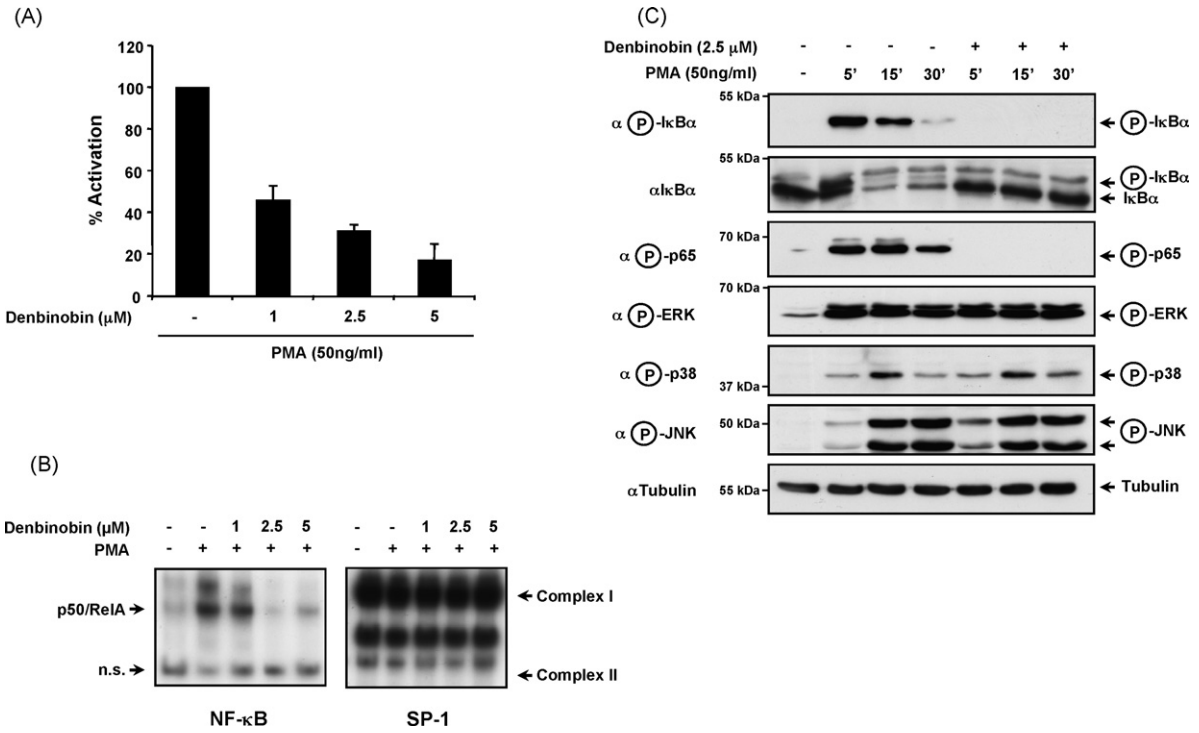

Fig. 1. Denbinobin inhibits NF-κB activation pathway in Jurkat T cells. (A) Denbinobin inhibits NF-κB-dependent reporter gene expression induced by PMA. Jurkat cells were transiently transfected with the KBF-Luc plasmid for 24 h. After transfection, the cells were treated with denbinobin at the indicated concentrations and PMA (50 ng/ml) for 6 h. The cell lysated were assayed for luciferase activity. Results are represented as percentage of activation considering the luciferase activity induced by PMA alone as 100% activation. (B) Denbinobin inhibits the binding of NF-κB to DNA. Nuclear extracts of untreated Jurkat cells or PMA cells treated with PMA in the absence or presence of denbinobin for 30 min were prepared, and then assayed for NF-κB and SP-1 binding to DNA using EMSA. (C) Western blot analysis of IκBα phosphorylation and degradation, p65 phosphorylation (Ser 536) and MAPKs activation (p-ERK, p-38, and p-JNK) performed in Jurkat cells pre-incubated with denbinobin (2.5 μM) and stimulated with PMA for the indicated periods of time.

phosphorylation, suggesting that denbinobin mediates its anti-NF-κB activity by targeting a step upstream of IκBα phosphorylation in the canonical pathway of NF-κB activation. Conversely, denbinobin did not interfere with the activation of the MAPKs; ERK, p38 and JNK (Fig. 1C).

Since the degradation of IκB proteins was shown to occur after signal-induced phosphorylation of IκB proteins at specific serine residues catalysed by IKKs present in the IKC [32], we investigated whether the prevention of IκBα phosphorylation and degradation was due to an impaired kinase activity of the IKC. Endogenous IKC was isolated by immunoprecipitation with an anti-IKKγ Ab, and its activity was analyzed by immune complex kinase assays using recombinant IκBα protein as substrate. Either PMA or TNFα-stimulation in Jurkat cells clearly induced an increase in IKK activity compared with unstimulated cells, and this activity was dose dependently inhibited in the presence of denbinobin (2.5 μM) (Fig. 2A). Since denbinobin impaired TNFα- and PMA-induced activation of IKK activity, obvious direct candidates for the inhibitory activity of denbinobin would be either the IKKs itself or, alternatively, other kinases upstream IKKs. This possibility was assessed in 293T cells transiently transfected with the KBF-luc plasmid alone or in combination with expression vectors encoding IKKα, IKKβ, TRAF-2, TRAF-6 and TAK1/TAB1. Fig. 2B shows that denbinobin could inhibit in a dose-dependent manner the NF-κB-dependent transcriptional activity induced by over-expression of TRAF-2, TRAF-6 and TAK1/TAB1, but not the activity induced by either IKKα or IKKβ.

In mammals, in vitro and over-expression studies suggest TAK1 is involved in TNFR1 and IL-1R/TLR-mediated signalling pathways upstream of IKKs and JNK [33]. Two mammalian TAK1 adaptor proteins, TAB1 and TAB2, were isolated as TAK1-interacting proteins by yeast two-hybrid screening and TAB1 interacts constitutively with TAK1 and induces TAK1 kinase activity when over-expressed [34]. To investigate the effects of denbinobin on TAK1 induced IKK activation in vivo we co-transfected 293T cells with plasmids encoding IKKα–GFP and IKKβ–GFP fusion proteins together with the expression vectors TAK1-HA and TAB1-myc. IKK–GFP fusion proteins were used to differentiate from endogenous IKKα/β proteins in which the phosphorylation is difficult to detect with the antibody used. Fig. 2C shows that TAK1 can phosphorylate both exogenous IKKα–GFP and IKKβ–GFP proteins (IKKβ–GFP migrates slower than IKKα–GFP) and also phosphorylate the endogenous IκBα protein. Incubation of transfected cells with denbinobin resulted in the inhibition of IKKβ–GFP, IKKα–GFP and IκBα phosphorylation in a dose-dependent manner. Over-expression of IKKβ–GFP and IKKα–GFP alone also led to some autophosphorylation of these kinases that was not affected by denbinobin (data not shown). Taken together, these results strongly suggest that denbinobin inhibits the NF-κB activation pathway by targeting the TAK1 kinase.

3.2. Denbinobin induces a sustained activation of MAPKs that is inhibited by N-acetyl cysteine

It has been shown that (5Z)-7-oxozeaenol, a selective TAK1 inhibitor, prevents p38 and JNK activation in IL-1 stimulated cells [17]. However, denbinobin did not inhibit PMA-induced p38, JNK and ERK phosphorylations (Fig. 1C). Therefore, we investigated whether denbinobin alone was sufficient to activate these MAPKs. Jurkat cells were incubated with denbinobin (5 μM) for the indicated times, and the phosphorylation/activation status of JNK, p38 and ERK was investigated by Western blots. As depicted in Fig. 3A, denbinobin induced a robust and sustained activation of the stress-activated kinases, JNK and p38. Denbinobin also

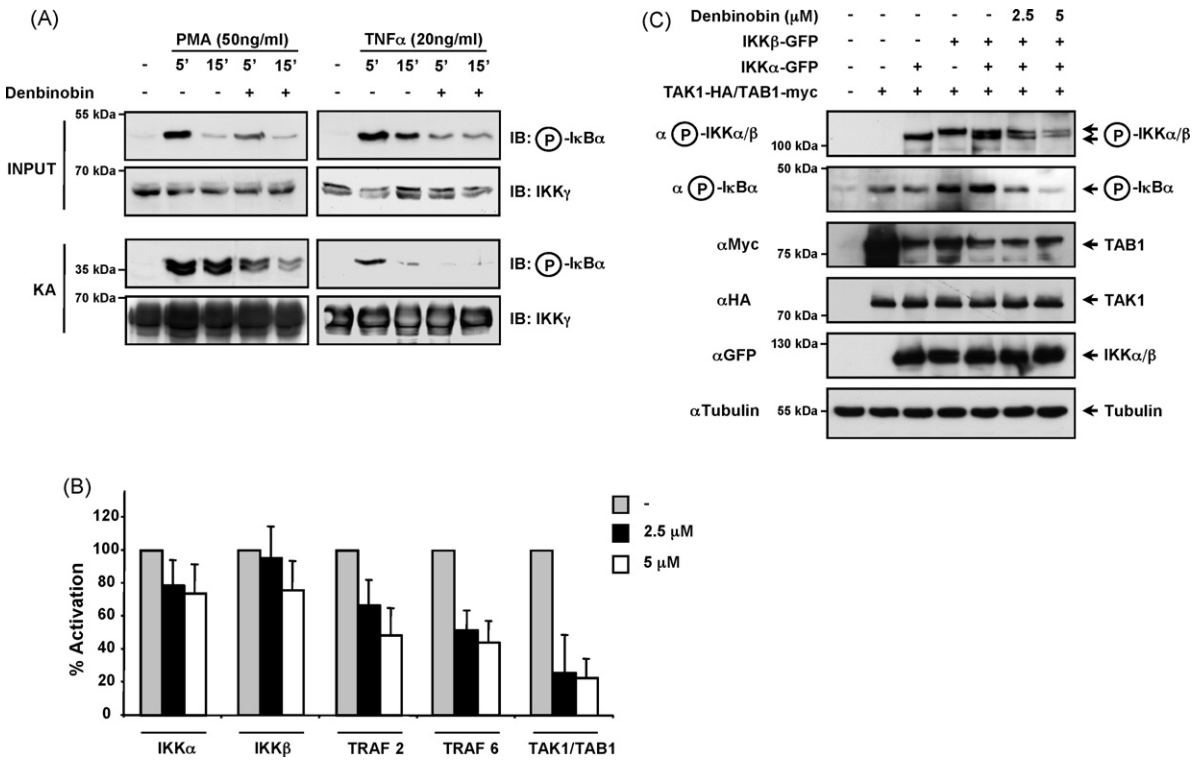

Fig. 2. (A) Denbinobin suppresses the TNFα and PMA-induced activation of IKK in vivo. Jurkat cells were treated with denbinobin (2.5 μM) for 10 min and the treated with either PMA or TNFα for the indicated times. Half of the cell lysates were subjected to Western blot for the detection of endogenous IκBα (Ser 32/36) phosphorylation (input) and the other half of the lysates was used for IKKγ immunoprecipitation and the kinase activity was monitored using recombinant GST-IκBα(1–54) as substrate (IP KA). IKKγ was also detected by immunoblots as a control of the immunoprecipitated fraction. (B) Effect of denbinobin on IKKα, IKKβ, TRAF-2, TRAF-6 and TAK1/TAB1 NF-κB-dependent transcription. 293T cells were transiently transfected with KBF-Luc alone or in combination with IKKα, IKKβ, TRAF-2, TRAF-6, or a pair of TAK1/TAB1 expression vectors. After 24 h of transfection cells were stimulated with different doses of denbinobin for 6 h and luciferase activity was assayed. The results show the percentage of activation and are representative of three different experiments. Grey bars represent transfected cells with kinase vectors and the KBF-Luc plasmid in the absence of denbinobin. (C) Denbinobin inhibits TAK1/TAB1-induced phosphorylation of exogenously expressed IKKα–GFP, IKKβ–GFP and endogenous IκBα phosphorylation (Ser 32/36). 293T cells were co-transfected with the indicated plasmids and 24 h later incubated with denbinobin at the indicated concentrations fro 6 h. Total cell extracts were analyzed by Western blot with specific antibodies.

activated ERK but to a lesser extent than JNK and p38. Since p38 and JNK are mainly activated by oxidative stress, we pre-incubated Jurkat cells with two different anti-oxidants, ambroxol (2-amino-3,5-dibromo-N-[trans-4-hydroxycyclohexyl]benzylamine) and *N*-acetyl cysteine (NAC), which reduce oxidant-mediated cell damage through different mechanisms [35,36]. Interestingly, NAC, but not ambroxol, completely inhibited denbinobin-induced phosphorylation and activation of p38, JNK and ERK. Since ambroxol is a weak inhibitor of superoxide-dependent auto-oxidation of substrates, it is likely that denbinobin induces MAPKs activation through generation of superoxides.

3.3. Denbinobin-induced reactive oxygen species, loss of mitochondrial membrane potential and activation of caspases in Jurkat cells

We have shown previously that quinone analogues induce apoptosis by generating high levels of intracellular ROS that, in

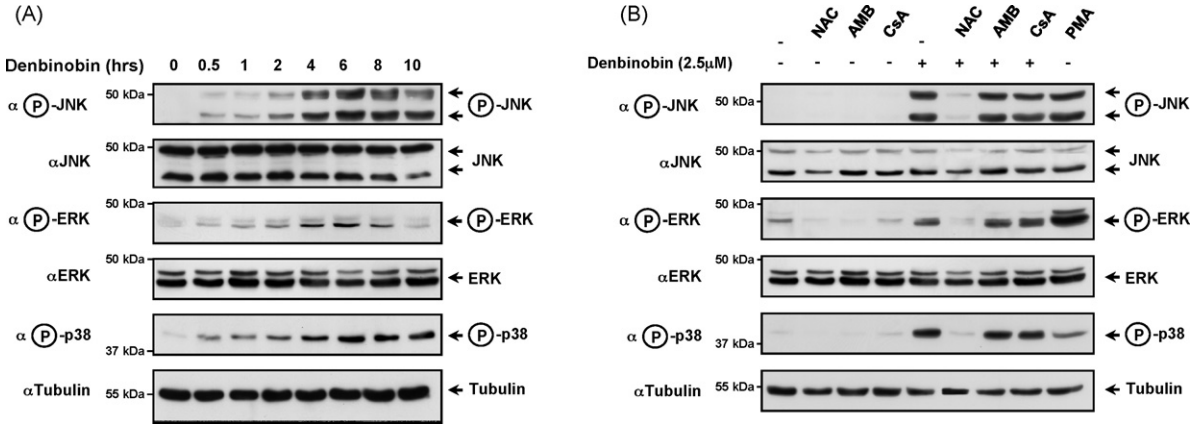

Fig. 3. (A) Effects of denbinobin on MAPKs phosphorylation. Jurkat cells were incubated with denbinobin (2.5 μM) during the indicated times and the phosphorylation of JNK (Thr 183/Tyr 185), ERK (Tyr 204) and p38 (Thr 180/Tyr 182), and the steady state levels of total MAPKs and α-tubulin were analyzed using specific antibodies by Western blots. (B) Deninobin induces MAPKs activation through a ROS-dependent pathway. Jurkat cells were pre-incubated with NAC (5 mM), ambroxol (1 mM), CsA (5 μM) during 15 min and then treated with denbinobin (2.5 μM) for 3 h. Total cell extracts were analyzed by Western blot as in (A).

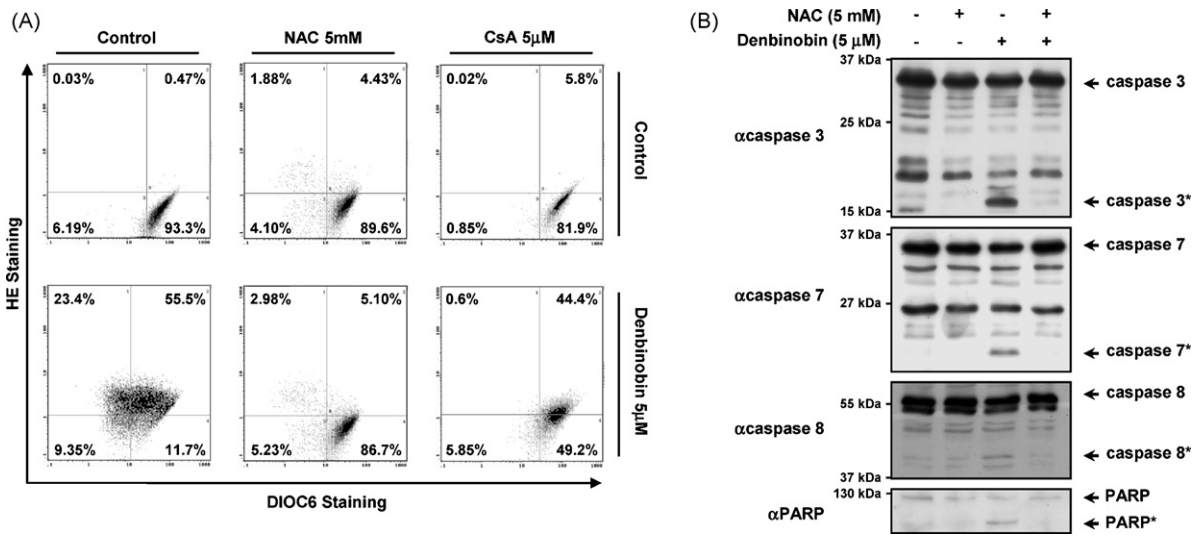

Fig. 4. (A) Effects of denbinobin in pre-apoptotic events. Jurkat cells were treated with denbinobin the in absence or presence of either NAC (5 mM) or CsA (5 μM). After 6 h treatment, the cells were collected and the simultaneous $\Delta\Psi_m$ disruption and ROS generation detected by cytofluorimetry. The results represent the percentage of cells obtained in biparametric histograms delimited by four compartments, namely, $\Delta\Psi_m^{high}/(HE \rightarrow Eth)^{low}$ (normal cells, 93.3%); $\Delta\Psi_m^{low}/(HE \rightarrow Eth)^{high}$ and $\Delta\Psi_m^{low}/(HE \rightarrow Eth)^{low}$ (denbinobin-treated cells; 23.4% and 9.35%), and $\Delta\Psi_m^{high}/(HE \rightarrow Eth)^{high}$ (denbinobin-treated cells; 55.5%). (B) Involvement of caspases activation in denbinobin-induced apoptosis. Jurkat cells were treated with denbinobin for 12 h in the presence or absence of NAC and the processing of PARP, caspases-3, -7 and -8 assessed by Western blot.

turn, induce mitochondria depolarization [37,38]. Thus, to investigate the role of ROS and mitochondria function in denbinobin-treated cells, we loaded Jurkat cells with a fluorochrome (dihydroethidium, DHE) that enters cells freely. Superoxide then oxidizes DHE into ethidium or a structurally similar product, which intercalates into DNA, producing red fluorescence (HE staining). The cells were treated as indicated for 6 h, and the percentage of cells expressing fluorescent Eth was determined by flow cytometry. Fig. 4A shows that cells treated with denbinobin had a very strong accumulation of intracellular ROS (78.9% of the cells) compared to untreated cells (0.5% of the cells). In parallel, the effects of denbinobin on the mitochondria inner transmembrane potential ($\Delta\Psi_m$) were investigated by using the fluorochrome $DiOC_6(3)$, a cationic probe that distributes passively between media, the cytosol and the mitochondria, where the final distribution of the fluorochrome depends mainly on the transmembrane potential. In Fig. 4A it is shown that compared to control cells (6.22% of the cells), denbinobin-treated cells have a lower $DiOC_6(3)$ fluorescence (32.75% of the cells), which corresponds with a drop in $\Delta\Psi_m$. Pre-treatment with NAC completely abrogated denbinobin-induced ROS production and disruption of the $\Delta\Psi_m$. Interestingly, pre-incubation with CsA, a potent inhibitor of the mitochondrial permeability transition pore (PTP) [39], completely inhibited the disruption of the $\Delta\Psi_m$ induced by denbinobin and partially inhibited ROS generation (50% of the cells). Accordingly, CsA did not prevent denbinobin-induced MAPKs activation. These results suggest that denbinobin can generate ROS from both mitochondrial and non-mitochondrial sources.

An immediate consequence of the $\Delta\Psi_m$ breakdown can be the release into the cytosol of pro-apoptotic factors (cytochrome *c* and AIF). Cytosolic cytochrome *c* next binds to and activates Apaf-1/caspase-9, triggering the activation of the effector caspases that cleaved poly(ADP-ribose) polymerase 1 (PARP-1) and initiating the apoptotic process [22]. To study the activation of the caspases pathway, we thus incubated cells with denbinobin for 6 h, a time at which the $\Delta\Psi_m$ dissipation was evident (Fig. 4A), and then evaluated the activation of caspases-3, -7 and -8 and the processing of PARP by Western blots. Fig. 4B shows that the treatment of Jurkat cells with denbinobin led to the processing and activation of these caspases, and that the processing of PARP was completely inhibited by pre-incubation with NAC.

Necrotic cell death has long been considered an accidental and uncontrolled mode of cell death, but evidence has been mounting that this process is a molecularly regulated event that is induced by ROS and disruption of the $\Delta\Psi_m$ [40]. To further discriminate whether denbinobin-induced cell death by necrosis or apoptosis, we treated cells with increasing concentrations of denbinobin for 12 h, and the cultures were then split for PI uptake and cell cycle analysis studies. Loss of plasma membrane integrity allows the uptake of PI, while intact plasma membranes exclude it. Therefore, PI uptake is a live/dead assay and does not distinguish between apoptotic and necrotic cell death. Conversely, cell cycle analysis makes it possible to assess the percentage of subdiploid cells, an indirect marker of apoptosis. As depicted in Fig. 5, denbinobin induced an increase in the percentage of death cells measured by PI uptake (Fig. 5, upper panel) that paralleled with the percentage of subdiploid cells (chromatinolysis) (Fig. 5, lower panel). These results clearly demonstrate that denbinobin induces apoptosis by a caspase-dependent pathway that is activated by an increase in the intracellular levels of ROS.

3.4. Denbinobin-induced apoptosis through an MAPK-independent pathway

Several lines of evidence suggest that prolonged JNK and p38 activation promotes apoptosis via the mitochondria-dependent pathway [10,11]. Therefore, using specific MAPKs chemical inhibitors, we investigated the role of JNK, p38 and ERK on denbinobin-induced apoptotic events. To this aim, Jurkat cells were pre-incubated for 30 min with PD98059, SP600125 and SB203085, inhibitors of MEK, JNK and p38, respectively. These inhibitors were used at the concentrations that specifically inhibited denbinobin-induced ERK, JNK and p38 phosphorylation in Jurkat cells (data not shown). We found that none of the inhibitors tested could prevent denbinobin-induced $\Delta\Psi_m$ dissipation (Fig. 6A), activation of caspases-3, -7 and -8 as well as PARP cleavage (Fig. 6B) in Jurkat cells. Furthermore, the inhibition of ERK and JNK could enhance denbinobin-induced $\Delta\Psi_m$ disruption and processing of caspases-3 and -8, an observation that suggests a

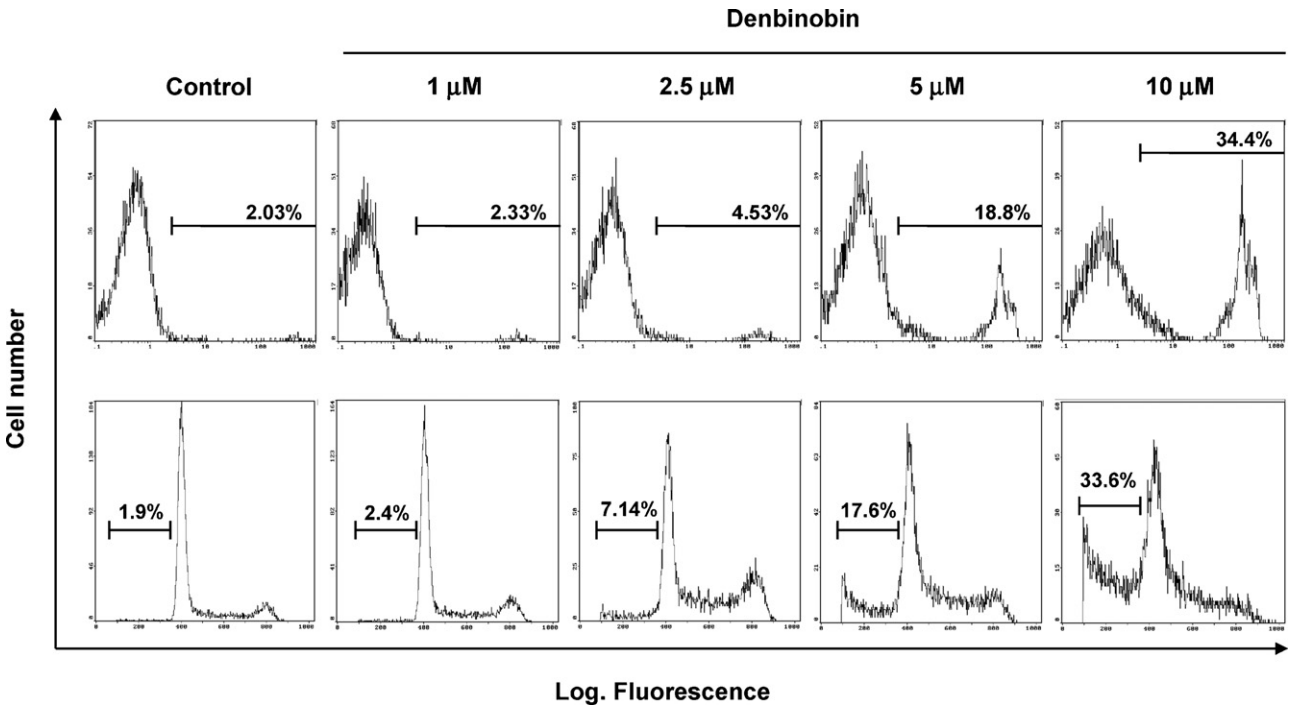

Fig. 5. Denbinobin induces cytotoxicity by apoptosis. Jurkat cells were treated with increasing concentrations of denbinobin for 12 h and half of the cells were analyzed by PI uptake (upper panel) and cell cycle analysis (lower panel) using flow cytometry. Results are representative of three different experiments.

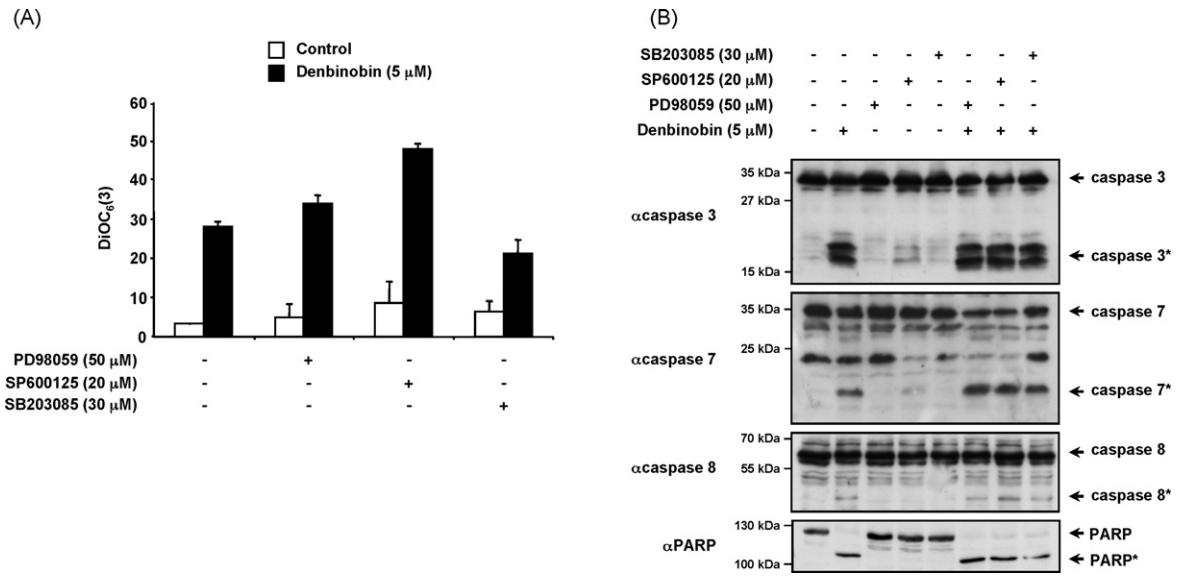

Fig. 6. Denbinobin induces $\Delta\Psi_m$ disruption and caspases activation by MAPK-independent pathways. (A) Jurkat cells were pre-incubated during 15 min with the indicated concentrations of the MAPKs inhibitors PD98059, SP600125 and SB203085 (ERK, JNK and p38 inhibitors, respectively), then treated with denbinobin (5 μM) for 3 h and the $\Delta\Psi_m$ analyzed by flow cytometry. The results are represented as the percentage of $DiOC_6(3)$ positive cells. (B) Jurkat T cells were pre-incubated with the indicated chemical MAPK inhibitors, and then treated with denbinobin (5 μM) for 12 h. The steady state of PARP, caspases-3, -7 and -8 in total cell extracts were analyzed by Western blot.

protective role for ERK and JNK on the apoptotic pathway activated by denbinobin.

3.5. Identification of the essential functional group in the phenanthrene ring of denbinobin

In order to identify the pharmacophore responsible for biological activities of denbinobin a series of analogues with different substituents on the phenanthrene ring were analyzed (Supplementary Table I). The IC50 anti-NF-κB activity of the compounds was analyzed using the 5.1 cell line and ROS generation was measured in Jurkat cell by flow cytometry. Compound **1**, a phenanthrenequinone having no substituents at the carbons analyzed, retains the biological activities of denbinobin (**2**) although some loss of anti-NF-κB activity was observed, removal of the OH group at C-3 (**3**) did not significantly affect the biological activities of denbinobin. In contrast, hydroxylation at C-8 in compound **3** (**18**) results in a complete loss of activity. Single methoxylation at C-6 (**5**), C-7 (**4**) or C-8 (**6**) showed similar activity to denbinobin (**2**) and enhanced the activity of compound **1**, which was further increased when double methoxylation was introduced in these carbons (**7**–**9**). In general, we observed that methoxylation at C-6, C-7 and C-8 enhanced the

biological activities of 1,4-phenanthrenequinones. Interestingly, methoxylation at C-3 is detrimental for the biological activities of this series since compound **10** that is compared to compound **4** lost the anti-NF-κB and pro-oxidant activities. While the detrimental activity of a single methoxylation at C-3 may be overcome by methoxylation at C-7 (**2** and **3**), single methoxylation at C-2 (**19** and **20**) and double methoxylation at C-3 and C-5 (**11**–**17**) and at C-2 and C-3 (**21**) are not active even in the presence of methoxy groups at positions C-6, C-7 and C-8. Next, to analyse the role of the quinone structures, we made substitutions at C-1 and C-4 preserving functional methoxy groups at C-7 and C-8. Reductive methylation at C-1 and C4 converted compounds **4** and **7** into the inactive compounds **22** and **23**. In contrast, inclusion of two acetoxy groups at C-1 and C-2 led to the active compound **24** that retains the same biological activities as compound **7** (Supplementary Table II). To further study if compound **24** is also endowed with the pro-apoptotic activities described above for denbinobin, we treated Jurkat cells with compounds **22**–**24** and found that only compound **24** was able to induce the activation of caspases-3 and -7 and the processing of PARP (Supplementary Fig. 1AS). Moreover, compound **24** also induced ROS production and disruption of the $\Delta\Psi_m$ (Supplementary Fig. 1BS).

4. Discussion

The use of plant extracts to alleviate human pathologies is centuries old, and continues nowadays. Thus, natural product research has paved the way to develop single agent drugs for the treatment of many diseases. In this context, both anti-oxidant and pro-oxidant natural products are of special interest for the development of new anticancer agents. ROS are a class of radical or non-radical oxygen-containing molecules that display high reactivity with lipids, proteins and nucleic acids, and it has been demonstrated that cancer cells produce high levels of ROS and are constantly under oxidative stress [41]. Thus, elevated rates of H_2O_2 generation have been detected in seven human cancer cell lines, and elevated levels of O_2^- in primary blood samples from patients with different types of leukemia have been reported [42]. Furthermore, elevated oxidative modifications in DNA, proteins, and lipids have been detected in various primary cancer tissues, further suggesting that cancer cells are inherently under oxidative stress due to an elevate rate of proliferation [43].

Recently, it has been suggested that the increase in ROS during antineoplastic therapy can induce the expression of anti-oxidants like SOD, but that the overproduction of ROS can, in turn, exhaust the capacity of SOD and other adaptive anti-oxidant defences, eventually inducing cell death. A "threshold concept" for cancer therapy has been proposed to explain the dual effects of oxygen radicals [43]. In cancer cells, if ROS levels reach the "threshold level" that overwhelms the anti-oxidant capacity, irreversible damage occurs and apoptosis is initiated. In general, tumour cells are very sensitive to pro-oxidant compounds like phenolic flavonoids and other inhibitory quinone analogues [37,38,44], and we have demonstrated that denbinobin and related phenanthrenequinones induce severe oxidative damage in leukemic cells.

The mechanisms by which denbinobin induces ROS is unknown, but we show that its quinone moiety is critical for this activity, and the activity of the diacetylated quinol **23** could be due to an easy saponification and oxidation to the corresponding 1,4-phenanthrenenequinone (compound **7**). In general, quinones can trigger a host of hazardous effects in vivo, including acute cytotoxicity, immunotoxicity, and carcinogenesis. The mechanisms by which quinones cause these effects can be quite complex. Thus, quinones are Michael acceptors, and cellular damage can occur through alkylation of crucial cellular proteins and/or DNA. Alternatively, quinones are highly redox active molecules which can redox cycle with their semiquinone radicals, leading to formation of reactive oxygen species, including superoxide, hydrogen peroxide, and ultimately the hydroxyl radical. Another interesting possibility is that denbinobin may function as an inhibitory quinone analogue that interferes with CoQ-dependent cellular redox systems at the mitochondria and at the plasma membrane. We suggest that the interference of denbinobin with the CoQ binding site of these systems could lead to a redirection of the normal electron flow in the complex, generating an excess of ROS. Although our data suggest that denbinobin induces ROS from both mitochondrial and extra-mitochondrial sources, we believe that the main target of denbinobin is the mitochondria respiratory chain. Since denbinobin-induced apoptosis is independent of JNK and p38 activation, it is likely that the pro-oxidative environment induces a direct oxidation of thiol groups in mitochondrial PT pores allowing its opening and, as a consequence, the free distribution of solutes at both sides of the inner mitochondrial membrane, triggering the apoptotic pathway [45]. This assumption is supported by the results obtained with the glutathione precursor NAC, which can prevent ROS generation, $\Delta\Psi_m$ dissipation and the activation of executor caspases. The role of PT in denbinobin-induced apoptosis was further confirmed by the observation that pre-incubation with CsA prevents denbinobin-induced $\Delta\Psi_m$ disruption. The mechanism by which denbinobin induces $\Delta\Psi_m$ dissipation is not limited to leukemic cells, since it has been shown that denbinobin also affects the functionality of the mitochondria and induces apoptosis in lung and colon cancer cell lines [7–9].

We have also demonstrated that denbinobin inhibits the activation of NF-κB, a key transcription factor that regulates the expression of anti-apoptotic and anti-oxidant genes such as SOD [46]. Therefore, the denbinobin-induced ROS increase is not balanced by NF-κB-regulated anti-oxidant cellular defences. SAR analyses with the denbinobin analogues indicate that the NF-κB inhibitory activity of denbinobin is mediated by its quinone ring and the inhibition of NF-κB is probably the result of ROS accumulation. Although mild oxidative stress is required for NF-κB activation, it has also been demonstrated that, under certain circumstances, a strong pro-oxidative environment may inhibit NF-κB signalling [23,24]. Kinase over-expression experiments suggest that TAK1 may be the major target for denbinobin in the inhibition of the NF-κB pathway. Although TAK1 is critically involved in stress-activated cell signalling [47,48] a redox regulation for the activity of this kinase has not been described yet. However, Omori et al. have shown that genetic ablation of TAK1-induced ROS accumulation in keratinocytes and promoted skin inflammation [49]. Interestingly, occasional exposure to field-grown orchids, as well as occupational contact with sawdust of phenanthrenequinones-containing tropical timbers, caused allergic contact dermatitis. In addition, animal models have demonstrated that phenanthrenequinones are potential allergens [50]. It will be therefore interesting to investigate whether phenathrenequinones induces skin inflammation by inhibiting TAK1 or by an immune mechanism that involves skin mast cells sensitization.

In conclusion, capitalizing on a series of mechanistic observation, we have elucidated the mechanism by which denbinobin, a constituent of the Chinese herb Shi-Hu and of some varieties of *Cannabis*, induces ROS and inhibits NF-κB in leukemic cells, highlighting the potential of this compound for the treatment of leukemias, identifying its structure–activity relationships, and providing a solid background for the further clinical developments of this and related phenanthrenequinones of plant origin.

Acknowledgments

This work was supported by the Junta de Andalucía Grant P06-CTS-01353, by the ISCIII-RETIC RD06/006 and by the MEyC grant

SAF2007-60305. The authors wish to thank Dr. M.L. Schmitz for providing plasmid reagents and to Carmen Cabrero-Doncel for her assistance with the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.01.004.

References

[1] Kovacs A, Vasas A, Hohmann J. Natural phenanthrenes and their biological activity. Phytochemistry 2008;69:1084–110.
[2] Lee YH, Park JD, Baek NI, Kim SI, Ahn BZ. In vitro and in vivo antitumoral phenanthrenes from the aerial parts of *Dendrobium nobile*. Planta Med 1995;61:178–80.
[3] Lin TH, Chang SJ, Chen CC, Wang JP, Tsao LT. Two phenanthraquinones from *Dendrobium moniliforme*. J Nat Prod 2001;64:1084–6.
[4] Chen HY, Shiao MS, Huang YL, Shen CC, Lin YL, Kuo YH, et al. Antioxidant principles from *Ephemerantha lonchophylla*. J Nat Prod 1999;62:1225–7.
[5] Sanchez-Duffhues G, Calzado MA, de Vinuesa AG, Caballero FJ, Ech-Chahad A, Appendino G, et al. Denbinobin, a naturally occurring 1,4-phenanthrenequinone, inhibits HIV-1 replication through an NF-kappaB-dependent pathway. Biochem Pharmacol 2008.
[6] Krohn K, Loock U, Paavilainen K, Hausen B, Schmalle HW, Kiesele H. Synthesis and electrochemistry of annoquinone-A, cypripedin methyl ether, denbinobin and related 1,4-phenanthrenequinones. ARKIVOC 2001;973–1003.
[7] Yang KC, Uen YH, Suk FM, Liang YC, Wang YJ, Ho YS, et al. Molecular mechanisms of denbinobin-induced anti-tumorigenesis effect in colon cancer cells. World J Gastroenterol 2005;11:3040–5.
[8] Kuo CT, Hsu MJ, Chen BC, Chen CC, Teng CM, Pan SL, et al. Denbinobin induces apoptosis in human lung adenocarcinoma cells via Akt inactivation, Bad activation, and mitochondrial dysfunction. Toxicol Lett 2008;177:48–58.
[9] Chen TH, Pan SL, Guh JH, Chen CC, Huang YT, Pai HC, et al. Denbinobin induces apoptosis by apoptosis-inducing factor releasing and DNA damage in human colorectal cancer HCT-116 cells. Naunyn Schmiedebergs Arch Pharmacol 2008;378:447–57.
[10] Nakano H, Nakajima A, Sakon-Komazawa S, Piao JH, Xue X, Okumura K. Reactive oxygen species mediate crosstalk between NF-kappaB and JNK. Cell Death Differ 2006;13:730–7.
[11] Temkin V, Karin M. From death receptor to reactive oxygen species and c-Jun N-terminal protein kinase: the receptor-interacting protein 1 odyssey. Immunol Rev 2007;220:8–21.
[12] Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell 2008;132:344–62.
[13] Hacker H, Karin M. Regulation and function of IKK and IKK-related kinases. Sci STKE 2006;2006:re13.
[14] Ninomiya-Tsuji J, Kishimoto K, Hiyama A, Inoue J, Cao Z, Matsumoto K. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. Nature 1999;398:252–6.
[15] Sato S, Sanjo H, Takeda K, Ninomiya-Tsuji J, Yamamoto M, Kawai T, et al. Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol 2005;6:1087–95.
[16] Chen ZJ, Bhoj V, Seth RB, Ubiquitin. TAK1 and IKK: is there a connection? Cell Death Differ 2006;13:687–92.
[17] Ninomiya-Tsuji J, Kajino T, Ono K, Ohtomo T, Matsumoto M, Shiina M, et al. A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase. J Biol Chem 2003;278:18485–90.
[18] Bartosz G. Reactive oxygen species: destroyers or messengers? Biochem Pharmacol)2008;(November 24).
[19] Gloire G, Legrand-Poels S, Piette J. NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 2006;72:1493–505.
[20] Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, et al. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. J Exp Med 1995;181:1661–72.
[21] Szabo I, Bathori G, Wolff D, Starc T, Cola C, Zoratti M. The high-conductance channel of porin-less yeast mitochondria. Biochim Biophys Acta 1995;1235:115–25.
[22] Crompton M, Barksby E, Johnson N, Capano M. Mitochondrial intermembrane junctional complexes and their involvement in cell death. Biochimie 2002;84:143–52.
[23] Korn SH, Wouters EF, Vos N, Janssen-Heininger YM. Cytokine-induced activation of nuclear factor-kappa B is inhibited by hydrogen peroxide through oxidative inactivation of I kappaB kinase. J Biol Chem 2001;276:35693–700.
[24] Moodie FM, Marwick JA, Anderson CS, Szulakowski P, Biswas SK, Bauter MR, et al. Oxidative stress and cigarette smoke alter chromatin remodeling but differentially regulate NF-kappaB activation and proinflammatory cytokine release in alveolar epithelial cells. FASEB J 2004;18:1897–9.
[25] Ichijo H. From receptors to stress-activated MAP kinases. Oncogene 1999;18:6087–93.
[26] Hatai T, Matsuzawa A, Inoshita S, Mochida Y, Kuroda T, Sakamaki K, et al. Execution of apoptosis signal-regulating kinase 1 (ASK1)-induced apoptosis by the mitochondria-dependent caspase activation. J Biol Chem 2000;275:26576–81.
[27] Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, et al. Requirement of JNK for stress-induced activation of the cytochrome *c*-mediated death pathway. Science 2000;288:870–4.
[28] Assefa Z, Vantieghem A, Garmyn M, Declercq W, Vandenabeele P, Vandenheede JR, et al. p38 mitogen-activated protein kinase regulates a novel, caspase-independent pathway for the mitochondrial cytochrome *c* release in ultraviolet B radiation-induced apoptosis. J Biol Chem 2000;275:21416–21.
[29] Hu R, Kong AN. Activation of MAP kinases, apoptosis and nutrigenomics of gene expression elicited by dietary cancer-prevention compounds. Nutrition 2004;20:83–8.
[30] Muller I, Niethammer D, Bruchelt G. Anthracycline-derived chemotherapeutics in apoptosis and free radical cytotoxicity (review). Int J Mol Med 1998;1:491–4.
[31] Sebald A, Mattioli I, Schmitz ML. T cell receptor-induced lipid raft recruitment of the I kappa B kinase complex is necessary and sufficient for NF-kappa B activation occurring in the cytosol. Eur J Immunol 2005;35:318–25.
[32] Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol 2000;18:621–63.
[33] Takaesu G, Surabhi RM, Park KJ, Ninomiya-Tsuji J, Matsumoto K, Gaynor RB. TAK1 is critical for I kappaB kinase-mediated activation of the NF-kappaB pathway. J Mol Biol 2003;326:105–15.
[34] Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, Ueno N, et al. TAB1: an activator of the TAK1 MAPKKK in TGF-beta signal transduction. Science 1996;272:1179–82.
[35] Gillissen A, Scharling B, Jaworska M, Bartling A, Rasche K, Schultze-Werninghaus G. Oxidant scavenger function of ambroxol in vitro: a comparison with N-acetylcysteine. Res Exp Med (Berl) 1997;196:389–98.
[36] Nowak D, Antczak A, Krol M, Bialasiewicz P, Pietras T. Antioxidant properties of ambroxol. Free Radic Biol Med 1994;16:517–22.
[37] Macho A, Blazquez MV, Navas P, Munoz E. Induction of apoptosis by vanilloid compounds does not require de novo gene transcription and activator protein 1 activity. Cell Growth Differ 1998;9:277–86.
[38] Macho A, Sancho R, Minassi A, Appendino G, Lawen A, Munoz E. Involvement of reactive oxygen species in capsaicinoid-induced apoptosis in transformed cells. Free Radic Res 2003;37:611–9.
[39] Crompton M, McGuinness O, Nazareth W. The involvement of cyclosporin A binding proteins in regulating and uncoupling mitochondrial energy transduction. Biochim Biophys Acta 1992;1101:214–7.
[40] Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, et al. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 2005;434:652–8.
[41] Szatrowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res 1991;51:794–8.
[42] Devi GS, Prasad MH, Saraswathi I, Raghu D, Rao DN, Reddy PP. Free radicals antioxidant enzymes and lipid peroxidation in different types of leukemias. Clin Chim Acta 2000;293:53–62.
[43] Kong Q, Beel JA, Lillehei KO. A threshold concept for cancer therapy. Med Hypotheses 2000;55:29–35.
[44] Galati G, O'Brien PJ. Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties. Free Radic Biol Med 2004;37:287–303.
[45] Costantini P, Chernyak BV, Petronilli V, Bernardi P. Modulation of the mitochondrial permeability transition pore by pyridine nucleotides and dithiol oxidation at two separate sites. J Biol Chem 1996;271:6746–51.
[46] Papa S, Bubici C, Zazzeroni F, Pham CG, Kuntzen C, Knabb JR, et al. The NF-kappaB-mediated control of the JNK cascade in the antagonism of programmed cell death in health and disease. Cell Death Differ 2006;13:712–29.
[47] Chen W, White MA, Cobb MH. Stimulus-specific requirements for MAP3 kinases in activating the JNK pathway. J Biol Chem 2002;277:49105–10.
[48] Huangfu WC, Omori E, Akira S, Matsumoto K, Ninomiya-Tsuji J. Osmotic stress activates the TAK1–JNK pathway while blocking TAK1-mediated NF-kappaB activation: TAO2 regulates TAK1 pathways. J Biol Chem 2006;281:28802–10.
[49] Omori E, Morioka S, Matsumoto K, Ninomiya-Tsuji J. TAK1 regulates reactive oxygen species and cell death in keratinocytes, which is essential for skin integrity. J Biol Chem 2008;283:26161–8.
[50] Hausen BM, Elsasser B, Krohn K, Loock U. Structure–activity relationships in allergic contact dermatitis. III. The sensitizing capacity of substituted phenanthrenequinones: a quantum-mechanical approach. Am J Contact Dermat 2003;14:82–9.