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Research Paper

Ingenol esters induce apoptosis in Jurkat cells through an AP-1 and NF-κB independent pathway

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Abstract

Background: Ingenol derivatives have received constant and multidisciplinary attention on account of their pleiotropic pattern of biological activity. This includes activation of protein kinase C (PKC), tumour-promotion, anticancer, and anti-HIV properties, and the possibility of dissecting co-cancerogenic and clinically useful activities has been demonstrated. Certain ingenol esters show powerful anticancer activity, and a structure–activity relationship model to discriminate between their apoptotic and non-apoptotic properties has been developed.

Results: The polyhydroxylated southern region of ingenol was selectively modified, using the anticancer and PKC activator ingenol 3,20-dibenzoate (IDB) as a lead compound. The evaluation of IDB analogues in apoptosis assays showed strict structure–activity relationships, benzoylation of the 20-hydroxyl being required to trigger apoptosis through a pathway involving caspase-3 and occurring at the specific cell cycle checkpoint that controls the S–M phase transition. Conversely, a study on the activation of the PKC-dependent transcription factors AP-1 and NF-κB by IDB analogues showed significant molecular flexibility, including tolerance to changes at the 3- and 20-hydroxyls. IDB-induced apoptosis was independent of activation of PKC, since it was not affected by treatment with the non-isoform-selective PKC inhibitor GF 109230X0.

Conclusions: Remarkable deviations from the tumour-promotion pharmacophore were observed for both the apoptotic and the PKC-activating properties of IDB analogues, showing that ingenol is a viable template to selectively target crucial pathways involved in tumour promotion and development. Since the apoptotic and the PKC-activating properties of ingenoids are mediated by different pathways and governed by distinct structure–activity relationships, it is possible to dissect them by suitable chemical modification. In this context, the esterification pattern of the 5- and 20-hydroxyls is critical.

Keywords: Ingenol; Protein kinase C; Apoptosis; Nuclear factor κB; Activator protein-1

1. Introduction

The use of spurges (*Euphorbia* spp.) to treat tumours is well documented in Greek and Roman medical literature [1], and modern studies have shown that these plants are still employed to treat cancerous conditions in traditional medicine in many areas of the world [1]. These historical and ethnobotanical clues have been backed up by the discovery of the powerful antineoplastic activity of ingenol 3,20-dibenzoate (IDB) [2], and certain semi-synthetic ingenoids are among the most powerful cytotoxic agents known, with an IC_{50} in the sub-nanomolar range [3]. Tumour promotion [4], skin irritancy [5,6], protein kinase C (PKC) activation [7,8], VCAM-1 inhibition [9], nerve growth factor promotion [10], as well as pro-inflammatory [4,11], molluscicide [12], and antiviral activities [13] have also been documented for ingenoids, testifying to the relevance of this class of diterpenoids for biomedical research. A pleiotropic pattern of activity is not uncommon for tumour promoters. Nevertheless, a clear separation between co-cancerogenic and clinically useful activities has been observed for ingenoids [13,14], validating the parent

Abbreviations: AIM, activation immediate marker; AP-1, activator protein-1; EMSA, electrophoretic mobility shift assay; IDB, ingenol 3,20-dibenzoate; NF-κB, nuclear factor κB; PKC, protein kinase C

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polyol as a versatile template for the induction/modulation of bioactivity. Not unexpectedly, ingenol has also received constant attention within the synthetic community [15], although its total synthesis is yet to be achieved.

Ingenol esters can display both co-carcinogenic and antitumour activity [16], and IDB, the archetypal antitumour ingenoid, can indeed activate responses mediated by PKC [7,8,17], the main target of tumour-promoting phorboid esters. However, while IDB shows inflammatory activity comparable to that of phorbol esters, it only retains a marginal fraction of their tumour-promoting activity [7], and similar differences were observed in other pairs of ingenol 3- and 3,20-diesters [7]. The mechanism underlying this paradoxical behaviour is largely unknown, as are the critical structural features responsible for the dissection of the co-carcinogenic and the antitumour activities. These findings underscore our limited knowledge of the ingenoid pharmacophore, while the cell type-dependent role of PKC adds an additional layer of complexity to this issue. Thus, while IDB induces apoptosis in thymocytes through a PKC-independent pathway [17], PKC activation by phorbol esters is crucial to activate the apoptotic machinery in other cell types [18].

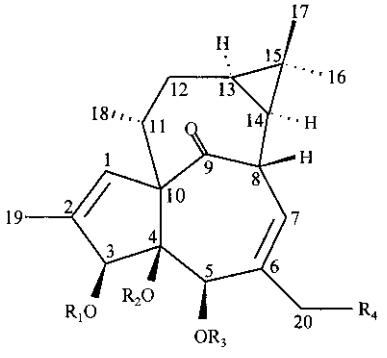
PKC consists of several subfamilies of serine/threonine-specific protein kinases [19], Ca^{2+}-dependent classical PKC (cPKC: α, β_1, β_2 and γ), Ca^{2+}-independent novel PKC (nPKC: δ, ε, η and θ) and atypical PKC (aPKC: χ, ι, ξ), which play a central role in signal transduction in eukaryotes [20–22]. Some of their molecular targets are transcription factors which regulate gene expression, such as the activator protein-1 (AP-1) and the nuclear factor κB (NF-κB), both involved in the regulation of cell proliferation and apoptosis [23,24].

The evaluation of IDB analogues modified at C-20 has shown that cytotoxicity and PKC activation can be dissected [25], and similar results were reported regarding the anti-HIV activity and PKC activation properties of phorbol esters [26]. These findings have rekindled interest in phorboids as drug leads, encouraging mechanistic studies on their anticancer and apoptotic activities. We report here that different pathways, critically dependent on the oxygenation pattern at C-5 and C-20, mediate the apoptotic and PKC-activating properties of IDB.

2. Results

2.1. Ingenol 20-benzoates induce apoptosis in Jurkat cells

The polyhydroxylated southern region plays a key role in the biological activity of ingenoids [25,33,34]. The apoptotic activity of the parent polyol (**1**), IDB (**2**), and analogues variously substituted at C-3, C-5 and C-20 (compounds **3**–**7**, Fig. 1) was investigated in the leukaemia Jurkat cell line. The cells were incubated with 10 μM concentration of each compound for 48 h, and the hypodiploidy (i.e., loss of fragmented DNA) was detected, using propidium iodide (PI) staining as a marker for apoptosis.



Compound	R_1	R_2	R_3	R_4
1	H	H	H	OH
2	Bz	H	H	OBz
3	H	H	H	OBz
4	Bz	H	H	OH
5	H	H	H	F
6	Bz	H	H	F
7	Bz	H	Bz	F
8	- CO -		H	OBz
9	- CS -		H	OBz

Fig. 1. Chemical structures of **1** (ingenol), **2** (ingenol 3,20-dibenzoate), **3** (ingenol 20-benzoate), **4** (ingenol 3-benzoate), **5** (20-deoxy-20-fluoroingenol), **6** (20-deoxy-20-fluoroingenol 3-benzoate), **7** (20-deoxy-20-fluoroingenol 3,5-dibenzoate), **8** (ingenol 20-benzoate 3,4-carbonate); **9** (ingenol 20-benzoate 3,4-thiocarbonate).

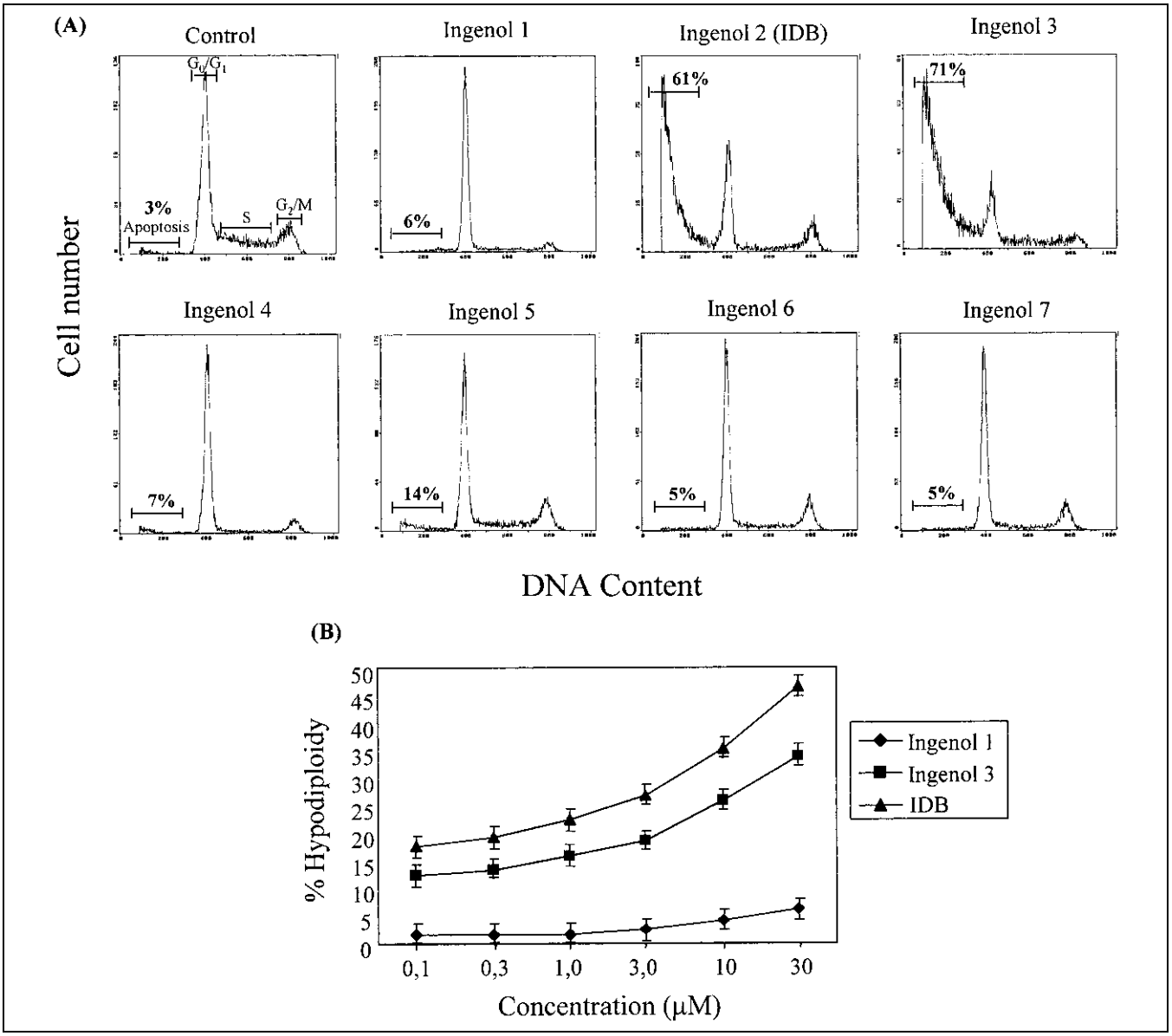


Fig. 2. Induction of apoptosis in Jurkat cells treated with ingenol and derivatives. (A) Jurkat cells were treated with a concentration of 10 μM of the compounds for 48 h, and the cell cycle was analysed by PI staining. Bars indicate the percentage of subdiploid cells (DNA-fragmented cells). (B) Hypodiploidy induced by ingenols is dose-dependent. Jurkat cells were treated with the indicated concentrations of ingenols **1**, **2** (IDB) and **3** for 24 h and the cell cycle was analysed as above. Values are means ± S.D. of three independent experiments.

Fig. 2A shows that only **2** and **3** were able to induce a strong increase in the percentage of hypodiploid cells (61.3% and 71.3% respectively). Within the series investigated, these are the only compounds where the 20-hydroxyl is benzoylated. The apoptosis induced by these two compounds was dose-dependent, and concentrations ranging from 0.1 to 30 μM mediated a progressive increase in the percentage of subdiploid cells after 24 h of culture. In contrast, ingenol itself (**1**) did not induce significant apoptosis even at the higher doses (Fig. 2B). Since apoptosis can also be detected after 24 h of treatment, doses of 10 μM and 24 h treatment were generally used throughout.

2.2. Apoptosis induced by IDB in Jurkat cells is mediated by caspase-3 activation and occurs mainly at the S phase of the cell cycle

The caspase family consists of postaspartate-cleaving cysteine proteases which have been shown to be required for apoptosis in a number of experimental systems [35,36]. To establish the involvement of caspases in IDB-mediated apoptosis of Jurkat cells, we preincubated the cells with the tetrapeptide caspase inhibitors Ac-DEVD-cmk or Z-YVAD-cmk before IDB treatment. Z-YVAD-cmk, which was reported to specifically inhibit caspase-1 and related enzymes, had a limited effect on the increase in the per-

centage of subdiploid cells induced by IDB (10 μM). In contrast, the same dose of Ac-DEVD-cmk, which is more specific for the inhibition of caspase-3, a member of the CED-3 subfamily of caspases, was an effective inhibitor of apoptosis induced by IDB.

The involvement of cell cycle control in apoptosis induced by IDB in Jurkat cells was studied by double staining with PI and FITC-4-deoxy-2-uridine triphosphate (FITC-dUTP) as described in Section 5. This gives information on the cell cycle phase where DNA fragmentation occurs. Fig. 3B shows that most DNA fragmentation was detected in the S phase of the cell cycle in Jurkat cells treated for 24 h with IDB at 10 μM concentration. A small but significant percentage of cells undergoing DNA fragmentation at both G_0/G_1 and M phases of the cell cycle was also detected.

2.3. *Activation of the AP-1 and NF-κB transcription factors by ingenol and its esters*

In order to study the role of ingenoids in the activation of PKC-inducible transcriptions factors, we stimulated Jurkat cells with IDB (10 μM) for 3 h and nuclear extract was obtained and analysed by electrophoretic mobility shift assay (EMSA) using an AP-1-specific ^{32}P-labelled oligonucleotide. In Fig. 4A we show that IDB increased the binding of AP-1 to DNA and that it was completely abolished by a 100-fold concentration of cold AP-1 oligonucleotide in the binding reaction, but not by the presence of irrelevant oligonucleotides (NF-κB and SP-1). Next, we stimulated Jurkat cells either with phorbol myristate acetate (PMA, 50 ng/ml) or with increasing doses of ingenols **1**, IDB, **3**, **5** and **6** for 3 h as indicated, and nuclear extracts were obtained and analysed by EMSA. As shown in Fig. 4B, although to a different extent, all compounds increased the AP-1 binding to DNA. To correlate these results with transcriptional activities, Jurkat and HeLa cells were transiently co-transfected with equimolar concentrations of both RSV-β-Gal and AP-1-Luc plasmids. Twenty-four hours after transfection, cells were treated with PMA or ingenols **1** and IDB for 6 h, and transactivation was determined and expressed as fold induction. Fig. 4C shows that both compounds were effective in the induction of AP-1 transactivation in both cell lines, IDB being more potent than the parent polyol, as expected from the binding data. In order to evaluate whether another inducible transcription factor had also been activated by ingenols, we carried out kinetics experiments in Jurkat cells stimulated with either IDB, ingenol **1** or ingenol **5** (10 μM each), and in Fig. 5A it is shown that all

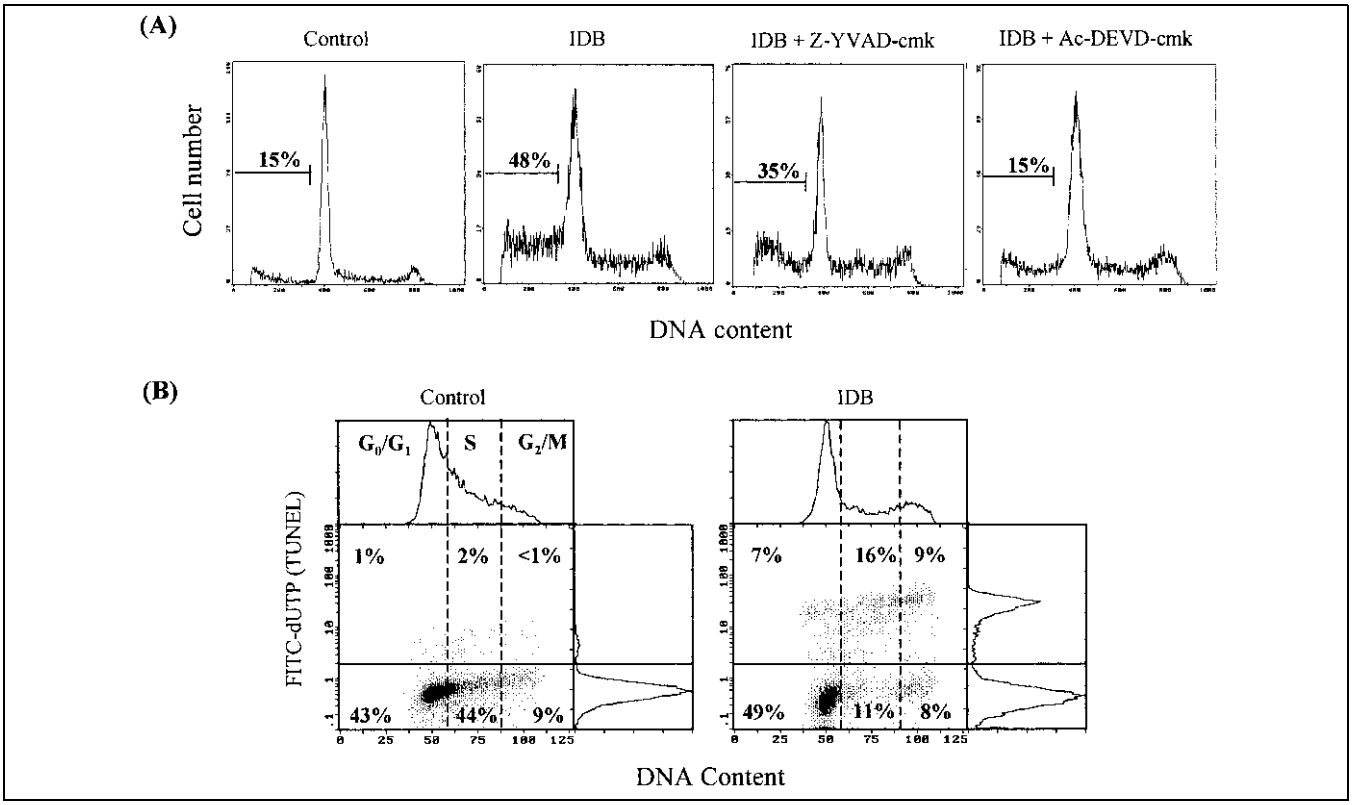


Fig. 3. (A) The caspase-3 inhibitor Ac-DEVD-cmk inhibits IDB-induced apoptosis. Jurkat cells were treated with IDB (10 μM) in the presence or absence of the caspase-1 inhibitor Z-YVAD-cmk (100 μM) and the caspase-3 inhibitor Ac-DEVD-cmk (100 μM) for 24 h, and the percentage of subdiploid cells was detected using flow cytometry. (B) S–M phase dependence for apoptosis induced by IDB. Jurkat cells were stimulated with IDB (10 μM) for 24 h and the cell cycle and the DNA strand breaks analysed by the TUNEL method using flow cytometry. Results are representative of three independent experiments.

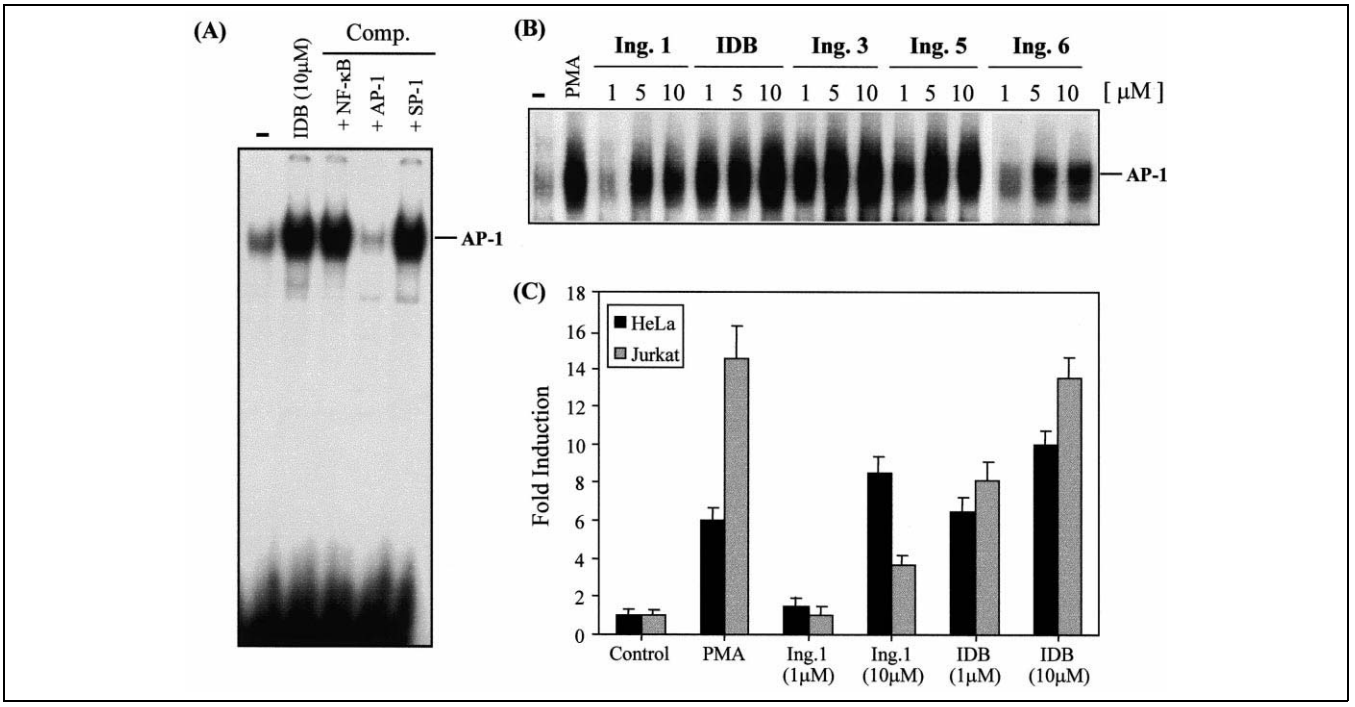


Fig. 4. Activation of AP-1 by ingenols. (A) Nuclear extracts were prepared from Jurkat cells treated with IDB (10 μM) for 3 h and the binding of AP-1 to DNA was performed with a ^{32}P end-labelled AP-1-specific double-stranded oligonucleotide in the presence or absence of a 50-fold excess of cold AP-1, NF-κB and SP-1 oligonucleotides. (B) Jurkat cells were stimulated with phorbol myristate acetate (PMA, 50 ng/ml) and the indicated concentrations of ingenols **1**, **2** (IDB), **3**, **5** and **6** for 3 h, and the binding of AP-1 was done as described above. The result for ingenol **6** is a shorter exposure of a retardation gel from a separate experiment. (C) Induction of AP-1 transcriptional activity by ingenols. Jurkat and HeLa cells were transiently co-transfected with equimolar concentrations of the plasmids RSV-βGal and AP-1-Luc, and 24 h later stimulated with different concentrations of IDB and ingenol **1** or with PMA (50 ng/ml). After 6 h stimulation, the cells were lysed and the luciferase activity measured and represented as fold induction after normalisation with the measure of β-Gal activity. Values are means ± S.D. of three independent experiments in each cell line.

ingenols tested induced an increase in the binding of NF-κB in nuclear extracts from stimulated Jurkat cells. The specificity of the binding was studied by supershift and cold competition experiments, and we identified that the heterodimer p50/p65 is the main complex activated by ingenols in Jurkat cells. The NF-κB binding results correlate well with the transactivation experiments performed in HeLa cells, where we found that IDB was more potent than either ingenol **1** or **5** to transactivate the KBF-Luc reporter plasmid (Fig. 5B). Altogether, these results show that both pro-apoptotic and non-apoptotic ingenoids can activate the AP-1 and the NF-κB transcription factors.

2.4. PKC is not involved in the apoptotic activity of IDB

IDB can activate several isoforms of PKC in both lymphoid and non-lymphoid cells [8,17–18]. Thus, to study whether this enzyme is involved in NF-κB activation and apoptosis, we transfected HeLa cells with the KBF-Luc plasmid. Twenty-four hours after transfection, the cells were stimulated for 6 h with PMA (10 ng/ml), ingenol (**1**) (10 μM) or IDB (10 μM) in the presence or absence of GF109203X (0.01 μM), a non-isoform-selective PKC inhibitor [37]. GF109203X completely inhibited NF-κB transactivation induced by PMA, ingenol and IDB (Fig. 6A). Similarly, this inhibitor prevented the binding of NF-κB to DNA in Jurkat cells stimulated with IDB (10 μM) for 90 min (Fig. 6B).

Next, we studied if the inhibition of PKC by GF109203X could affect the apoptosis induced by IDB in Jurkat cells. Fig. 6C shows that apoptosis induced by IDB was not mediated by PKC activation, since the PKC inhibitor did not reduce the percentage of hypodiploid cells observed after 18 h stimulation with a pro-apoptotic concentration of IDB; rather, it increased the apoptosis induced by IDB.

2.5. Activation of NF-κB and AP-1 by ingenoids requires a free hydroxyl at C-5

In our set of ingenoids, modifications of the 20-hydroxyl do not affect the capacity to activate NF-κB and AP-1 transcription factors. On the other hand, the apoptotic activity depends on the modification of this position, since only **2** (IDB) and **3** are pro-apoptotic. To investigate the role of the 5-hydroxyl on the transcriptional activities of ingenoids, the non-apoptotic compound **7**, where the 5-hydroxyl is esterified with benzoic acid and the 20-hydroxyl is replaced by a fluorine to prevent acylotropic migration from the 5- to the 20-hydroxyl, was investigated. Thus, Jurkat cells were stimulated with either PMA or increasing doses of **7** for 3 h, and nuclear extracts studied for DNA

binding of NF-κB and AP-1. Fig. 7A shows that, unlike its 5-debenzoyl derivative, **7** does not activate NF-κB binding and it is a very weak activator of AP-1. To compare the lack of binding of NF-κB and AP-1 with the transcriptional activity, the effect of the selected compounds was investigated in the K562-AIM cell line, which contains the luciferase gene driven by the activation immediate marker (AIM/CD69) gene promoter stably transfected [38]. This promoter is highly responsive to NF-κB and AP-1 transcription factors [38]. Thus, K562-AIM cells were stimulated with either PMA (10 ng/ml), ingenol **3**, IDB (**2**) or ingenol **7** (10 μM) for 6 h, and we measured the luciferase activity as described above. As expected, IDB and **3** were as effective as PMA to transactivate the AIM promoter, while **7** was practically inactive in this assay. Taken together, these results suggest that the 5-hydroxyl is critical for PKC activation by IDB and its analogues.

2.6. Hydroxylation at C-3 and C-4 is not required for apoptosis and NF-κB activation

The esterification of the 3-hydroxyl with lipophilic acids is essential for tumour promotion by ingenol esters. To evaluate the role of the hydroxyl groups at C-3 and C-4 in the apoptotic activity, the cyclic carbonate **8** and thiocarbonate **9** were synthesised (Fig. 1). Jurkat cells were treated with these compounds (10 μM) for 24 h and the induction of apoptosis determined by flow cytometry. Fig. 8A shows that both ingenols induce apoptosis in Jurkat cells to a similar extent, as measured by the percentage of hypodiploid cells (33.6 vs. 37.8%, respectively). Moreover, when NF-κB activation was evaluated in transiently transfected HeLa cells, both compounds were equally potent to activate the NF-κB reported gene (Fig. 8B). These results reinforce the hypothesis that benzoylation at position C-20 is required for ingenol-induced apoptosis.

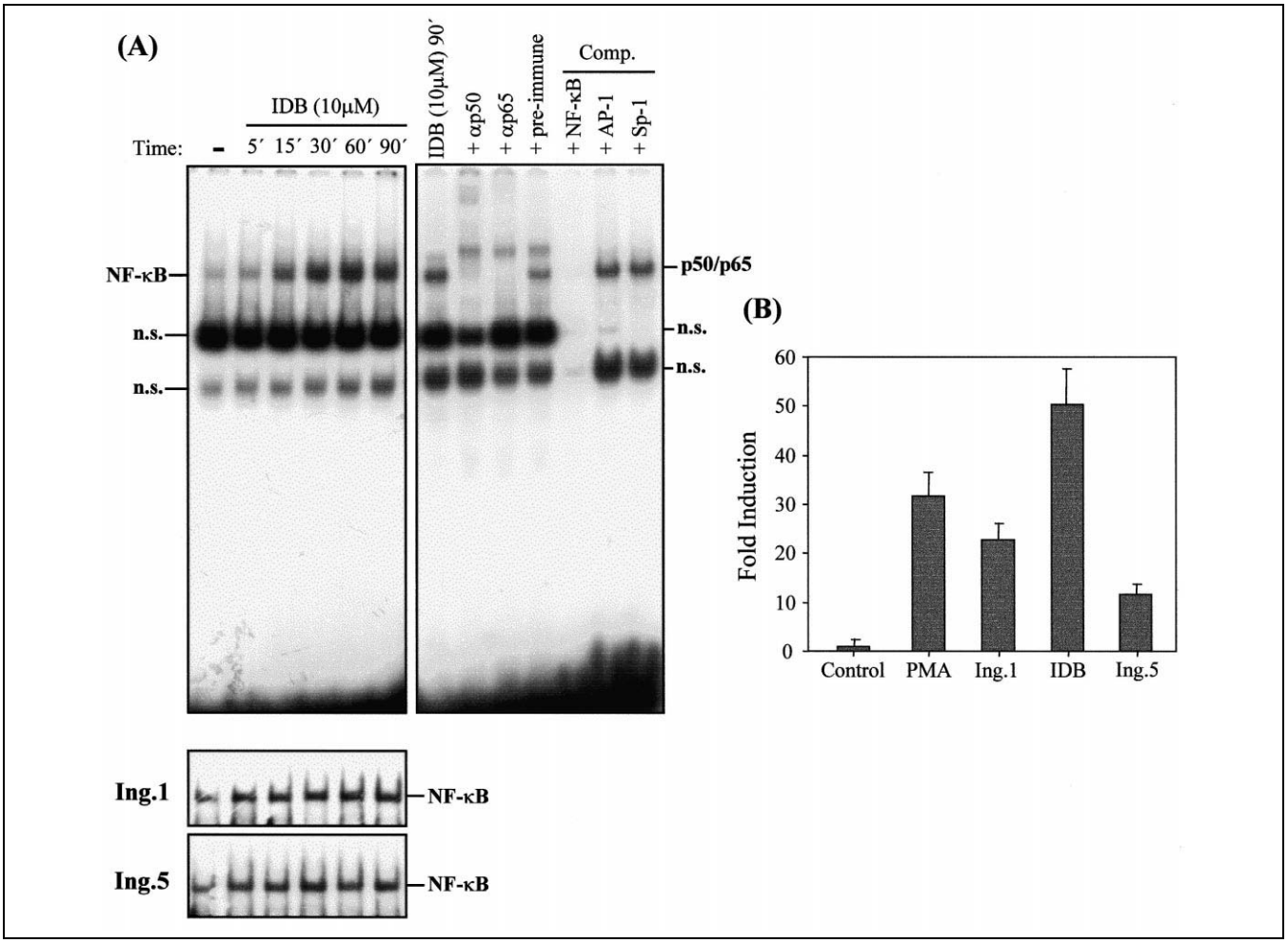


Fig. 5. Induction of NF-κB by ingenols. (A) Jurkat cells were treated with IDB (**2**), ingenol **1** and **5** (10 μM) for the indicated times, and the binding of NF-κB to DNA in nuclear extract was analysed by EMSA. The binding specificity of NF-κB was studied by supershift with specific antiserum and cold competition. (B) HeLa cells were transiently co-transfected with equimolar concentrations of the plasmids RSV-βGal and KBF-Luc and 24 h later stimulated with IDB, ingenol **1** and **5** (10 μM) or with PMA (50 ng/ml). After 6 h stimulation, the cells were lysed and the transcriptional activity represented as indicated in Fig. 4.

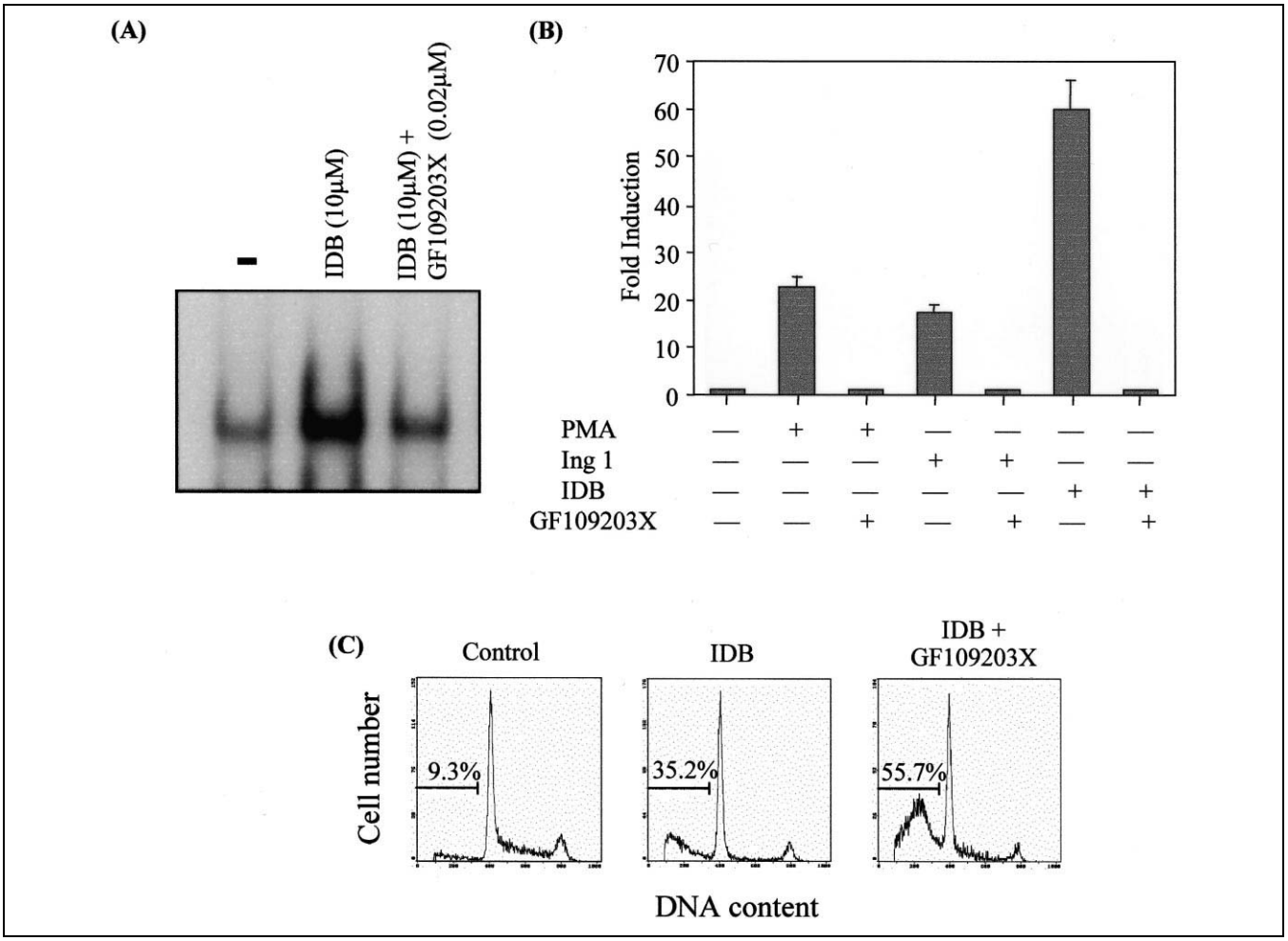


Fig. 6. The PKC inhibitor GF109203X prevents NF-κB from binding to DNA induced by IDB and the transcriptional activity of NF-κB induced by ingenol (**1**) and IDB (**2**). (A) Jurkat cells were stimulated with 10 μM of IDB for 90 min in the presence or absence of the PKC inhibitor (a section of the EMSA gel is shown). (B) HeLa cells were transiently transfected with the KBF-luc plasmid, and 24 h later stimulated for 6 h with PMA (50 ng/ml), **1** (10 μM) or **2** (10 μM) in the presence or absence of the PKC inhibitor (0.02 μM). (C) GF109203X does not affect IDB-induced apoptosis. Jurkat cells were treated with IDB (10 μM) with or without the PKC inhibitor (0.02 μM) and cell cycle analyses performed by PI staining and flow cytometry.

3. Discussion

Despite the variety of biological end-points of ingenoids, clear structure–activity relationships have emerged only as regards tumour promotion, which requires the presence of a lipophilic ester group at C-3 and a free hydroxyl at C-20 [11]. A binding model based on a three-fold interaction with PKC mediated by the 3-ester carbonyl, the 9-keto group, and the 20-hydroxyl has emerged [39,40]. However, the biological activity of the parent polyol and that of several 3,20-diesters suggests that alternative fits to PKC exist [4], while large differences in binding between simplified analogues and the natural derivatives [41] underscore the relevance of structural elements different from those highlighted by the postulated pharmacophore for PKC interaction.

Within the set of compounds investigated, only those bearing a benzoate at C-20 (compounds **2**, **3**, **8**, **9**) could induce apoptosis in Jurkat cells. The lack of activity of ingenol 3-benzoate (**4**) showed that the induction of apoptosis by IDB could not be mediated by enzymatic loss of the 20-ester group, which would generate an inactive compound. Moreover, the activity of IDB, ingenol 20-benzoate (**3**), ingenol 3,4-carbonate 20-benzoate (**8**), and ingenol 3,4-thiocarbonate 20-benzoate (**9**) shows that the induction of apoptosis is relatively insensitive to changes at C-3, a substantial and dramatic deviation from the tumour-promotion pharmacophore.

Deviation from the tumour-promotion pharmacophore was also evidenced as regards PKC-mediated NF-κB and AP-1 activation. Although we have not measured directly the translocation and activation of nPKC, the activation of both transcription factors through nPKC has been documented. Thus, in the AP-1 binding assays the 20-deoxy-20-fluoro derivatives **5** and **6** showed an activity comparable to that of their corresponding hydroxylated compounds (**1** and **4**, respectively), while the 20-esters **2** and **3** were active, as were **8** and **9**, where the 3-hydroxyl was

part of a carbonate (**8**) or a thiocarbonate ring (**9**). Interestingly, benzoylation of the 5-hydroxyl of the 20-deoxy-20-fluoro derivative **7** led to a dramatic decrease of activity, suggesting that the 5-hydroxyl is critical for PKC binding underlying both AP-1 and NF-κB activation.

The affinity of IDB for PKC was quantified by inhibition of [^{3}H]PDBU binding to PKC-α, which only showed a modest activity [41]. To explain the exceedingly high inflammatory activity of this compound, in vivo enzymatic hydrolysis to the corresponding 3-monobenzoate, a highly irritant activator of cPKC, was suggested [41]. However, the lack of evidence for the in vivo hydrolysis of the 20-ester group, the different profile of tumour-promoting activity of IDB and ingenol-3-benzoate, as well as recent data from the study of calcium-independent induction of apoptosis in thymocytes [17], suggested that IDB targets those PKC isoforms different from cPKC isoforms. Thus, IDB was identified as a selective activator of PKC-ε, although further studies have demonstrated that IDB can interact with almost all isoforms of novel PKC [17]. Since these nPKCs do not require calcium for activation, the observation that neither IDB nor ingenol 3-benzoate mobilises calcium intracellularly (data not shown) further supports the view that non-classical PKCs mediate the activation of NF-κB and AP-1 transcription factors by these compounds.

Apoptosis induced by ingenoid 20-benzoate depends on the activation of caspase-3, and occurs mainly at the S phase of the cell cycle. The discovery of different structure–activity relationships for apoptotic and PKC-mediated responses suggests that the two processes are mechanistically distinct. In accordance with this, the PKC inhibitor GF109203X does not reduce hypodiploidy induced by IDB; rather, it increased the apoptosis induced by IDB, showing that PKC activation may be involved in the anti-apoptotic activity. This is consistent with the fact that PKC activation in Jurkat cells makes them resistant to apoptosis induced by the death receptor FAS [42]. Thus, depending on the cell type, non-apoptotic PKC-binding ingenoids can behave as tumour promoters in at least two ways: either by activating a set of PKC-dependent genes involved in cellular growth and differentiation, or, alternatively, by rendering the cells resistant to apoptosis mediated by the death receptor Fas/APO-1 and by NF-κB-dependent anti-apoptotic genes [43–45].

Phorboid-containing plants have been used to treat tumours for centuries [1], and PMA, the archetypal tumour-promoting phorboid, has been clinically investigated as an anticancer agent [46]. It is therefore ironic that most studies on these essential probes for cell biology have been focused instead on their skin-irritant and tumour-promoting activities. The discovery that the apoptotic and the PKC-activating properties of IDB are mediated by different pathways and governed by distinct structure–activity relationships should foster investigations aimed at a better mechanistic understanding of the anticancer potential of phorboids. In this context, the identification of the molecular targets underlying their antitumour activity and the dissection of anticancer and PKC-mediated activities by suitable structural modifications will be essential to obtain

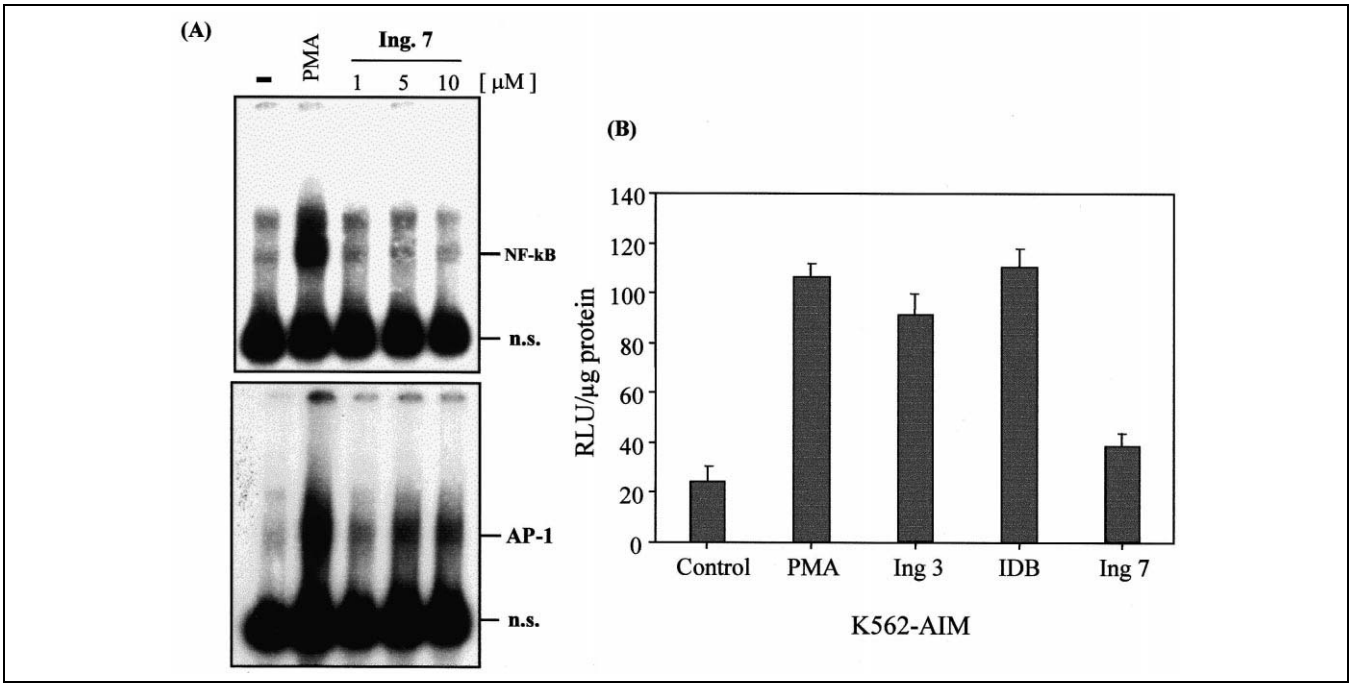


Fig. 7. (A) 20-Deoxy-20-fluoroingenol-3,5-dibenzoate (**7**) does not activate NF-κB or AP-1 binding to DNA in Jurkat cells. (B) Ingenol (**1**) and IDB (**2**), but not **7**, stimulate AIM gene transcription in K562 cells.

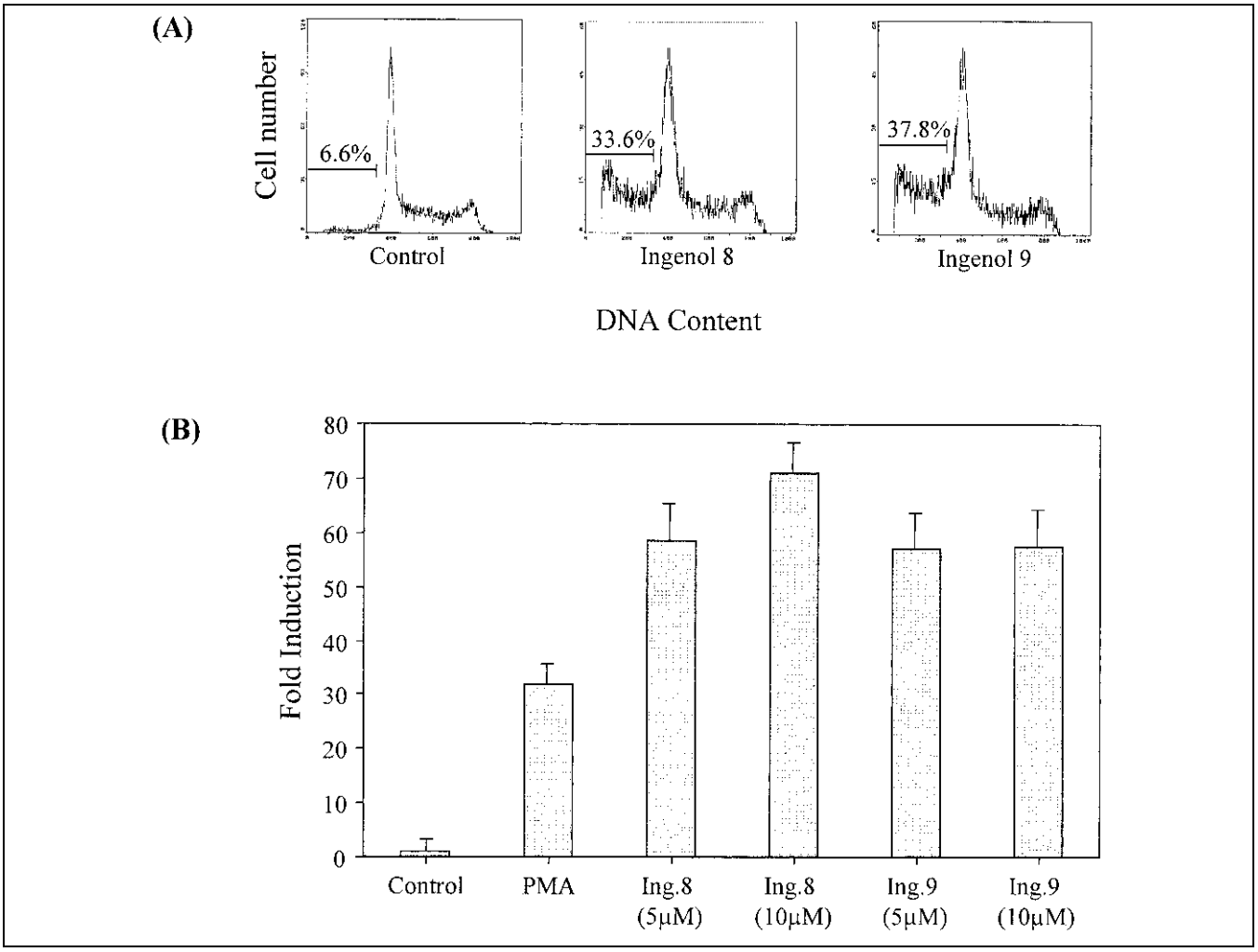


Fig. 8. Carbonatation and thiocarbonatation of ingenol 20-benzoate do not affect its biological activities. (A) Jurkat cells were treated with a concentration of 10 μM of the compounds for 24 h, and the cell cycle was analysed by PI staining. Bars indicate the percentage of subdiploid cells (DNA-fragmented cells). (B) HeLa cells were transiently transfected with an NF-κB-dependent luciferase reporter plasmid, and 24 h later stimulated with ingenols **1**, **8** and **9** at the indicated concentration. After 6 h stimulation, the cells were lysed and the luciferase activity measured. The transcriptional activity was expressed as fold induction. Values are means ± S.D. of two independent experiments.

non-pleiotropic drug candidates amenable to pharmaceutical development.

4. Significance

Ingenoids are a structurally diverse class of diterpenes endowed with a pleiotropic pattern of biological activity and paradoxical tumour-promoting and anticancer properties. The polyhydroxylated southern region has a key role in the biological activity of these compounds, and we have developed a structure–activity relationship model to evaluate the apoptotic and the non-apoptotic properties of ingenoids. The induction of apoptosis and the activation of the PKC-dependent transcription factors NF-κB and AP-1 turned out to be mediated by different signal pathways. Esterification of the 20-hydroxyl was required to induce apoptosis, while a free 5-hydroxyl was critical for PKC activation, evidencing significant deviations from the tumour-promotion pharmacophore for both activities. The finding that the apoptotic and the PKC-activating properties of IDB are mediated by different pathways and governed by distinct structure–activity relationships validates ingenol as a lead structure to develop new anticancer drugs.

5. Materials and methods

5.1. Cell lines and reagents

Jurkat cells (ATCC, Rockville, MD, USA) were maintained in exponential growth in RPMI 840 medium (Bio-Whittaker, Verviers, Belgium) and the cervix cancer HeLa cells (ATCC) in Dulbecco's modified Eagle's medium (Bio-Whittaker). The culture media were supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine and the antibiotics penicillin and strep-

tomycin (Gibco, Paisley, UK). K562-AIM cells were maintained in complete RPMI medium containing G418 (100 μg/ml). [γ-^{32}P]ATP (3000 Ci/mM) was purchased from ICN (Costa Mesa, CA, USA). The cell-permeable inhibitors of caspase-1 (Z-YVAD-cmk) and caspase-3 (Ac-DEVD-cmk) were from Bachem (Switzerland). FITC-dUTP and terminal deoxynucleotide transferase (TdT) were from Boehringer Mannheim (Germany). The PKC inhibitor GF109203X was from Biomol (Plymouth Meeting, PA, USA). All other reagents not cited above or later were from Sigma Chemical Co. (Barcelona, Spain). Ingenol was obtained from the seeds of the caper spurge [27].

5.2. Synthesis of ingenoids

Compounds **2**–**7** are known, and were prepared according to literature procedures [25,28]. Compounds **8** and **9** were synthesised as follows (synthesis of **8** as representative): they were added to a cooled (0°C) solution of ingenol 5,20-acetonide [28] (100 mg, 1.26 mM) in dry CH_2Cl_2 (10 ml), pyridine (5 ml) and excess phosgene (1.0 ml of a 20% toluene solution, 10 mM, 38 mol. equiv.). After being stirred at 0°C for 30 min the reaction was worked up by dilution with hexane–ether (3:1, 100 ml) and sequential washing with water and brine. Removal of the solvent gave the crude 3,4-carbonate 5,20-acetonide, which was taken up in 10 ml of 10^{-3} M methanolic $HClO_4$. After being stirred for 45 min at room temperature, the reaction was worked up by the addition of sat. aq. $NaHCO_3$, concentration at reduced pressure and partition between water and EtOAc. The organic phase was washed with brine, dried ($MgSO_4$) and evaporated, to give ingenol 3,4-carbonate as a colourless foam. For the selective benzoylation, the latter was dissolved in dry CH_2Cl_2 (1 ml), and the cooled (−18°C) solution was treated with *N*-3-dimethylaminopropyl-*N*′-ethylcarbodiimide hydrochloride (EDC, 74 mg, 0.39 mM), 4-dimethylaminopyridine (DMAP, 47 mg, 0.39 mM), and benzoic acid (47 mg, 0.39 mM). The solution was stirred at −18°C overnight and worked up by dilution with EtOAc and sequential washing with 5% NaOH, water, and brine. After removing the solvent, the crude product was purified by column chromatography on silica gel (hexane–EtOAc 9:1 as eluant) to give 66 mg (53% from ingenol 5,20-acetonide) **8** as a colourless amorphous foam. $[\alpha]_D^{25}$ −35° (CH_2Cl_2, *c* 0.8); IR (ν_{max}, liquid film) cm^{-1}: 3474, 1833, 1715, 1603, 1453, 1385, 1282, 1123, 1030; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.00 (Bz-AA′), 7.58 (Bz-C), 7.44 (Bz-BB′), 6.29 (d, *J* = 4.8 Hz, H-7), 6.07 (s, H-1), 5.21 (s, H-3), 4.90 (d, *J* = 12.6 Hz, H-20a), 4.70 (d, *J* = 12.8 Hz, H-20b), 3.74 (m, H-8), 3.48 (s, H-5), 2.33 (m, H-11), 2.12 (m, H-12a), 1.91 (s, H-19), 1.85 (m, H-12b), 1.13 (s, H-16), 1.06 (s, H-17), 0.96 (d, J = 6.9 Hz, H-18), 0.87 (m, H-14), 0.74 (m, H-13). HRMS: 478.5342 (calc. for $C_{28}H_{30}O_7$: 478.5336). For the synthesis of **9**, thiocarbonyldiimidazole was substituted for phosgene, carrying out the thiocarbonylation in refluxing toluene for 90 min. The overall yield from ingenol 5,20-acetonide was 34%. **9** was obtained as a colourless oil, $[\alpha]_D^{25}$ −28° (CH_2Cl_2, *c* 0.6); IR (ν_{max}, liquid film) cm^{-1}: 3474, 1809, 1725, 1285, 1262, 1111, 1030, 655; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (Bz-AA′), 7.60 (Bz-C), 7.46 (Bz-BB′), 6.31 (d, *J* = 4.1 Hz, H-7), 6.12 (s, H-1), 5.48 (s, H-3), 5.03 (d, *J* = 12.8 Hz, H-20a), 4.76 (d, *J* = 12.8 Hz, H-20b), 4.05 (s, H-5), 3.72 (m, H-8), 2.34 (m, H-11), 2.12 (m, H-12a), 1.94 (s, H-19), 1.87 (m, H-12b), 1.18 (s, H-16), 1.09 (s, H-17), 1.00 (d, *J* = 6.8 Hz, H-18), 0.79 (m, H-13 and H-14). HRMS: 494.597 (calc. for $C_{28}H_{30}O_6S$: 494.6002).

5.3. Determination of nuclear DNA loss and cell cycle analysis

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 phosphate-buffered saline (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 room temperature. The cell cycle was analysed by cytofluorimetry as previously described [29]. With this method, low molecular weight DNA leaks from the ethanol-fixed apoptotic cells and the subsequent staining make it possible to determine the percentage of subdiploid cells (sub-G_0/G_1 fraction).

5.4. Detection of DNA strand breaks by the TUNEL method

The percentage of apoptotic cells was alternatively measured by the TUNEL method as previously described, with minor modifications. Briefly, cells (1×10^6) were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C, washed twice in PBS and permeabilised in 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min. Fixed cells were washed three times in PBS and resuspended in a final volume of 50 μl of TUNEL buffer (0.3 nmol FITC-dUTP, 3 nmol dATP, 50 nmol $CoCl_2$, 5 U TdT, 200 mM potassium cacodylate, 250 μg/ml bovine serum albumin (BSA) and 25 mM Tris–HCl pH 6.6). The cells were incubated for 1 h at 37°C and then washed twice in PBS and analysed by flow cytometry. To determine both the DNA strand breaks and the cell cycle, TUNEL-stained cells were counterstained with PI and treated with RNase as described above prior to cytofluorimetric analysis. In this method, fixation in formaldehyde prevents the leaking of low molecular weight DNA from apoptotic cells, and thus the cell cycle distribution estimates both apoptotic and non-apoptotic cells [30].

5.5. Isolation of nuclear extracts and mobility shift assays

Jurkat cells were cultured at 2×10^6/ml and stimulated with the agonists in complete medium as indicated. Cells were then washed twice with cold PBS and proteins from nuclear extracts isolated as previously described [31]. Protein concentration was determined by the Bradford method (Bio-Rad, Richmond, CA, USA). For the EMSA assay, the consensus oligonucleotide probes: NF-κB, 5′-AGTTGAGGGGACTTTCCCAGG-3′; AP-1, 5′-CGCTTGATGAGTCAGCCGGAA-3′, were labelled with [γ-^{32}P]ATP (Amersham, Freiburg Germany). The binding reaction mixture contained 3 μg of nuclear extract, 0.5 μg poly-(dI-dC) (Pharmacia Fine Chemical, Piscataway, NJ, USA), 20 mM HEPES pH 7, 70 mM NaCl, 2 mM dithiothreitol (DTT), 0.01% NP-40, 100 μg/ml BSA, 4% Ficoll, and 100 000 cpm of end-labelled DNA fragments in a total volume of 20 μl. When indicated, 0.5 μl of rabbit anti-p50, anti-p65 or preimmune serum [32] was added to the standard reaction before the addition of the radiolabelled probe. Dr. Alain Israël (Institut Pasteur, France) kindly provided the anti-NF-κB sera. For cold competition, a 100-fold excess of the double-stranded oligonucleotide competitor was added to the binding reaction. After 30 min incubation at 4°C, the mixture was electrophoresed through a native 6% polyacrylamide gel containing 89 mM Tris–borate, 89 mM boric acid and 1 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 XAR film at −80°C.

5.6. Transient transfections and luciferase activity

HeLa (10^5/ml) or Jurkat cells (2×10^6/ml) were transiently transfected using either the lipofectamine reagent (Life Technologies, Madrid, Spain) or the Superfect reagent (Qiagen, Germany) respectively, according to the manufacturer's recommendations. To determine AP-1-dependent transcription, Jurkat or HeLa cells were transfected with the AP-1-Luc reporter plasmid (0.5 μg/ml) which was constructed by inserting three copies of an SV40 AP-1 binding site into the *Xho* site of pGL-2 promoter vector (Promega, Madison, WI, USA), and to determine NF-κB-dependent transcription, HeLa cells were transfected with the KBF-Luc (0.5 μg/ml) reporter plasmid which contains three copies of an NF-κB binding site (from the MHC promoter) fused to a minimal SV40 promoter driving luciferase [32]. Twenty-four hours after transfection the cells were stimulated with the indicated agonists for 6 h and then lysed in 25 mM Tris–phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured in a luminometer (Lumat Berthold, USA) following the instructions of the luciferase assay kit (Promega). The background obtained with the lysis buffer was subtracted in each experimental value. All the experiments were repeated at least three times. Transfection efficiencies were normalised by co-transfection with the β-galactosidase expression plasmid RSV-β-galactosidase, and β-galactosidase levels were determined using a commercial kit (Promega).

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References

[1] J.L. Hartwell, Plants used against cancer. A survey, Lloydia 32 (1969) 153–205.
[2] S.M. Kupchan, I. Uchida, A.Q.R. Branfman, R.G.Jr. Dailey, B.Y. Fei, Antileukemic principles isolated from *Euphorbiaceae* plants, Science 191 (1976) 571–572.
[3] D. Kamimura, K. Yamada, K. Tokuhisa, H. Watanabe, Preparation of ingenol diterpenoids as antitumours. Jpn. Kokai Tokyo Koho JP 08,245,505, 1996, Chem. Abstr. 126 (1997) 60180.
[4] B. Sorg, R. Schmidt, E. Hecker, Structure/activity relationships of polyfunctional diterpenes of the ingenane type. I Tumour promoting activity of homologous, aliphatic 3-esters of ingenol and of delta 7,8-isoingenol-3-tetradecanoate, Carcinogenesis 8 (1987) 1–4.
[5] G. Fürstenberger, E. Hecker, On the active principles of the spurge family (Euphorbiaceae). XI. [1] The skin irritant and tumour promoting diterpene esters of Euphorbia tirucalli L. originating from South Africa, Z. Naturforsch. [C] Biosci. 40 (1985) 631–646.
[6] E.H. Seip, E. Hecker, Skin irritant ingenol esters from Euphorbia esula, Planta Med. 46 (1982) 215–218.
[7] C.M. Hasler, G. Acs, P.M. Blumberg, Specific binding to protein kinase C by ingenol and its induction of biological responses, Cancer Res. 52 (1992) 202–208.
[8] G. Zhang, M.G. Kazanjetz, P.M. Blumberg, J.H. Hurley, Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester, Cell 81 (1995) 917–924.
[9] D. Kamimura, K. Yamada, Preparation of 13-hydroxyingenols as VCAM-1 formation inhibitors, Jpn. Kokai Tokyo Koho JP 07,258,168, 1995, Chem. Abstr. 124 (1996) 87430r.
[10] T. Tsuji, K. Yamaguchi, D. Kamimura, S. Kondo, Nerve growth factor formation promotors containing diterpenes from Euphorbia. Jpn. Kokai Tokyo Koho JP 07,149,633, Chem. Abstr. 123 (1995) 218433y.
[11] F.J. Evans, S.E. Taylor, Pro-inflammatory, tumour-promoting and anti-tumour diterpenes of the plant families Euphorbiaceae and Thymelaeaceae, Fortschr. Chem. Org. Naturst. 44 (1983) 1–99.
[12] C.L. Zani, A. Marston, M. Hamburger, K. Hostettmann, Molluscicidal milliamines from Euphorbia milii var. Hislopii, Phytochemistry 34 (1993) 89–95.
[13] M. Fujiwara, K. Ijichi, K. Tokuhisa, K. Katsuura, S. Shigeta, K. Konno, G.Y. Wang, D. Uemura, T. Yokota, M. Baba, Mechanism of selective inhibition of human immunodeficiency virus by ingenol triacetate, Antimicrob. Agents Chemother. 40 (1996) 271–273.
[14] A. Marston, E. Hecker, On the active principles of the Euphorbiaceae VI: Isolation and biological activities of seven milliamines from Euphorbia milii, Planta Med. 47 (1983) 141–147.
[15] S. Kim, J.D. Winkler, Approaches to the synthesis of ingenol, Chem. Soc. Rev. 26 (1997) 387–399.
[16] H. Itokawa, Y. Ichihara, K. Watanabe, K. Takeya, An antitumour principle from Euphorbia lathyris, Planta Med. 55 (1989) 271–272.
[17] A. Asada, Y. Zhao, S. Kondo, M. Iwata, Induction of thymocyte apoptosis by Ca2+-independent protein kinase C (nPKC) activation and its regulation by calcineurin activation, J. Biol. Chem. 273 (1998) 28392–28398.
[18] S.G. Weller, I.K. Klein, R.C. Penington, W.E. Karnes Jr., Distinct protein kinase C isozymes signal mitogenesis and apoptosis in human colon cancer cells, Gastroenterology 117 (1999) 848–857.
[19] Y. Nishizuka, Protein kinase C and lipid signaling for sustained cellular responses, FASEB J. 9 (1995) 484–496.
[20] W.J. Ryves, A.T. Evans, A.R. Olivier, P.J. Parker, F.J. Evans, Activation of the PKC-isotypes alpha, beta 1, gamma, delta and epsilon by phorbol esters of different biological activities, FEBS Lett. 288 (1991) 5–9.
[21] G. Werlen, E. Jacinto, Y. Xia, M. Karin, Calcineurin preferentially synergizes with PKC-theta to activate JNK and IL-2 promoter in T lymphocytes, EMBO J. 17 (1998) 3101–3111.
[22] E.M. Genot, P.J. Parker, D.A. Cantrell, Analysis of the role of protein kinase C-alpha, -epsilon and -zeta in T cell activation, J. Biol. Chem. 270 (1995) 9833–9839.
[23] F. Mercurio, A.M. Manning, Multiple signals converging on NF-κB, Curr. Opin. Cell Biol. 11 (1999) 226–232.
[24] M. Karin, Z. Liu, E. Zandi, AP-1 function and regulation, Curr. Opin. Cell Biol. 9 (1997) 240–246.
[25] G. Appendino, G.C. Tron, G. Cravotto, G. Palmisano, R. Annunziata, G. Baj, N. Surico, Synthesis of modified ingenol esters, Eur. J. Org. Chem. 4 (1999) 345–352.
[26] S. El-Mekkawy, M.R. Meselhy, N. Nakamura, M. Hattori, T. Kawahata, T. Otake, 12-*O*-acetylphorbol-13-decanoate potently inhibits cytopathic effects of human immunodeficiency virus type 1 (HIV-1) without activation of protein kinase C, Chem. Pharm. Bull. 47 (1999) 1346–1347.
[27] G. Appendino, G.C. Tron, G. Cravotto, G. Palmisano, J. Jakupovic, An expeditious procedure for the isolation of ingenol from the seeds of Euphorbia lathyris, J. Nat. Prod. 62 (1999) 76–79.
[28] R. Bagavathi, B. Sorg, E. Hecker, Ingenol from seeds of Euphorbia lathyris L. and preparation of (9*R*)(9*S*)-9-deoxo-9-hydroxyingenol with some corresponding 3- and 9-esters, Z. Naturforsch. 46 (1991) 1425–1433.
[29] M.A. Hotz, J. Gong, F. Traganos, Z. Darzynkiewicz, Flow cytometric detection of apoptosis: comparison of the assays of in situ DNA degradation and chromatin changes, Cytometry 7 (1994) 237–244.

[30] A. Macho, C. Lucena, M.A. Calzado, M. Blanco, I. Donnay, G. Appendino, E. Muñoz, Phorboid 20-homovanillates induce apoptosis through a VR1-independent mechanism, Chem. Biol. 7 (2000) 483–492.

[31] A. Macho, M.V. Blázquez, P. Navas, E. Muñoz, Selective induction of apoptosis by vanilloid compounds does not require the novo gene transcription and AP-1, Cell Growth Differ. 6 (1999) 155–165.

[32] M. Kieran, V. Blank, F. Logeat, J. Vanderkerckhove, F. Lottspeich, O. Le Bail, M.B. Urban, P. Kourilsky, P.A. Bauerle, A. Israel, The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the rel oncogene product, Cell 62 (1990) 1007–1018.

[33] H.J. Opferkuch, E. Hecker, On the active principles of the spurge family (Euphorbiaceae). IV. Skin irritant and tumour promoting diterpene esters from Euphorbia ingens E.Mey, J. Cancer Res. Clin. Oncol. 103 (1982) 255–268.

[34] M. Hergenhahn, S. Kusumoto, E. Hecker, On the active principles of the spurge family (Euphorbiaceae). V. Extremely skin-irritant and moderately tumour-promoting diterpene esters from Euphorbia resinifera Berg, J. Cancer Res. Clin. Oncol. 108 (1984) 98–109.

[35] G. Kroemer, B. Dallaporta, M. Rescherigon, The mitochondrial death/life regulator in apoptosis and necrosis, Annu. Rev. Physiol. 98 (1998) 619–642.

[36] N.A. Thornberry, Y. Lazebnik, Caspases: enemies within, Science 281 (1998) 1312–1316.

[37] D. Toullec, P. Pianetti, H. Coste, P. Belleverque, T. Grand-Perret, M. Ajakane, V. Baudet, P. Boissin, E. Boursier, F. Loriolle, The bisindolymaleimide GF 109203X is a potent inhibitor of protein kinase C, J. Biol. Chem. 266 (1991) 15771–15781.

[38] M. López-Cabrera, E. Muñoz, M.V. Blázquez, M.A. Ursa, A.G. Santis, F. Sánchez-Madrid, Transcriptional regulation of the gene encoding the human C-type lectin leukocyte receptor AIM/CD69 and functional characterization of its tumour necrosis factor-alpha-responsive elements, J. Biol. Chem. 270 (1995) 21545–21551.

[39] G. Krauter, C.-W. Von der Lieth, R. Schmidt, E. Hecker, Structure/activity relationships of polyfunctional diterpenes of the tigliane type. A pharmacophore model for protein-kinase-C activators based on structure/activity studies and molecular modelling of the tumour promoters 12-*O*-tetradecanoylphorbol 13-acetate and 3-*O*-tetradecanoylingenol, Eur. J. Biochem. 242 (1996) 417–427.

[40] Y. Kishi, R.R. Rando, Structural basis of protein kinase C activation by tumour promoters, Acc. Chem. Res. 31 (1998) 163–172.

[41] J.D. Winkler, B.-C. Hong, A. Bahador, M.G. Kazanietz, P.M. Blumberg, Synthesis of ingenol analogues with affinity for protein kinase C, Bioorg. Med. Chem. Lett. 3 (1993) 577–580.

[42] T.H. Holmström, S.C. Chow, I. Elo, E.T. Coffey, S. Orrenius, L. Sistonen, J.E. Eriksson, Suppression of Fas/APO-1-mediated apoptosis by mitogen-activated kinase signalling, J. Immunol. 160 (1998) 2626–2636.

[43] Z.-G. Liu, H. Hsu, D.V. Goeddel, M. Karin, Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death, Cell 87 (1996) 565–576.

[44] L.P. Kane, V.S. Shapiro, D. Stokoe, A. Weiss, Induction of NF-κB by the Akt/PKB kinase, Curr. Biol. 9 (1999) 601–604.

[45] Q.L. Deveraux, J.C. Reed, IAP family of proteins-suppressors of apoptosis, Genes Dev. 13 (1999) 239–252.

[46] Z.T. Han, X.X. Zhu, R.Y. Yang, J.Z. Sun, G.F. Tian, X.J. Liu, G.S. Cao, H.L. Newmark, A.H. Conney, R.L. Chang, Effect of intravenous infusion of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in patients with myelocytic leukemia: Preliminary studies on therapeutic efficacy and toxicity, Proc. Natl. Acad. Sci. USA 95 (1998) 5357–5361.