Protein tyrosine kinase inhibitors prevent didemnin B-induced apoptosis in HL-60 cells

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Abstract Didemnin B induces rapid apoptosis in human promyeloid HL-60 cells with an optimal concentration of 1 μM (Grubb et al. (1995) Biochem. Biophys. Res. Commun. 215, 1130–1136), but little is known about how it does so. In order to determine whether protein tyrosine phosphorylation is involved in this rapid induction of apoptosis, HL-60 cells were pre-treated with tyrosine kinase inhibitors for 1 h before didemnin B treatment. Genistein, 2,5-dihydroxycinnamic acid methyl ester, and a range of tyrphostins inhibit didemnin B-induced apoptotic morphology in a concentration-dependent manner. DNA fragmentation induced by didemnin B is also inhibited by genistein, 2.5-dihydroxycinnamic acid methyl ester, and tyrphostins.

Key words: Apoptosis; Didemnin B; Tyrosine phosphorylation; Tyrosine kinase inhibitor

1. Introduction

Apoptosis is a form of physiological cell death which appears to be under highly complicated regulation, similar to other cellular processes such as cell growth and differentiation [1]. Apoptosis can occur through different signalling pathways even in a single cell type. Some inducers of apoptosis may require a period of days before any apoptotic effect is seen while others induce apoptosis within only a few hours [2].

Didemnin B, a branched cyclic peptolide which is currently under clinical trial as an anticancer agent [3,4], has been shown to induce apoptosis in human promyeloid HL-60 cells very rapidly. 100% apoptosis is reached after 2–3 h treatment with 1 μM didemnin B [5]. The mechanism by which didemnin B induces apoptosis is unknown. Studies on the cellular biochemical effects of didemnin B have shown that it is an inhibitor of protein, DNA, and RNA synthesis, and that it can induce growth arrest in any phase of the cell cycle if used at concentrations of 10^{-7} M and above [6]. Immunosuppressive activity has been ascribed to didemnin B similar to that of cyclosporin A [7], and didemnin B has also been shown to suppress phosphorylation of an epidermal cytosolic protein (p100) as does cyclosporin A [8]. However, these effects do not necessarily explain its ability to induce apoptosis since cyclosporin A has been shown not to induce such rapid apoptosis in HL-60 cells (Grubb and Lawen, unpublished). Neither is inhibition of protein synthesis likely to be the mechanism of induction since didemnin B induces apoptosis at a concentration that is much lower than that required to inhibit in vitro protein synthesis [9].

Protein tyrosine phosphorylation is often an early event in signal transduction pathways that are involved in the regulation of cell growth and differentiation [10]. Previously, tyrosine phoshorylation of proteins has been shown to be involved in some types of apoptosis, however, understanding the role of such proteins is complicated by the fact that the use of specific tyrosine kinase inhibitors has been shown to regulate apoptosis both positively and negatively [11,12]. In this study a range of tyrosine kinase inhibitors has been used to investigate the requirement for protein tyrosine phosphorylation in didemnin B-induced apoptosis.

2. Materials and methods

2.1. Materials

Didemnin B was kindly supplied by UpJohn Laboratories, Kalamazoo, MI, USA. Genistein, 2,5-dihydroxycinnamic acid methyl ester, and 123 bp DNA ladder were purchased from Sigma, Sydney, Australia and tyrphostins 1, 25, B42, B44−, B46, B48, B50+, and B56 from Calbiochem, San Diego, CA, USA.

2.2. Cell culture

Human promyeloid HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μg/ml uridine, and 5 mM Hepes (pH 7.4) at 37°C in the presence of 5% CO_2.

2.3. Determination of nuclear morphology

Treatment of HL-60 cells with didemnin B and tyrosine kinase inhibitors was performed at a cell density of about 2×10^5 cells/ml. Didemnin B was dissolved in ethanol, and tyrosine kinase inhibitors were dissolved in DMSO before dilution in medium. At the end of each incubation period, 200 μl aliquots of cells were removed and centrifuged onto glass slides coated with 0.01% (w/v) poly-L-lysine at 2000 rpm for 5 min in a cytospin. Cells attached to the slides were fixed in ethanol/acetic acid (3:1) for 5 min, rinsed in distilled water, and then stained with 1 μg/ml 4′,6-diamidino-2-phenylindole·2HCl for 5 min. Slides were mounted using anti-fading mounting medium (10 mg/ml *p*-phenyldiamine in 90% glycerol, pH 9.0) and nuclear morphology was analyzed under UV light (280 nm) through a Nikon microphot-FX fluorescence microscope. Percentage apoptosis was determined by counting at least 400 nuclei from each slide.

2.4. Analysis of DNA fragmentation

HL-60 cells were treated with didemnin B and tyrosine kinase inhibitors as described above. To extract cytosolic fragmented DNA, 2×10^6 cells were lysed after one wash in ice-cold PBS by resuspending cells in 100 μl lysis buffer (5 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.5% Triton X-100). After 10 min on ice, samples were centrifuged for 15 min at 4°C at $14000\times g$. SDS and RNAse A were added to the supernatant fractions to final concentrations of 1% and 0.2 mg/ml, respectively. After 2 h at 56°C, proteinase K was added to a final concentration of 0.5 mg/ml and incubated at 37°C for 2 h. DNA was precipitated with the addition of 0.1 volume 10 M ammonium acetate and 2 vols. cold ethanol at −20°C. Washes in 70% ethanol and 100% ethanol were followed by resuspension of the DNA in TBE (45 mM Tris-borate, pH 7.4, 1 mM EDTA) plus 7% sucrose. To extract total DNA, 10^6 cells were resuspended in 50 mM Tris-HCl pH 7.4, 10 mM EDTA, and 100 mM NaCl and incubated for 1 h at 50°C in the

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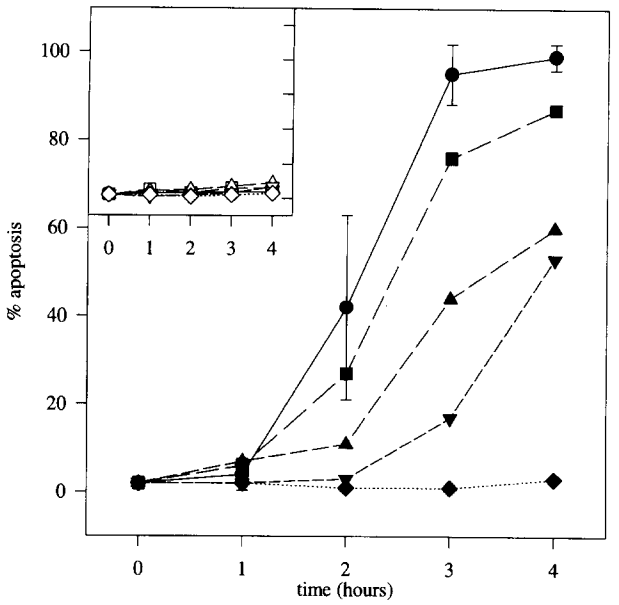

Fig. 1. Inhibition of didemnin B-induced apoptosis by genistein. The percentage apoptosis induced by 1 μM didemnin B after 1 h pretreatment with different genistein concentrations is plotted versus time where 0 hours is the time of addition of didemnin B. The genistein concentrations used were 0 (●,○), 25 (■,□), 50 (▲,△), 75 (▼,▽), and 100 μg/ml (◆,◇). The inset shows the controls (open symbols) without addition of didemnin B; closed symbols represent data from didemnin B-treated incubations. Data represent the mean of at least two experiments. For clarity, the standard deviation is indicated by error bars only for 0 and 100 μg/ml genistein of the didemnin B-treated cells.

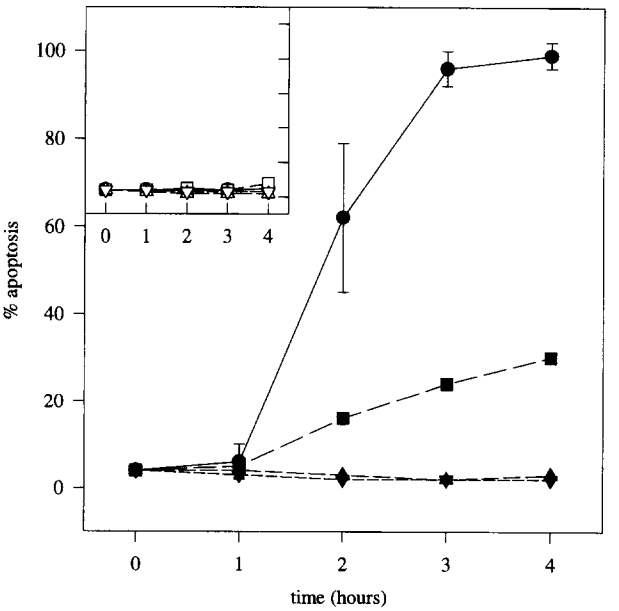

Fig. 2. Inhibition of didemnin B-induced apoptosis by 2,5-dihydroxycinnamic acid methyl ester. Percentage apoptosis induced by 1 μM didemnin B after 1 h pretreatment with different concentrations of 2,5-dihydroxycinnamic acid methyl ester is plotted versus time where 0 hours is the time of addition of didemnin B. The 2,5-dihydroxycinnamic acid methyl ester concentrations used were 0 (●,○), 10 (■,□), 25 (▲,△), and 50 μg/ml (▼,▽). The inset shows the controls (open symbols) without addition of didemnin B; closed symbols represent data from didemnin B-treated incubations. Data represent the mean of at least two experiments. The standard deviation is indicated by error bars for 0 and 50 μg/ml 2,5-dihydroxycinnamic acid methyl ester of the didemnin B-treated cells.

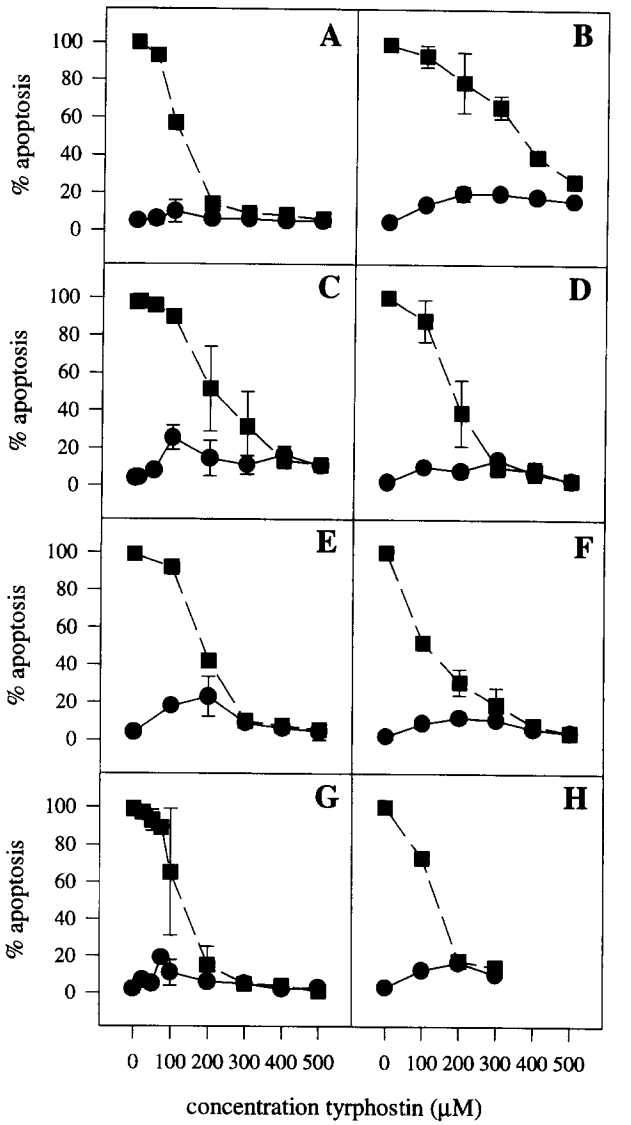

Fig. 3. Inhibition of didemnin B-induced apoptosis by tyrphostins. HL-60 cells were pretreated for 1 h with tyrphostins 1 (A), 25 (B), B42 (C), B44− (D), B46 (E), B48 (F), B50+ (G), and B56 (H), and percentage apoptosis was determined after a further 3 h incubation in the absence (●) or presence (■) of 1 μM didemnin B. The data represent the mean of at least two experiments with error bars showing the standard deviation.

presence of 0.1% SDS and 200 μg/ml proteinase K. After 5 min RNAse treatment at 37°C, the DNA was extracted by standard phenol chloroform procedure and ethanol precipitation. The dry DNA pellet was resuspended in distilled water with addition of 7% sucrose. The DNA was electrophoresed in a 0.8% agarose gel and then visualized with a UV transilluminator after staining the gel in 2 μg/ml ethidium bromide for 10–15 min.

3. Results and discussion

To investigate the possible role of tyrosine phosphorylation in the pathway of induction of apoptosis by didemnin B, HL-60 cells were treated with genistein prior to didemnin B treatment. Genistein is a tyrosine kinase inhibitor which acts as an analogue for the ATP substrate of tyrosine kinases [13]. HL-60 cells were pretreated for 1 h with 25, 50, 75, and 100 μg/ml genistein followed by 1 μM didemnin B, and samples were taken each hour for 4 h to determine morphologically the

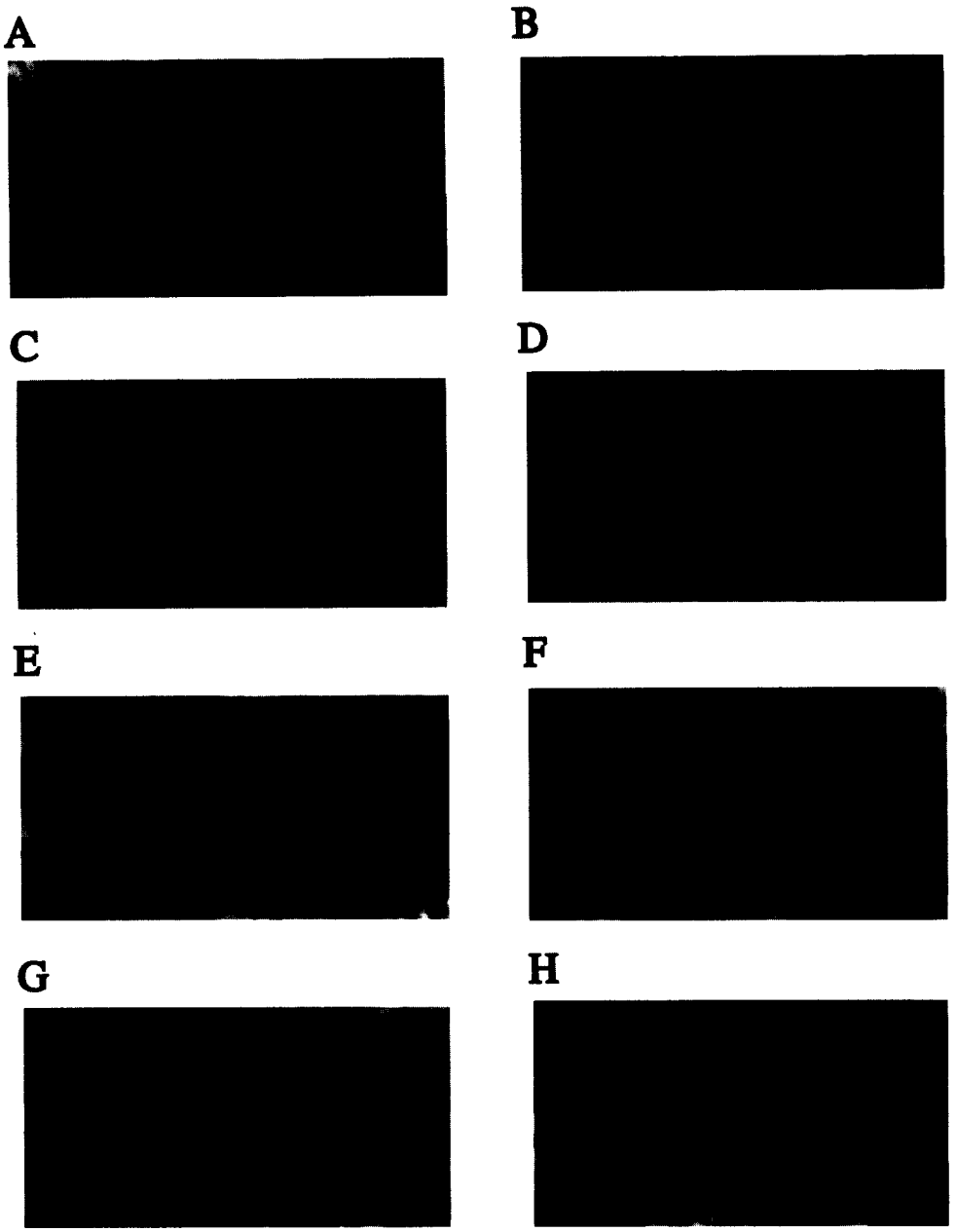

Fig. 4. Effect of tyrosine kinase inhibitors on didemnin B-induced cell morphology. Cells were prepared for fluorescence microscopy as described in section 2. Cells were either treated with the inhibitors alone (left panel) or with inhibitors and didemnin B (right panel). The nuclear morphology of normal (A) and apoptotic (B) HL-60 cells is shown after control treatment (A) and 3 h treatment with 1 μM didemnin B (B). Cells were pretreated with 100 μg/ml genistein (C,D), 25 μg/ml 2,5-dihydroxycinnamic acid methyl ester (E,F), and 300 μM tyrphostin 1 (G,H) for 1 h, and then treated with 1 μM didemnin B (D,F,H) for a further 3 h.

percentage of apoptosis. Genistein clearly inhibits didemnin B-induced apoptosis in a concentration-dependent manner (Fig. 1).

Although genistein is considered to be specific for tyrosine protein kinases [13], it has been reported to have other non-specific effects on cell biochemistry [14]. In order to exclude any non-specific effects of genistein and to confirm the involvement of tyrosine kinases we determined whether didemnin B-induced apoptosis could be inhibited by other tyrosine kinase inhibitors as well. Therefore, we tested 2,5-dihydroxycinnamic acid methyl ester and a range of inhibitors known as tyrphostins, which are more specific for tyrosine kinases than is genistein [15]. 2,5-Dihydroxycinnamic acid methyl ester (Fig. 2) and the tyrphostins (Fig. 3) prevent didemnin B-induced apoptosis in a dose-dependent manner. Examples of the apoptotic morphology of HL-60 cells after 3 h didemnin B treatment with and without pretreatment with the various tyrosine kinase inhibitors are given in Fig. 4. The cells pretreated with the inhibitors exhibit nuclear morphologies similar to control cells and are thus prevented from undergoing apoptosis upon didemnin B treatment. Without inhibitor treatment didemnin B induces apoptosis in all cells under the conditions used in these experiments (Fig. 4B).

As an additional proof for apoptosis, DNA laddering was examined. HL-60 cells treated with didemnin B with or without pretreatment with genistein, 2,5-dihydroxycinnamic acid methyl ester, tyrphostin 1, and tyrphostin B50+ were lysed, and their DNA was subjected to analysis by agarose gel elec-

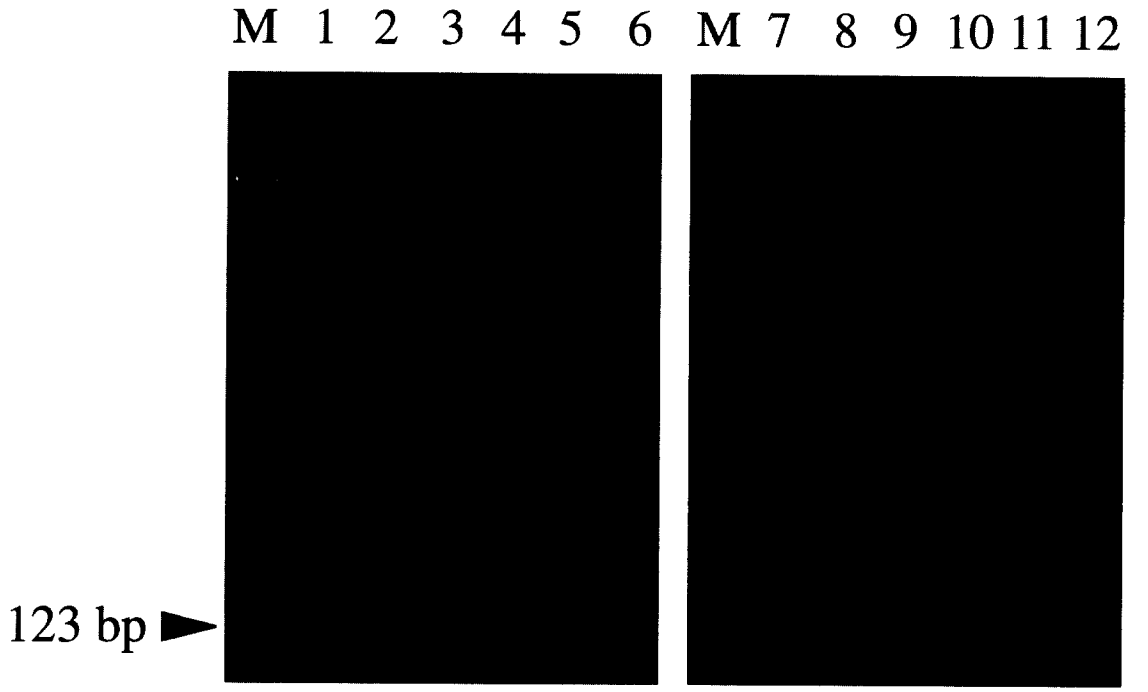

Fig. 5. Effect of tyrosine kinase inhibitors on DNA laddering induced by didemnin B. DNA extracted from HL-60 cells treated with ethanol alone as control (lanes 1,7), 1 μM didemnin B for 2 h (lanes 2,8), and 2 h of 1 μM didemnin B after 1 h pretreatment with 100 μg/ml genistein (lanes 3,9), 25 μg/ml 2,5-dihydroxycinnamic acid methyl ester (lanes 4,10), 300 μM tyrphostin 1 (lanes 5,11), and 300 μM tyrphostin B50+ (lanes 6,12) was separated by gel electrophoresis on a 0.8% agarose gel and stained with ethidium bromide as described in section 2. Lanes 1–6, fragmented cytosolic DNA extracted from the supernatant after cell lysis; lanes 7–12, crude total DNA indicating similar amounts of chromosomal DNA in control and inhibitor treated cells. A 123 base pair ladder was used as a standard.

trophoresis. While cells treated with didemnin B showed a DNA ladder of multiples of approx. 200 base pairs, cells pretreated with tyrosine kinase inhibitors showed no such laddering pattern (Fig. 5). It is unexpected that tyrphostin 1 would inhibit didemnin B-induced apoptosis since tyrphostin 1 has been shown to be ineffective as an inhibitor of the EGF receptor kinase [15] and is, therefore, often used as a negative control. However there is a large range of tyrosine kinase inhibitors, including tyrphostin 1, which inhibit didemnin B-induced apoptosis, indicating an involvement of a tyrosine kinase. Whether this kinase is an yet unknown (or an yet untested for tyrphostin inhibition) or whether the effect of tyrphostin 1 is different from tyrosine kinase inhibition has yet to be shown.

Tyrosine phosphorylation has been shown to play a role in the induction of apoptosis in other reports, however, the nature of the regulation of apoptosis by tyrosine phosphorylation is complicated. Tyrosine protein kinase inhibitors have been shown to induce apoptosis in HL-60 cells within 18 h [11], and our results show that they can inhibit apoptosis in HL-60 cells in a much shorter period of treatment. Herbimycin A, another tyrosine kinase inhibitor, has been shown to inhibit TNF-and dexamethasone-induced apoptosis in U937 cells and rat thymocytes respectively [12,16]. However, tyrosine phosphorylation regulates TNF-and didemnin B-induced apoptosis differently. In TNF-induced apoptosis, only the DNA fragmentation is prevented by tyrosine kinase inhibition; the morphology is still apoptotic, and herbimycin A can be added well after commencement of TNF-treatment without losing effectiveness [12]. In contrast, in didemnin B-induced apoptosis both apoptotic morphology and DNA fragmentation are inhibited by tyrosine kinase inhibition, and we have observed that genistein must be added well before didemnin B treatment for maximal inhibition of apoptosis. Didemnin B-induced apoptosis is similar to apoptosis induced by ionizing radiation which also occurs rapidly and can be inhibited by pretreatment with tyrosine kinase inhibitors [17]. It is evident that tyrosine phosphorylation is involved in the regulation of some apoptotic pathways, including that initiated by didemnin B, however details of these pathways are not yet known. It is likely that tyrosine phosphorylation can regulate different steps in different regimens of apoptotic cell death.

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