Antineoplastic cyclic astin analogues kill tumour cells via caspase-mediated induction of apoptosis

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Astins, a family of cyclopentapeptides isolated from the roots of a medicinal plant Aster tataricus (Compositae), show antitumour activity. Their chemical structures consist of a 16-membered ring system containing a unique β,γ-dichlorinated proline [Pro(Cl₂)], other non-coded amino acid residues, and a cis conformation in one of the peptide bonds. The β,γ-dichlorinated proline residue is considered to play an important role in their antineoplastic activities in vitro on nasopharynx carcinoma (KB) cells and in vivo on sarcoma 180 ascites and P388 lymphocytic leukaemia in mice. The acyclic astins without Pro(Cl₂) do not show antitumour activity against S-180 ascites in vivo, suggesting that the cyclic nature of astins plays an important role in their antitumour activities. We synthesized new astin-related cyclopeptides differing from the natural product for the presence of some non-proteinogenic amino acid residues: Aib, Abu, -S(β⁰)-hPhe and a peptide bond surrogate (SO₂-NH-) and we tested for their antitumour effect. We observed cytotoxic effects of the newly synthesized cyclic astins, but not with the acyclic analogues astins. We also observed that the cyclic astin induced apoptosis in a human papillary thyroid carcinoma cell line (NPA cell line) and that apoptosis was associated with activation of caspases. The caspase family inhibitor, Z-Val-Asp-(OMe)-FMK, protected NPA cells from cyclic analogue astin-induced apoptosis. To determine which caspase was specifically activated, we assayed caspase activity in astin-treated cells in the presence of specific caspase 8, 9 or 3 inhibitors, i.e. Z-IETD-FMK, Z-LEHD-FMK Z-DEVd-FMK, which inhibit caspases 8, 9 and 3, respectively. The data presented here show selective antineoplastic properties of the newly synthesized cyclic astins, and suggest, for the first time, a mechanism for their antineoplastic action through the activation of apoptotic pathway.

Introduction

The possibility to develop new drugs effective on cancer is one of the main problems connected with cancer therapy. The development of new tumour specific drugs is therefore greatly needed.

Astins, A-I, are a family of natural antitumour cyclopentapeptides isolated from the roots of Aster tataricus (Compositae) and characterized by a 16-membered ring system containing a unique β,γ-dichlorinated proline [Pro(Cl₂)] and the presence of several unencoded amino acid residues. The antitumour activity of astins was first observed, several years ago, by Morita and coworkers (1,2). The astin backbone conformation together with a cis 3,4-dichlorinated proline residue has been considered to play an important role in the antineoplastic activity in vitro, on nasopharynx carcinoma (KB) cells, and in vivo on sarcoma 180A and P388 lymphocytic leukaemia (3,4). The acyclic astins without Pro(Cl₂) did not show antitumour activity against S-180 ascites cells in vivo (2,4), suggesting that the cyclic nature of astins plays an important role in the antitumour activities (5). Although the antineoplastic activity of the natural astins has been screened both in vitro and in vivo, their mechanism of action has never been investigated. In order to improve our knowledge of the structural and conformational properties influencing the bioactivity in this class of compounds, we synthesized new astin-related cyclopeptides differing from the natural product in the presence of non-proteinogenic amino acid residues and a peptide bond surrogate (SO₂-NH-), and analysed for their antineoplastic activity. We have shown earlier that the antitumour activity of one of them, astin 3, is comparable in vitro with that of natural astins A and B used by Morita and coworkers (1,2) when tested on a papillary human thyroid tumour cell line (NPA) (6).

In the present study we have extended these observations to several human tumour cell lines of different origin and degree of malignancy, and also investigated the mechanism used by the astins to kill the cells.

It is well documented that most anticancer agents induce apoptosis, and it has been observed that several peptides (7), in their antineoplastic action, promote apoptosis. A series of enzymes known as caspases are involved in apoptotic cell death (8-11). Caspases are cysteine proteases that are expressed as inactive pro-enzymes and are normally classified as signalling or effector caspases (12,13). Caspases are synthesized as relatively inactive zymogens that become activated by scaffold-mediated transactivation or by cleavage upstream proteases in an intracellular cascade (11).

Abbreviations: AFC, 7-amino-4-trifluoromethyl-coumarin; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; FMK, fluoromethylketone; Hoechst 33258, 2′(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl) - 2,5′-bi (1H-benzimidazol)-6-(1-methyl-4piperazinyl)benzimidazole trihydrochloride; MTI, 3-(4,5-dimethy1imidazol-2-yl) 2,5-diphenyltetrazolium bromide (thiazolyl blue); PI, propidium iodide; Z-DEVD-FMK, Z-Asp-(OMe)-Glu-(OMe)-Val-Asp(OMe)-fluoromethylketone; Z-IETD-FMK, Z-Ile-Glu-Thr-Asp-(OMe)-fluoromethylketone; Z-LEHD-FMK, Z-Leu-Glu-(OMe)-Asp-(OMe)-fluoromethylketone; Z-DEVd-FMK, Z-Val-Asp-(OMe)-fluoromethylketone; Pro(Cl₂), β,γ-dichlorinated proline.
The data presented show, for the first time, that the antitumour effect of the cyclic astins depends on the activation of the apoptotic machinery by the subsequent activation of signalling caspases 8 and 9 and consequently activation of effector caspase 3. We determined the specificity of caspase induction by measuring their activity in the presence of selective inhibitors and showing that the caspase family inhibitor protected cells from apoptotic death.

Materials and methods

Astell

We synthesized cyclic analogue astins and linear analogue astin as previously described (6). Natural astins were kindly provided by Dr Morita.

Reagents

Culture media and supplements were purchased from Life Technologies (Ponsley, UK), foetal calf serum from Hyclone Lab (Logan, UT, USA), Trypan blue from Flow Laboratories (Irvin, Scotland), MTT, Hoechst 33342, and Etoposide from Sigma Chemical Company (St Louis, MO, USA). Caspase 3 and caspase 8 fluorometric assay kits were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), caspase 9 fluorometric assay kit, APC, FMK negative control, and inhibitors: Z-VD-FMK, Z-IETD-FMK, Z-LEHD-FMK and Z-DEVd-FMK from Alexis Biochemicals (San Diego, CA, USA) and Death Detection Elixa Kit Plus from Roche Diagnostics (Mannheim, Germany), respectively.

Cell lines

NPA and ARO cell lines, a gift of Dr. A.Fusco, derived respectively from human papillary and anaplastic thyroid carcinomas are tumorigenic when injected into nude mice (14,15). SK-OV-3 cell line, a gift of Dr C. De Lorenzo, was derived from human ovary adenocarcinoma (16). All these cell lines were grown in the Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum containing glutamine (2 mM), streptomycin (50 μg/ml) and penicillin (50 U/ml). The SK-BR-3, human breast carcinoma cell line (16) and A-431, human epithelial carcinoma cell line (16), kindly provided by Dr C. De Lorenzo, were cultured in RPMI 1640, supplemented with 10% foetal calf serum containing glutamine (2 mM), streptomycin (50 μg/ml) and penicillin (50 U/ml).

Cell viability assays

For cell viability analysis, tumour cell lines (NPA, ARO, SK-BR-3, SK-OV-3, A-431) were seeded in 96-well plates (5 × 10^3/well in 150 μl of medium) and incubated for different times in the presence or absence of astin 3. Astin was added to a final concentration of 10^5 μM to the culture medium 24 h after plating, and the cells were incubated for an additional 24, 48 and 72 h period. At the end of the treatments, a colorimetric assay based on the ability of live, but not dead, tumour cells to reduce a tetrazolium base compound (MTT) to a blue formazan product was used (17,18). MTT was added and the mixture was allowed to incubate for 4 h. During this period, precipitation of tetrazolium blue crystals, DMSO (100 μl of 140 nM annexin V-FITC. At the end of the incubation, 5 μl of PI (50 μg/ml) was added to each sample and analysed by flow cytometry (Becton Dickinson, San Jose, CA, USA).

For apoptotic analyses, 10^4 NPA cells were plated in triplicate on different cover glasses inserted in separate wells of a 24-well plate; 24 h after plating, cyclic astin 3 (10^5 nM) and linear astin 4 (10^5 nM) were added. After 24, 48 and 72 h of additional treatment, the medium was removed, the cells fixed for 20 min in 4% formaldehyde, washed in phosphate-buffered saline (PBS) and permeabilized for 5 min in 0.1% Triton X-100. The cells then were washed with PBS and stained for 30 min with Hoechst 33258 immunofluorescent reagent at a concentration of 0.5 μg/ml in PBS. The stained cells were observed under a Zeiss Axioshot microscope and photographed.

To analyse the time dependency of apoptotic DNA fragmentation, NPA cells were plated in 96-well plates (10^4/well in 100 μl medium) and 24 h later, astin 3 was added to the culture medium (10^5 μM) and incubated for an additional 24, 48 and 72 h. Etoposide was used as a positive control. Apoptosis was measured in triplicate samples using the Cell Death Detection Elixa Plus Kit. This photometric sandwich enzyme-immunassay determines cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) upon induction of cell death. During incubation, the antihistone antibody binds to the histone component of the apoptotic nucleosomes and links the immunocomplex to the streptavidin-coated plates through its biotinylatin. The amount of nucleosome material by peroxidase activity was determined spectrophotometrically at 405 nm with 2′,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate (Microplate reader, Biorad, Milano, Italy).

Determination of caspase activation

Cells were treated with cyclic and linear analogue astins for various time durations. At the end of each treatment, activities of caspase 3, 8, and 9 were measured with fluorometric protease assay kits according to the manufacturer’s instructions. Briefly, after induction of apoptosis, NPA cells were harvested and collected by centrifugation. The pelleted cells were taken up in lysis buffer. Lysates were incubated for 1 h at 37°C with the specific fluorescent substrate. The caspase 3, 8 and 9 kits assay the activity of caspase 3, which recognizes the Asp–Glu–Val–Asp (DEVD) sequence, the activity of caspase 8, which recognizes the Ile–Glu–Thr–Asp (IETD) sequence, and the activity of caspase 9, which recognizes the Leu–Glu–His–Asp (LEHD) sequence, respectively. Fluorescence derived from release of AFC was followed using a spectrophotometer at 400 nm excitation and 505 nm emission.

Caspase inhibitors

Caspase inhibitors irreversibly bind to the caspase active sites because the peptide recognition sequence is linked to a fluoromethylketone (FMK). A dose of 50 μM Z-Val-Ala-Asp-(OMe)-fluoromethylketone (Z-VD-FMK, added every 24 h) was used as a general upstream caspase inhibitor for DNA fragmentation assay. A dose level of 10 μM, Z-IETD-FMK, Z-LEHD-FMK and Z-DEVD-FMK, was used as caspase 8, 9 and 3 selective inhibitors, respectively. All samples were analysed with a spectrophotometer at 400 nm excitation and 505 nm emission.

Results

Cytotoxic effect of cyclic astin 3

The antitumour effect of different classes of natural astins (chlorine- and Thr-containing cyclic pentapeptides) has been previously observed (2) but the mechanism of action has never been evaluated.

We synthesized novel analogue astins, similar to the natural products, to better define the structural and conformational properties influencing the bioactivity and analysed them for their antineoplastic activity. The cyclic astin analogues exerted antineoplastic activity whereas the linear astins did not show any activity (5), confirming that the cyclic conformation is crucial for their antineoplastic effect. Among the different novel astins the most effective was astin 3 (or peptide III) in which the carbonyl in the --HPhe-Abu peptide bond was replaced by a -SO2- group showing an increased backbone flexibility (6) as shown in Figure 1.

Figure 2 shows the antiproliferative effect exerted, in vitro, by astin 3 on several human neoplastic cell lines of different origin and different degree of malignancy; when compared with untreated control cells at the same point. As shown, the
antitumour activity of antineoplastic cyclic astin analogues

To better define the antitumour effect of the astins, cell viability analysis has been performed on the same cell lines comparing the effect of cyclic versus linear astins. Figure 3 shows that linear astin 4 did not inhibit the survival of NPA cell line. On the other hand, cyclic natural astin B or newly synthesized astin 3 significantly inhibited survival and growth. The cytotoxic effect has been evaluated using the trypan blue exclusion analysis. Basically, similar results were observed using, in parallel, the MTT assay (data not shown).

Cyclic astin 3 induces apoptosis and a general caspase inhibitor protects thyroid carcinoma cells from cell death

Since the mechanism of antineoplastic action of astins has never been investigated, and since it is well documented that most anticancer agents induce apoptosis, we investigated the action of our newly synthesized astins on modulating apoptosis. To identify the mode of cell death, we treated NPA cells with cyclic astin 3, linear astin 4 and natural astin B. Etoposide was used as a positive control because it has been reported to facilitate apoptosis (22). Figure 4 shows the effect of annexin V-FITC/PI that discriminates between apoptotic and necrotic cells; annexin V binds only the phosphatidylserine of apoptotic cells. The apoptotic cell count with astin 3 and natural astin B after 24 h treatment is comparable with the count observed after treatment with etoposide, whereas the apoptotic cell count after the ineffective linear astin 4 treatment is similar to that of untreated control cells.

To confirm that cell death was attributable to activation of apoptosis pathways, we treated NPA cells with astins 3 and 4, using again etoposide as a positive control. Cells were examined for the presence of apoptotic nuclear bodies by fluorescence microscopy using Hoechst 33258 staining. A clear induction of apoptosis was observed in NPA cells treated with cyclic astin 3, as revealed after 24 and 48 h of treatment in the form of a large number of nuclei with apoptotic bodies (Figure 5B and C). In contrast, no sign of apoptosis was detectable in untreated cells (negative control, Figure 5A) or in NPA cells treated with linear astin 4 (data not shown). A similar degree of apoptotic cells was observed for NPA cells treated with etoposide, which was employed as a positive control (Figure 5D and E).

To measure apoptosis we also used an ELISA-based assay. As shown in Figure 6, apoptotic nucleosomes were detected after treatment of NPA cells with cyclic astin 3 or with etoposide as a positive control; the maximum effect occurred 48 h after the treatment with etoposide or astin 3. If astin 3 induced a specific apoptosis, then DNA fragmentation should be
partially or fully prevented in cells cotreated with astin 3 and caspase family inhibitors. To address this question, we cotreated NPA cells with cyclic astin 3 and Z-VAD-FMK, a general upstream inhibitor of caspases. The inhibitor was replenished daily when the treatment exceeded 24 h.

Z-VAD-FMK fully decreased astin 3 or etoposide induced DNA fragmentation showing that the caspase family inhibitor protected NPA treated cells from apoptotic death.

**Astin 3 triggers caspase cascades in NPA cell line**

Since apoptosis is executed by caspases, to assess its role in astin-induced apoptosis, we analysed the kinetics of caspase activation using substrates specific for caspases 8, 9, and 3. In these assays the spectrophotometer detection of AFC fluorophore, cleaved from the specific synthetic substrate, is a measure of caspase activity. Caspase 8 activity increased at 6 h and peaked at 24 h. Its activity declined thereafter and reached the baseline at 48 h, as shown in Figure 7A. Caspase 9 increased slowly at 6 h and peaked at 36 h and this protease decreased progressively until 72 h, as shown in Figure 7B. Caspase 3 activity consistent with its downstream role, increased slowly at 24 h, peaked at 48 h and declined drastically at 72 h, as shown in Figure 7C. No caspase activation was detected with untreated cells (control) or in cells treated with linear astin 4 (data not shown). These results suggest that apoptosis, induced by cyclic astin 3, in the NPA cells, is associated with activation of the caspase pathway.

To further assess the specificity of caspases in cyclic astin-induced apoptosis, selective inhibitors of the three caspases were used as a control of specificity. As shown in Figure 8 the specific caspase inhibitors Z-IETD-FMK, Z-LEHD-FMK and Z-DEVD-FMK stopped the activation of caspases 8, 9, and 3 respectively.

To determine whether caspase-8 activated procaspase 9 directly, we blocked the activation of caspase 8 by specific Z-IETD-FMK inhibitor and evaluated the activation of both caspases 9 and 3. As shown in Figure 9 (A and B), inhibition of
caspase 8 prevented activation of caspase 9 or 3, indicating that caspase 8 was upstream of caspases 9 and 3.

Moreover, by blocking caspase 9, by specific Z-LEHD-FMK inhibitor, no activation of effector caspase 3 was observed (Figure 9C and D). Caspase 8 was activated, under the same conditions showing further that, in our NPA cell system, the main apoptotic pathway activated included sequential activation of caspases 8, 9 and 3.

Discussion

Peptides from different natural or newly synthetic sources have been used as therapeutic, diagnostic and vaccines for a long time. Plant extracts or products as the active ingredient are normally used as a source of novel compounds from which drugs active against several different tumours are derived. It is well documented that most anticancer agents induce apoptosis, raising the intriguing possibility that a defect in apoptotic programmes contributes to treatment failure (25). Apoptosis was initially described by its morphological characteristics including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (26). Apoptosis is now thought to be a gene-directed programme regulated by factors that influence cell survival as well as those that control proliferation and differentiation. It can be considered an intrinsic suicide programme of the cell, and for this reason it is viewed as a mechanism to limit tissue injury (27).

Cyclic astins, a family of cyclopentapeptides isolated from the roots of a medicinal plant, _A. tataricus_ (Compositae), have been implicated as antitumour substances and this activity depends on their cyclic nature (2). To assess if the structure and conformational properties of astins influence their bioactivity we synthesized new astin related cyclopeptides (6) and analysed them for their antineoplastic activity.

We used a series of malignant cell lines derived from human tumours of different histogenesis to analyse the effect of the newly synthesized cyclic astin 3, and observed that all cell lines analysed were inhibited in their growth by the action of astin 3, whereas no toxic effect was observed with newly synthesized linear astin 4.

To understand the nature of the selective cell death induced specifically by the action of astin 3, the most sensitive NPA cell line was analysed for apoptosis. We observed DNA fragmentation, chromatin condensation and exposure of phosphatidylserine, all typical signs of apoptosis activation. The presence of specific apoptotic nuclear bodies was analysed by fluorescence microscopy after Hoechst 33258 staining (Figure 5B and C). A quantitative estimation of the percentage of cyclic astin induced apoptosis gave values of ~40% of apoptotic cells (Figure 4) and these data were confirmed by flow cytometry (data not shown). To confirm that the phenomenon observed was specific, the cells, after treatment with astins, were also analysed for the presence of apoptotic nucleosomes (Figure 6). When the same cells were treated with the acyclic analogue astin, according with the data reported in literature, they did not show any change in the normal morphology and number, suggesting that the cyclic nature of astins plays an important role in their antitumour activities (5).

Since different approaches have shown the activation of the apoptotic programme we tried to understand the mechanism of this activation involved in the analogue astin treated tumour cells. Apoptosis a genetically programmed, morphologically distinct form of cell death can be triggered by a variety of physiological and pathological stimuli. Studies performed over the past 10 years have demonstrated that proteases play critical
roles in initiation and execution of this process (11–13). The caspases, a family of cysteine-dependent aspartate-directed proteases, are responsible for many of the biochemical and morphological changes associated with apoptosis (28). Initiation of the apoptosis response involves ‘initiation’ caspases, such as caspase 8 which may induce apoptosis directly activating ‘effector’ caspases such as caspases 6, 7 and 3 (29,30) or caspase 9 subsequent to the involvement of mitochondria that can then activate the same effector caspases 6, 7 and 3 (30–32). Moreover, it has also been reported that chemotherapeutic drugs can activate caspase 8, which produce mitochondrial responses, suggesting that crosstalk between apoptotic signals can occur (33–36).

The data presented show that, in our NPA cell system, the activation of cyclic analogue astin follows the mechanism involving the subsequent caspases 8, 9 and 3 activation and the specificity of their activity has been demonstrated using the specific caspases 8, 9 and 3 inhibitors or the caspase family inhibitors, Z-VAD-FMK that blocked caspases activation and protected NPA cells from apoptotic death. In addition on blocking caspase 8 no activation of caspase 9 or 3 has been observed and on blocking activated caspase 9 no activation of caspase 3 occurred while caspase 8 is still active, showing again that in our system the main apoptotic pathway activated by the cyclic analogue astin is the pathway involving the sequential activation of caspases 8, 9 and 3. It is possible that the cascade originated by activation of caspase 8 induced by astines may propagate to mitochondria (caspase 9) and other organelles (caspase 3).

Additional experiments are necessary to better define the interaction between cyclic astin analogues and tumour cells and their involvement in the specific activation of the apoptotic pathways. Moreover, these data suggest the possibility of utilizing synthetic analogue astins as a potential new chemotherapeutic drug.

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References


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