Cancer Chemother Pharmacol (1997) 39: 223–226

ORIGINAL ARTICLE

Hilary J. Anderson · John E. Coleman
Raymond J. Andersen · Michel Roberge

Cytotoxic peptides hemiasterlin, hemiasterlin A and hemiasterlin B induce mitotic arrest and abnormal spindle formation

Received: 21 September 1995 / Accepted: 28 May 1996

Abstract *Purpose:* Hemiasterlin, hemiasterlin A and hemiasterlin B are newly isolated cytotoxic tripeptides with potential as antitumor drugs. We wished to determine their mechanism of cytotoxicity. *Methods:* We studied their effect on cell survival, cell cycle progression, and microtubule morphology in MCF-7 human mammary carcinoma cells. *Results:* At the nanomolar concentrations at which they were cytotoxic, the peptides induced arrest in mitotic metaphase. Hemiasterlin A produced abnormal mitotic spindles like those produced by the microtubule inhibitors taxol, nocodazole and vinblastine at low concentrations. At high concentrations hemiasterlin A did not cause microtubule bundling like taxol, but caused microtubule depolymerization like nocodazole and vinblastine. *Conclusions:* The hemiasterlins probably exert their cytotoxic effect by inhibiting spindle microtubule dynamics.

Key words Cancer · Microtubules · Mitosis · Taxol · Vinblastine

This work was supported by grants from the British Columbia Health Research Foundation to M. R. and from the National Cancer Institute of Canada and the Natural Sciences and Engineering Research Council of Canada to R. J. A. M. R. is a Scholar of the Medical Research Council of Canada

H. J. Anderson · M. Roberge (✉)
Department of Biochemistry and Molecular Biology,
2146 Health Sciences Mall, University of British Columbia,
Vancouver, British Columbia, V6T 1Z3, Canada
Tel. (604) 822-2304; Fax (604) 822-5227
E-mail michel@otter.biochem.ubc.ca

J. E. Coleman · R. J. Andersen
Departments of Chemistry and Oceanography,
University of British Columbia, Vancouver, British Columbia, Canada

Introduction

The hemiasterlins are a family of potent cytotoxic peptides recently isolated from marine sponges [3, 15]. Hemiasterlin A is a tripeptide composed of the unusual amino acids trimethyltryptophan, *tert*-leucine and *N*-methyl homo vinylogous valine, and is closely related to hemiasterlin and hemiasterlin B (Fig. 1). This group of peptides is active in vitro against murine leukemia P388, human mammary carcinoma MCF-7, human glioblastoma/astrocytoma U373, human ovarian carcinoma HEY, and in vivo against P388 cells in mice [3]. The mechanism of cytotoxicity of this family of compounds is unknown.

In this study we examined the effects of hemiasterlin A, hemiasterlin and hemiasterlin B on cell survival and cell cycle progression in human mammary carcinoma MCF-7 cells and suggest that they exert their cytotoxic effect by binding to tubulin and inhibiting spindle microtubule dynamics.

Materials and methods

MCF-7 cells were grown as a monolayer at 37 °C in a humidified atmosphere containing 10% CO_2 in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin and streptomycin, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 1% MEM nonessential amino acids, 1 μg/ml bovine insulin, 1 μg/ml hydrocortisone, 1 ng/ml human EGF and 1 ng/ml β-estradiol. The hemiasterlins, geodiamolide B, taxol and nocodazole (Sigma) were dissolved in dimethyl sulfoxide (DMSO) and stored at –20 °C. Vinblastine (David Bull Laboratories, Victoria, Australia) was used as a 1 mg/ml solution in physiological saline.

Cytotoxicity was measured using a microculture tetrazolium assay [4]. MCF-7 cells were grown at low density in multiwell plates and treated with different concentrations of compounds for 20 h. The compounds were then washed away and the cells allowed to grow in normal medium for 3–5 days before assay.

For mitotic arrest determination, MCF-7 cells were treated with different concentrations of compounds for 20 h, prepared for chromosome spreads using a previously described method [5] and the percentage of mitotic cells determined by fluorescence microscopy.

For tubulin immunofluorescence, MCF-7 cells were grown on poly-L-lysine-coated coverslips, treated with the compounds for 20 h,

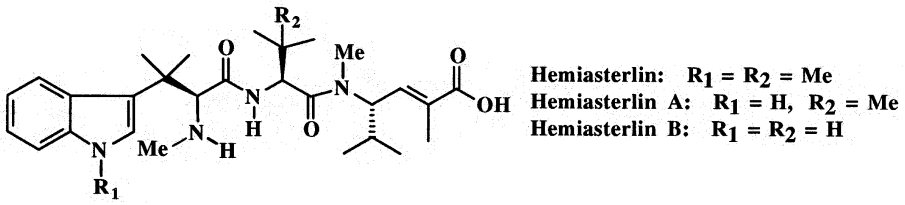

Fig. 1 Chemical structure of the hemiasterlins

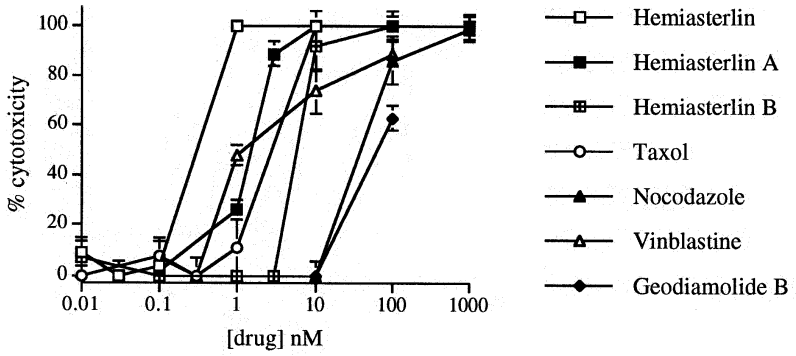

Fig. 2 Cytotoxicity of the hemiasterlins, microtubule inhibitors and geodiamolide B. Mean and standard deviation of quadruplicate measurements are shown for each point

centrifuged at 200 *g*, rinsed with PBS (10 m*M* sodium phosphate, pH 7.4, 150 m*M* NaCl), fixed with 3.7% formaldehyde in PBS and rinsed in KB (10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.1% Triton X-100, 0.1% BSA). The coverslips were then incubated with monoclonal antibody E7 to β-tubulin (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, Iowa) at a dilution of 1/30 in KB for 30 min at room temperature. After two rinses of 10 min each in KB, the coverslips were incubated with CY3-conjugated goat antimouse IgG secondary antibody (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. They were then rinsed in KB, stained with the DNA dye bisbenzimide, mounted on slides in 10% PBS in glycerol, and photographed on Kodak TMax 400 film with a Zeiss Axiophot microscope.

Results

We first determined the cytotoxicity of the hemiasterlins towards MCF-7 human mammary carcinoma cells. Cells were treated for the duration of one cell cycle (20 h) with different concentrations of the compounds or the vehicle DMSO and cytotoxicity was determined after 3–5 days using a microculture tetrazolium assay. DMSO had no effect. Hemiasterlin killed MCF-7 cells half-maximally at 0.5 n*M*, hemiasterlin A at 2 n*M*, and hemiasterlin B at 7 n*M* (Fig. 2). Geodiamolide B, a cytotoxic cyclodepsipeptide also found in the marine sponge *Cymbastela* sp. [3], was also tested and found to be less potent, killing cells half-maximally at 80 n*M*.

Since many antitumor drugs affect the transition between particular phases of the cell cycle, we next examined the effect of the hemiasterlins on mitosis. Cycling cells were treated with different concentrations of the hemiasterlins or DMSO for 20 h and the percentage of mitotic cells was determined microscopically. DMSO had no effect. All three hemiasterlins induced a concentration-dependent accumulation of cells in mitotic metaphase, with hemiasterlin having a half-maximal effect at 0.5 n*M*, hemiasterlin A at 2.5 n*M*, and hemiasterlin B at 28 n*M* (Fig. 3). Since each compound caused mitotic arrest and cytotoxicity in the same concentration range, it is likely that mitotic arrest is an important determinant of their cytotoxic activity. Geodiamolide B, by contrast, failed to block cells in mitosis, even at 300 n*M* (Fig. 3).

The most common compounds causing mitotic arrest are the microtubule inhibitors. We therefore compared the cytotoxic and antimitotic activity of the hemiasterlins with the activity of taxol, vinblastine and nocodazole, as representatives of the major classes of microtubule inhibitors. Taxol, vinblastine and nocodazole showed half-maximal cytotoxicity at 5 n*M*, 2 n*M*, and 63 n*M* (Fig. 2) and half-maximal ability to arrest cells in mitosis at 7 n*M*, 15 n*M* and 42 n*M*, respectively (Fig. 3). The hemiasterlins are therefore potent compounds, with hemiasterlin and hemiasterlin A being more potent in both capacities than the microtubule inhibitors.

We next compared the morphology of cells treated with hemiasterlin A with those treated with the microtubule inhibitors. We treated cells with concentrations of

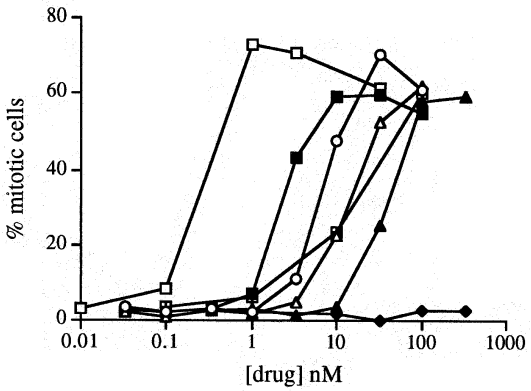

Fig. 3 Antimitotic activity of the hemiasterlins, microtubule inhibitors and geodiamolide B. For an explanation of the symbols, see Fig. 2

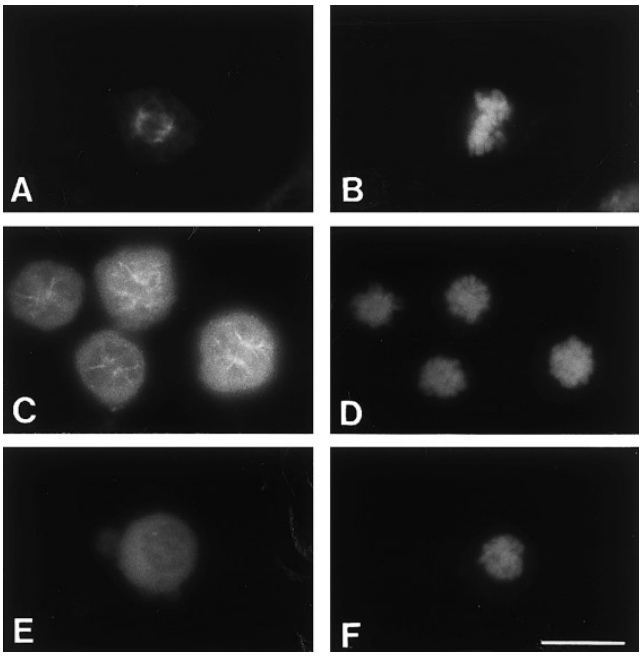

Fig. 4A–E Microtubule and chromosome distribution after 20 h incubation with no drug (**A, B**), 2 n*M* hemiasterlin A (**C, D**) or 10 n*M* hemiasterlin A (**E, F**). **A, C, E** antitubulin immunofluorescence; **B, D, F** DNA staining with the dye bisbenzimide. *Bar* 26 μm

hemiasterlin A, taxol, vinblastine or nocodazole that produce half-maximal or maximal mitotic arrest and examined the morphology of their mitotic spindles by indirect immunofluorescence using a monoclonal antibody to β-tubulin and the distribution of their chromosomes using the fluorescent DNA dye bisbenzimide. The results are shown in Fig. 4. In the presence of hemiasterlin A at 2 n*M* no completely normal spindles were seen. Some cells showed relatively minor abnormalities in which a bipolar spindle was present, but the astral microtubules were considerably longer than normal and the chromosomes were not completely confined to the metaphase plate. Most commonly cells had multiple asters, and the chromosomes were distributed in a spherical mass (Fig. 4C, D). Half-maximal concentrations of taxol, vinblastine and nocodazole produced the same types of abnormal spindle as hemiasterlin A (not shown). Hemiasterlin A at 10 n*M*, the lowest concentration causing maximal mitotic arrest in MCF-7 cells, caused microtubule depolymerization in mitotic cells (Fig. 4E, F). This was also the case for high concentrations of vinblastine and nocodazole (not shown). Taxol at high concentrations had a quite different effect, causing bundling of cytoplasmic microtubules in interphase cells and very dense multiple asters in mitotic cells.

Discussion

The hemiasterlins are chemically novel tripeptides [3, 15] with promising potential for development as antitumor drugs. They show potent in vitro cytotoxicity against human solid tumor cell lines and show equally potent cytotoxicity against murine leukemia P388 both in vitro and in vivo [3]. Preliminary data indicate that the effective in vivo dose against P388 in mice is at least two orders of magnitude lower than the toxic dose (T. M. Allen, personal communication). In this study, we showed that at nanomolar concentrations these compounds are cytotoxic to MCF-7 cells, cause mitotic arrest, and at least one, hemiasterlin A, produces abnormal mitotic spindles.

A broad array of drugs can arrest cells in mitosis, producing abnormal mitotic spindles. Some of these bind tubulin directly, such as the well-studied microtubule inhibitors taxol, vinblastine and nocodazole used here for comparative purposes [9, 10]. They do so at distinct sites, the taxol, vinca and colchicine domains respectively. At the low concentrations at which they cause mitotic arrest, all three produce abnormal spindles [9, 10], as does hemiasterlin A. At higher concentrations each of the three drugs produces a different set of microtubule morphologies. Vinblastine and nocodazole depolymerize microtubules [9], as does hemiasterlin A. At even higher concentrations vinblastine can assemble tubulin dimers into nearly crystalline arrays called vinblastine paracrystals [9]. In contrast, at high concentrations taxol enhances microtubule assembly and produces thick bundles of microtubules [10]. Hemiasterlin A at high concentrations does not produce paracrystals or microtubule bundles.

In recent years many other naturally occurring cytotoxic compounds have been isolated that bind tubulin, causing mitotic arrest. Their structures are extremely varied, including peptides such as phomopsin A and the dolastatins [7], ustiloxin [12], and cryptophycin [11], as well as nonpeptide compounds such as maytansine, rhizoxin, halichondrin B, homohalichondrin B and the halistatins [7], curacin A [2], the spongistatins [1], discodermolide [6], steganacin, combrestatin, and podophyllotoxin [14]. In vitro these compounds bind in or near the taxol [6], vinca [1, 7, 11, 12], or colchicine domains [2, 14], and are the subject of structure-activity studies. Their effects on microtubule morphology vary at high concentrations, as for taxol, vinblastine and nocodazole. However, what these diverse drugs appear to have in common, no matter where or how they bind to tubulin, is their ability, at the low concentrations at which they arrest cells in metaphase, to disrupt the delicate control of microtubule dynamics that is essential for the conversion of the interphase microtubule network into the precisely organized mitotic spindle and for the spindle to carry out its function of arranging and sorting chromosomes [9, 10].

Other drugs that can arrest cells in mitosis are inhibitors of protein phosphatases 1 and 2A [8, 13]. They also produce multiple asters with elongated microtubules [13, 16, 17], but probably act indirectly on microtubules by promoting the phosphorylation of proteins such as microtubule-associated proteins that control microtubule dynamics. Hemiasterlin A does not inhibit protein phosphatases 1 or 2A in vitro (C. Holmes, personal communication). Although the hemiasterlins could act indirectly on microtubules through another, unknown, mechanism, they

are more likely to be microtubule inhibitors. In vitro binding studies should resolve this issue.

The hemiasterlins are small molecules with simpler structures than all known microtubule inhibitors, promising ease of chemical synthesis and structure-function studies. Cytotoxic drugs that cause metaphase arrest through disrupting microtubule dynamics are already valuable chemotherapeutic agents. It would be useful to identify new drugs of this type that act at lower concentrations, thereby reducing the possibility of pleiotropic and possibly deleterious effects, that are effective against multidrug-resistant tumor cells, and that can be produced cheaply. The hemiasterlins might fit these criteria.

References

1. Bai R, Taylor GF, Cichacz ZA, Herald CL, Kepler JA, Pettit GR, Hamel E (1995) The spongistatins, potently cytotoxic inhibitors of tubulin polymerization, bind in a distinct region of the vinca domain. Biochemistry 34: 9714
2. Blokhin AV, Yoo HD, Geralds RS, Nagle DG, Gerwick WH, Hamel E (1995) Characterization of the interaction of the marine cyanobacterial natural product curacin A with the colchicine site of tubulin and initial structure-activity studies with analogues. Mol Pharmacol 48: 523
3. Coleman JE, de Silva ED, Kong F, Andersen RJ, Allen TM (1995) Cytotoxic peptides from the marine sponge *Cymbastela* sp. Tetrahedron 39: 10653
4. Goodwin CJ, Holt SJ, Downes S, Marshall NJ (1995) Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XXT and MTS. J Immunol Methods 179: 95
5. Guo XW, Th'ng JPH, Swank RA, Anderson HJ, Tudan C, Bradbury EM, Roberge M (1995) Chromosome condensation induced by fostriecin does not require $p34^{cdc2}$ kinase activity and histone H1 hyperphosphorylation, but is associated with enhanced histone H2A and H3 phosphorylation. EMBO J 14: 976
6. Haar E ter, Kowalski RJ, Hamel E, Lin CM, Longley RE, Gunasekera SP, Rosenkrantz HS, Day BW (1996) Discodermolide, a cytotoxic marine agent that stabilizes microtubules more potently than taxol. Biochemistry 35: 243
7. Hamel E (1992) Natural products which interact with tubulin in the vinca domain: maytansine, rhizoxin, phomopsin A, dolastatins 10 and 15 and halichondrin B. Pharmacol Ther 55: 31
8. Ishida Y, Furukawa Y, Decaprio JA, Saito M, Griffin JD (1992) Treatment of myeloid leukemic cells with the phosphatase inhibitor okadaic acid induces cell cycle arrest at either G1/S or G2/M depending on dose. J Cell Physiol 150: 484
9. Jordan MA, Thrower D, Wilson L (1992) Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. J Cell Sci 102: 401
10. Jordan MA, Toso RJ, Thrower D, Wilson L (1993) Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc Natl Acad Sci USA 90: 9552
11. Kerksiek K, Mejillano MR, Schwartz RE, Georg GI, Himes RH (1995) Interaction of cryptophycin 1 with tubulin and microtubules. FEBS Lett 377: 59
12. Li Y, Koiso Y, Kobayashi H, Hashimoto Y, Iwasaki S (1995) Ustiloxins, new antimitotic cyclic peptides: interaction with porcine brain tubulin. Biochem Pharmacol 49: 1367
13. Roberge M, Tudan C, Hung SMF, Harder KW, Jirik FR, Anderson H (1994) Antitumor drug fostriecin inhibits the mitotic entry checkpoint and protein phosphatases 1 and 2A. Cancer Res 54: 6115
14. Sackett DL (1993) Podophyllotoxin, steganacin and combrestatin: natural products that bind at the colchicine site of tubulin. Pharmacol Ther 59: 163
15. Talpir R, Benayahu Y, Kashman Y, Pannell L, Schleyer M (1994) Hemiasterlin and geodiamolide TA; two new cytotoxic peptides from the marine sponge *Hemiasterella minor* (Kirkpatrick). Tetrahedron Lett 35: 4453
16. Van Dolah FM, Ramsdell JS (1992) Okadaic acid inhibits a protein phosphatase activity involved in formation of the mitotic spindle of GH4 rat pituitary cells. J Cell Physiol 152: 190
17. Vandré DD, Wills VL (1992) Inhibition of mitosis by okadaic acid: possible involvement of a protein phosphatase 2A in the transition from metaphase to anaphase. J Cell Sci 101: 79–91