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The influence of cell growth media on the stability and antitumour activity of methionine enkephalin

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Abstract: Studies with cultured tumour cell lines are widely used *in vitro* to evaluate peptide-induced cytotoxicity as well as molecular and biochemical interactions. The objectives of this study were to investigate the influence of the cell culture medium on peptide metabolic stability and *in vitro* antitumour activity. The degradation kinetics of the model peptide methionine enkephalin (Met-E, Tyr-Gly-Gly-Phe-Met), demonstrated recently to play an important role in the rate of proliferation of tumour cells *in vitro* and *in vivo*, were investigated in cell culture systems containing different amounts of fetal bovine serum (FBS). The influence of enzyme inhibitors (bestatin, captopril, thiorphan) on the Met-E degradation was also investigated. The results obtained in the Dulbecco's modified Eagle medium containing 10% FBS indicated a rapid degradation of Met-E ($t_{1/2} = 2.8$ h). Preincubation of the medium with a mixture of peptidase inhibitors reduced the hydrolysis of Met-E, as shown by the increased half-life to 10 h. The *in vitro* activity of Met-E against poorly differentiated cells from lymph node metastasis of colon carcinoma (SW620) and human larynx carcinoma (HEp-2) cells was determined. Tumour cells were grown for 3 weeks prior to the experiment in a medium supplemented with 10%, 5% or 2% FBS. Statistically significant to mild or no suppression of cell proliferation was observed in all cultures. In both cell lines, a significant suppression of cell growth by a combination of peptidase inhibitors and Met-E, compared with cells exposed to the peptide alone and cells grown in the absence of Met-E, was observed. This study indicated that caution must be exercised in interpreting the antiproliferative effects of peptide compounds in conventional drug-response assays.

Keywords: bestatin; captopril; cytotoxicity *in vitro*; DMEM; fetal bovine serum; methionine enkephalin; protease inhibitors; thiorphan

INTRODUCTION

A remarkable number of peptides of various size and structure are increasingly making their way into clinical application [1, 2]. More specifically, promising lead candidates are being discovered by modification of natural products, phage display and combinatorial chemistry, and several peptide compounds are in clinical trials. Although cancer treatment is a major focus of biotechnological research and development [3], there are many other potential applications for peptide therapeutics such as their use as antimicrobial agents [4], in Alzheimer's [5] and Creutzfeldt-Jakob [6] diseases or to control malaria [7]. Peptides exert their action on cells in a variety of ways. In the majority of cases, peptides bind to a cell surface protein (a receptor or a channel) and act either by inducing or inhibiting one or multiple signal transduction pathways. In other cases, peptides may be internalized and act by binding to an intracellular protein. During the past decade proof of the principle that peptide receptors can be used successfully for *in vivo* targeting of human cancers has been provided [8]. At the diagnostic level, due to the observation that peptide receptors are expressed in large quantities in certain tumours, radiolabelled peptides can be exploited for the localization of tumours and their metastasis. Peptides are also being used in cancer therapy to generate drugs for enhancing cellular uptake, drug treatment and vaccination.

Discovering which peptide binds to a specific protein(s) is a key to the therapeutic application of peptides. In the search for active peptide compounds, phage display technology is extremely useful because it provides a direct link between the peptide and its encoding nucleotide sequence (for a review, see [9]). However, *in vitro* studies on established cell lines or primary cell cultures still play an important role in designing and optimizing therapeutic protocols [10]. Currently, *in vitro* assays of drugs use mainly semi-automated methods in which tumour cells, in suspension or adherent to multi-wells, are treated for 24–72 h. At the end of the treatment, cell growth inhibition is evaluated by adding the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) [11] or sulforhodamine B [12] and the resulting stain is quantified using a spectrophotometer. Such assays are

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undoubtedly useful in preclinical studies because of the short time (2–4 days) required to perform them and the use of machines to quantify accurately the results. Dose-response and time-response curves must also be defined for each compound in order to identify the concentration and the exposure time capable of inhibiting cell growth by 50% (IC_{50}). The antiproliferative activity is evaluated against a panel of cancer cell lines in a culture medium, such as Dulbecco's modified Eagle medium (DMEM), containing usually 10%–30% fetal calf (FCS) or fetal bovine serum (FBS).

Some non self-explanatory results, and the differences observed between the *in vitro* and *in vivo* anticancer activity of small peptide compounds, led us to assume that caution must be exercised in the identification of a potent peptide compound in a cell culture medium containing either FBS or FCS. This paper is an attempt to illustrate differences in the bioactivity of peptide compounds under different *in vitro* conditions. Our current interest in peptide-based anticancer drugs, centred on opioid peptides, led us to investigate the influence of the medium on the *in vitro* stability and antiproliferative activity on different human cancer cell lines by using methionine enkephalin (Met-E, Tyr-Gly-Gly-Phe-Met) as the model peptide compound in the presence and absence of enkephalin-degrading enzyme inhibitors. Met-E belongs to a large family of endogenous opioid peptides playing a role in analgesia, stress, gastrointestinal, renal and hepatic functions, and immunological responses [13, 14]. Recent *in vitro* and *in vivo* studies with endogenous opioid peptides have shown that particularly Met-E was capable of enhancing immune function in patients with cancer or AIDS [15]. Of great interest also is the fact that Met-E acts as a native opioid growth factor, playing a role in cell proliferation and tissue organization during development, cancer, wound healing and angiogenesis [16].

MATERIALS AND METHODS

Materials

Met-E, methionine enkephalin sulfoxide [Met(O)-E], bestatin, captopril and thiorphan were purchased from Sigma Chemical Co. (St Louis, USA). Dulbecco's modified Eagle medium (Gibco BRL, Life Technologies, Paisley, UK) and trypsin-EDTA were Institute of Immunology Inc. (Zagreb, Croatia) products. Fetal bovine serum (heat inactivated) was obtained from Gibco BRL, Life Technologies. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide, trifluoroacetic acid (TFA), and dimethylsulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany).

Cell Culture

Poorly differentiated cells from lymph node metastasis of colon carcinoma (SW620) and human larynx carcinoma (HEp-2) cells were obtained from the Institute Rudjer Bošković, Division of Molecular Medicine, Zagreb, Croatia. Cells were grown as monolayers in tissue culture flasks (250 ml, BD Falcon, Germany) in DMEM supplemented with 10%, 5% and 2% of FBS, respectively, 2 mM glutamine, 100 U penicillin and 0.1 mg streptomicin. Cells were cultured in a humidified (95% air, 5% CO_2) CO_2 incubator (Shell Lab, Sheldon Manufacturing, USA) at 37 °C. The trypan blue dye exclusion method was used to assess cell viability.

Test Substances and Mixtures

Met-E was dissolved in DMEM. The final concentration in the MTT test was 10^{-4} M. Bestatin, captopril and thiorphan were dissolved in 0.05 M phosphate buffer, pH 7.4, as a 2×10^{-3} M stock and kept frozen in aliquots at −20 °C. IM-1 represents a mixture of bestatin, captopril and thiorphan at a final concentration of 10 μM, each. IM-2 represents a mixture of bestatin (final concentration: 30 μM), captopril and thiorphan (final concentration: 10 μM, each). All solutions were prepared immediately before each experiment.

Degradation of Met-E in Cell Culture Media

For stability studies 4 ml of DMEM, with 4.5 g glucose/l, containing different concentrations (0%, 2%, 5% or 10%) of FBS, and Met-E at a concentration of 8×10^{-4} M, were incubated under sterile conditions at 37 °C in a teflon lined screw-cap test tube. At appropriate times three samples (100 μl) of the reaction mixture were taken and deproteinized by the addition of 20 μl of 48% aqueous TFA. The samples were briefly vortexed and frozen. The thawed samples were centrifuged for 10 min (15 000 × *g*) and the supernatants were analysed by RP-HPLC. When the protease inhibitors bestatin, captopril and thiorphan were included at various concentrations (IM-1, IM-2) (see legend to Figure 2 for details), they were preincubated with a DMEM solution containing either 10% or 5% FBS at 37 °C for 15 min prior to addition of Met-E. The concentration of Met-E in the incubation mixtures was determined using an external standard (*o*-hydroxyphenylacetic acid) by RP-HPLC on an Eurospher 100 reversed-phase C-18 analytical column (250 × 4 mm i.d., 5 μm), eluted at a flow rate of 0.5 ml/min with 40% MeOH/0.1% trifluoroacetic acid using a HP 1090 system equipped with a diode array detector. UV absorption detection was performed at 280 nm and 215 nm.

Cytotoxicity Assay

Cytotoxic effects on cell growth were determined using the MTT assay as described by Horiuchi *et al.* [17]. Tumour cells, 2×10^4 cells/ml, were placed onto 96-microwell plates (Costar, Cambridge, USA). Then 24 h later, the medium was replaced with a fresh medium containing well defined concentrations of Met-E, Met-E + IM-1, Met-E + IM-2, IM-1 and IM-2. In the experiments in which inhibitors and Met-E were combined, the cells were pretreated with either IM-1 or IM-2 for 1 min and, after that, Met-E was immediately added. At the end of 3, 6, 24 and 72 h treatment with test mixtures, the medium was removed. MTT (diluted in phosphate buffer solution) (40 μl) was added to all culture wells which were incubated for an additional 4 h. The optical density was determined on the ELISA Stat Fax 2100 microplate reader at 570 nm,

after dissolving formazan, generated by live cells, in DMSO. All experiments were performed at least three times, with three wells each. The control cells were grown under the same conditions without addition of the tested compound. Cell survival was calculated relative to the untreated control cells.

Statistics

The original results of the MTT tests were used for statistical analysis. The Kolmogorov-Smirnov test, a normality distribution test was applied. The differences between groups were assessed by a non-parametric Kruskal-Wallis test ($p < 0.05$). Statistical analyses were performed with Statistica 6 for Windows package.

RESULTS AND DISCUSSION

Stability of Met-E in Cell Culture Media

In order to evaluate the stability of Met-E in the cell culture medium DMEM, the peptide was incubated at 37 °C for up to 3 days in the absence or presence of different amounts of FBS. The time course of Met-E degradation was followed by RP-HPLC analysis at varying periods of incubation. As shown in Figure 1, Met-E incubated in the cell culture medium without added FBS displayed a reasonably good stability, with a very slow formation of a single catabolite (~7% after 3 days at 37 °C). The degradation product was isolated by RP-HPLC and identified as methionine enkephalin sulphoxide [Met(O)-E], thus demonstrating the susceptibility of the Met residue in the pentapeptide to oxidation in DMEM. Although sulphur oxidation of Met-E generated two diastereomeric sulphoxides, they were not separable under the RP-HPLC conditions used.

In experiments carried out in DMEM containing FBS (10%, 5% or 2%), the Met-E disappearance followed first-order kinetics. The extent of enzymatic hydrolysis as a function of incubation time is shown in Figure 1. The half-life values were calculated from the first-order rate constants obtained from semi-logarithmic plots. A marked degradation of Met-E was determined in the presence of 10% FBS ($t_{1/2} = 2.8$ h), i.e. under the conditions frequently used to study the effects of the drugs on human cancer cell growth.

The route of Met-E (Tyr-Gly-Gly-Phe-Met) degradation observed in mammalian systems involves an aminopeptidase action, cleaving the Tyr-Gly peptide bond, combined with endopeptidase actions, cleaving the Gly-Phe bond [18, 19]. The peptidase inhibitors bestatin, captopril and thiorphan were tested for their ability to inhibit the actions of peptidases contained in FBS. Bestatin is an effective inhibitor of aminopeptidase, while captopril and thiorphan inhibit the cleavage of the Gly-Phe peptide bond in enkephalins and related neuropeptides. Figure 2 summarizes the effects of a combination of protease inhibitors on the stability to

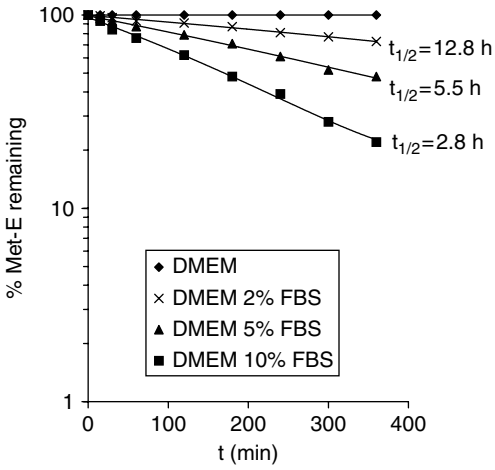

Figure 1 Kinetics of degradation of Met-E in the DMEM medium alone (♦) or in DMEM containing 2% (×), 5% (▲) or 10% (■) FBS at 37 °C. Each point represents the mean of three separate determinations.

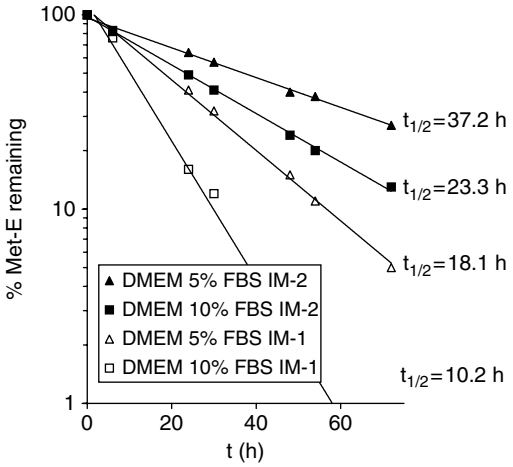

Figure 2 Combined effects of two different mixtures of peptidase inhibitors, bestatin, captopril and thiorphan, on degradation of Met-E in the DMEM medium containing either 10% (■, □) or 5% (▲, Δ) FBS. The mixture of peptidase inhibitors was given to the medium 15 min before the opioid peptide. Each point represents the mean of three separate determinations. IM-1 represents a mixture of bestatin, captopril and thiorphan at the final concentration of 10 μM each. IM-2 represents mixture of bestatin (final concentration 30 μM) whereas the concentration of captopril and thiorphan remained 10 μM.

enzymatic hydrolysis of Met-E in DMEM containing either 10% or 5% FBS. The presence of bestatin, thiorphan and captopril, at a final concentration of 10 μM each (IM-1), reduced the degradation of Met-E, as shown by the threefold increase in half-lives in both FBS containing media. The involvement of aminopeptidases in Met-E degradation, under the conditions studied, was demonstrated by the increasing concentration of bestatin to 30 μM in the mixture of peptidase inhibitors

(IM-2), whereas the concentration of thiorphan and captopril remained at 10 μM. As presented in Figure 2, an increased concentration of bestatin reduced significantly the degradation of Met-E, as evaluated by the increase in half-lives to 23 h and 37 h in media containing either 10% or 5% FBS, respectively. However, the cleavage of Met-E still proceeded, indicating the presence of different endo- and exopeptidase activities in FBS.

Cytotoxic Effects of Met-E on Human Tumour Cells

In this study two tumour cell lines (HEp-2 and SW620) were used as a model to investigate the effect of protease inhibitors (bestatin, captopril, thiorphan) on the growth-inhibitory effects of Met-E. The cytotoxic effects of Met-E at a concentration of 10^{-4} M on tumour cells were compared in the presence or absence of a mixture of inhibitors (IM-1 and IM-2). The cells were exposed to Met-E and/or inhibitors for 3, 6, 24 or 72 h. Tumour cells were grown for 3 weeks prior to the experiment in medium supplemented with 10%, 5% or 2% FBS. As expected, the cells grew slower in DMEM containing 2% FBS (Figure 3).

The cytotoxic effects of the Met-E differed between the two tumour cell lines tested. Statistically significant to mild or no suppression of cell proliferation was observed in all cultures (Figure 3). A time-course study showed no statistically significant differences in comparison with the control in HEp-2 and SW620 cells exposed to Met-E for 3, 6 or 24 h in media containing different amounts of FBS. However, after 72 h Met-E statistically significantly ($p \leq 0.05$) suppressed the proliferation of HEp-2 cells grown in media supplemented with either 5% or 2% FBS, but not that of SW620 cells.

In the inhibitory tests, the cells in DMEM, containing different amounts of FBS, were preincubated for a short time (1 min) with the mixture of peptidase inhibitors (IM-1) and then immediately exposed to Met-E. In

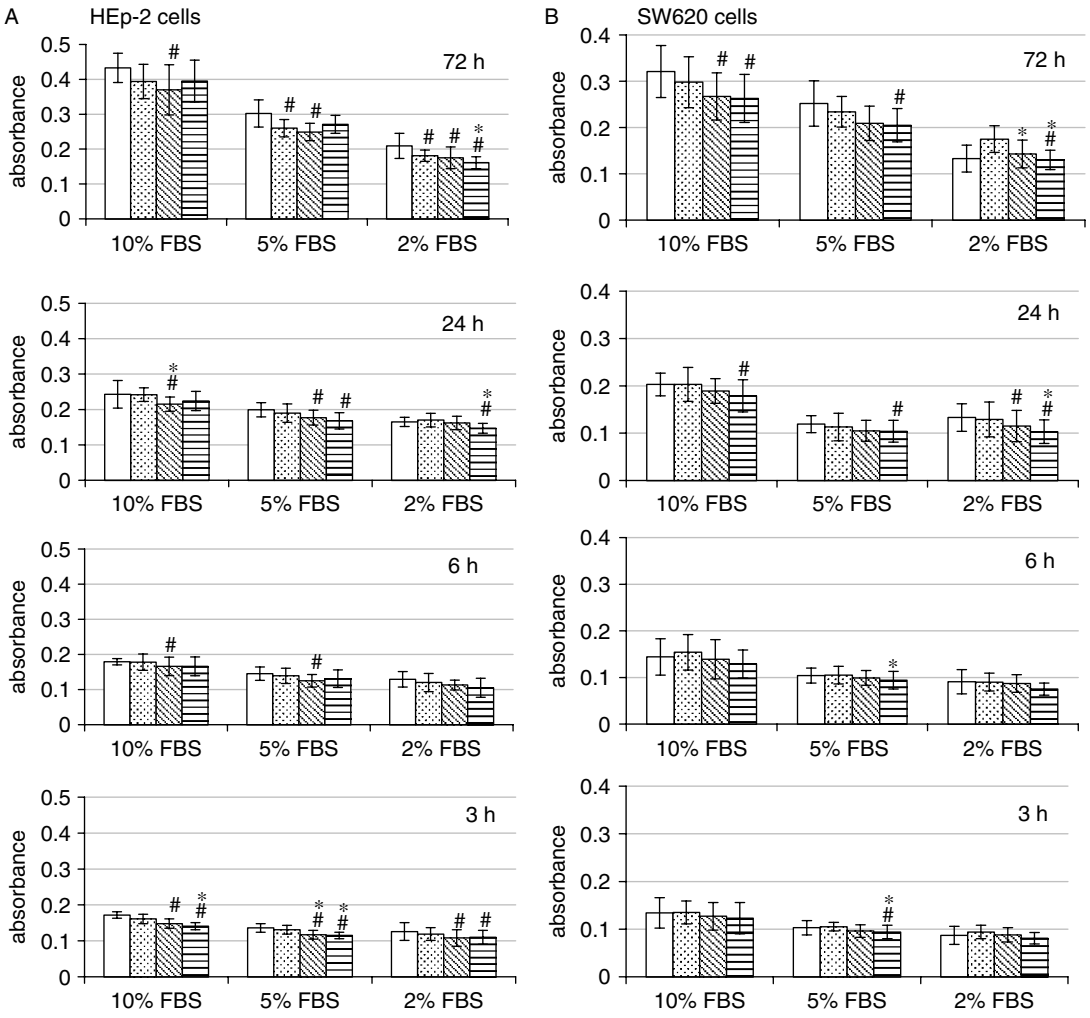

Figure 3 Cytotoxicity studies (MTT assay) on (A) HEp-2 and (B) SW620 cells treated with the test compounds in DMEM containing different concentrations of FBS for 72, 24, 6 and 3 h: control (□), Met-E (▣), Met-E + IM-1 (▨), Met-E + IM-2 (▤). Data are expressed as means of three individual experiments conducted in triplicate ±SD. # denotes statistically significant difference from control ($p \leq 0.05$), * denotes statistically significant difference from Met-E ($p \leq 0.05$).

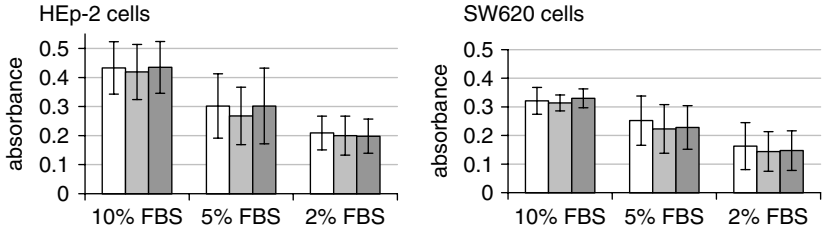

Figure 4 Growth inhibitory effects of the mixture of peptidase inhibitors on HEp-2 and SW620 cells treated for 72 h: control (□), IM-1 (▩), and IM-2 (■). Data are expressed as means of three individual experiments conducted in triplicate ±SD.

both lines a statistically significant suppression of cell growth by the combination of inhibitors and Met-E was observed, compared with cells exposed to the peptide alone and cells grown in the absence of Met-E (Figure 3). HEp-2 cells grown in media containing 10% and 5% FBS were especially sensitive to the combination treatment, showing a statistically significant difference from control even after 3 and 6 h incubation (Figure 3A). SW620 cells incubated in the presence of Met-E and IM-1 showed significant levels of growth suppression only for an incubation period of 72 h in the media containing either 10% or 5% FBS (Figure 3B). Next, examined was whether an increased concentration of bestatin in the mixture of peptidase inhibitors (IM-2) potentiates the antiproliferative effect of Met-E on the cell lines. Inhibitory effects on HEp-2 cells of the combination of IM-2 and Met-E were higher compared with Met-E, and statistically significant after an incubation period of 3 h in the media containing 10% and 5% FBS, and after 24 h and 72 h in the medium with 2% FBS. However, an increased concentration of bestatin in the IM-2 mixture showed only a weak augmentation of growth-inhibitory effects compared with exposure of HEp-2 cells to the Met-E and IM-1 combination. In fact, almost no differences in cell growth of HEp-2 lines from controls were observed after a 3 day treatment in media containing increased amounts of FBS (Figure 3A). In the SW620 cell-line, a combination of Met-E and IM-2 showed a significant inhibitory effect on cell growth even after treatment for 3 days. These results confirm the thesis of Van der Valk *et al.* [20] that serum is in general an extremely complex mixture of a large number of constituents and for research purposes, especially for cytotoxic studies, it is necessary to use serum-free media and alternatives for FBS.

Mixtures of the three peptidase inhibitors (IM-1, IM-2) reduced the growth of cancer cells after 3 days of incubation (up to 13%) (Figure 4). However, a shorter exposure to IM-2 did not inhibit the growth of both cell lines tested (data not shown).

Recent studies indicated that bestatin efficiently induced apoptosis of solid tumour cell lines in combination with death ligands, such as Fas ligand and TNF-α, although bestatin alone did not cause apoptosis in these cells [21]. These results suggested that the synergistic effects of bestatin on the effects of death ligands might be due to inhibition of the neutral aminopeptidase activity. On the other hand, aminopeptidase inhibitors can influence the related mRNA expression, since bestatin causes an increase in aminopeptidase expression in a time- and dose-dependent manner [22]. The precise role of membrane-associated peptidases in tumour growth and metastasis still needs to be defined. However, their enzymatic activity alters the biological activity of molecules that regulate tumour cell growth. Our findings add new aspects to observations on the influence of Met-E, a native opioid growth factor, on tumour cell proliferation. In both tumour cell lines investigated, a combination of Met-E and a mixture of peptidase inhibitors showed a synergistic inhibitory effect on cell growth compared with treatment with either agent alone. The growth-inhibitory effect by combination treatment was not time-dependent, suggesting that the observed augmenting effects are not only related to inhibition of Met-E degradation but also to specific actions of ligands in the mixture on the plasma membrane of tumour cells.

CONCLUSION

In order to be developed as therapeutic agents against cancer, peptide-based therapeutics should have strong tumoricidal activity and be short in length to ease the production. In the present study, by using Met-E as a substrate, it was shown that exposure of the peptide to the cell culture medium DMEM containing heat inactivated FBS resulted in a fast degradation. The rate of hydrolysis of Met-E was reduced significantly by co-incubation with a mixture of bestatin, captopril and thiorphan (IM-1 and IM-2) to inhibit the actions of peptidases contained in FBS.

The results from the antiproliferative assay on tumour cell lines HEp-2 and SW620 revealed that a mixture of protease inhibitors, in combination with Met-E, statistically significantly augmented the growth-inhibitory effect of the opioid pentapeptide. Inhibitors alone did not have any marked role on the proliferation of these cells. The observed increasing effect was not a time-dependent phenomenon, as 3 h exposure was

sufficient for the combination of Met-E and IM-1 or IM-2 to show an antiproliferative effect on both tumour cell lines.

In conclusion, the results indicate that, under the conditions frequently used to study effects of the drugs on human cancer cell growth, FBS and membrane-associated peptidases can rapidly cleave short peptide compounds. Investigations of the metabolic and chemical stabilities of peptides, as well as of the antiproliferative effects, in combination with peptidase inhibitors, should be useful in developing peptide-based anticancer therapeutics.

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