ELSEVIER

European Journal of Pharmacology 347 (1998) 95–99

ejp

Immunologic action of [Met^5]enkephalin fragments

Jan Kowalski *

Department of Clinical Pharmacology, Silesian University School of Medicine, ul. Medyków 18, 40-752, Katowice, Poland

Received 11 December 1997; revised 22 January 1998; accepted 27 January 1998

Abstract

The aim of this study was to evaluate the effects of [Met^5]enkephalin, des-Met-[Met^5]enkephalin, des-Tyr-[Met^5]enkephalin, and Tyr–Gly–Gly on natural killer cytotoxic activity and on concanavalin A- and lipopolisaccharide-stimulated proliferation of B and T cells in mice. Single i.p. injections of [Met^5]enkephalin, des-Met-[Met^5]enkephalin, and Tyr–Gly–Gly increased both natural killer cytotoxicity and proliferation of mitogen-stimulated B and T cells. These effects were inhibited by naloxone pretreatment, which suggests the opioid mechanism of the peptides studied. The rate of lymphocytic proliferation increases not only after single injection of [Met^5]enkephalin or its metabolites, but also after 3 or 7 days of treatment. Apart from the functional effects, [Met^5]enkephalin, des-Met-[Met^5]enkephalin, and Tyr–Gly–Gly increased the percentages of natural killer cells and T cells. The results of this study suggest that the immunomodulatory action of [Met^5]enkephalin may be mediated or enhanced by its N-terminal metabolites des-Met-[Met^5]enkephalin and Tyr–Gly–Gly. © 1998 Elsevier Science B.V.

Keywords: [Met^5]enkephalin; Des-Met-[Met^5]enkephalin; Tyr–Gly–Gly; Immunomodulation

1. Introduction

[Met^5]enkephalin is a neuropeptide that modulates the activity of central neurons, and it also may affect natural killer activity (Kay et al., 1984; Kraut and Greenberg, 1986; Kowalski, 1997) and T cell proliferation (Srisuchart et al., 1989; Kusnecow et al., 1989). When administered in vivo, [Met^5]enkephalin is broken down within minutes by amino- and carboxypeptidases that are present in both plasma (Kerr and Kenny, 1974; Hambrook et al., 1976) and immune cells' membranes (Roscetti et al., 1990). Therefore, it is not clear whether [Met^5]enkephalin alone or together with its active metabolites modulates the functions of immune cells.

The aim of this study was to evaluate the effects of [Met^5]enkephalin, and the products of [Met^5]enkephalin breakdown (des-Met-[Met^5]enkephalin, des-Tyr-[Met^5]enkephalin and Tyr–Gly–Gly), on the cytotoxic activity of natural killer cells and on the mitogen-induced proliferation of B and T cells.

2. Materials and methods

2.1. Animals

The experiment was performed on female C57Bl/6 mice aged 4–6 weeks and weighing 20 ± 1 g obtained from the Animal Farm of the Silesian University School of Medicine. They were kept 8 per cage at room temperature and under standard light conditions. They received a standard mouse chow and water ad libitum.

2.2. Agents

[Met^5]enkephalin, des-Met-[Met^5]enkephalin, des-Tyr-[Met^5]enkephalin, and Tyr–Gly–Gly were purchased from Sigma. ^{51}Cr used in cytotoxicity assay was provided by DuPont NEN. FITC anti-mouse CD45R/B220, 5E6 natural killer cell antigen and CD90 (Thy-1.2) were purchased from Phar-Mingen.

2.3. Culture medium

Culture medium RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 2 mM glutamine, 100 μg/ml streptomycin, 100

* Corresponding author. Tel.: +48-32-152-39-02; fax: +48-32-1523902.

UI/ml penicillin designated as complete medium was used.

2.4. Cytotoxicity assay

Spleens of opioid-treated and control mice were excised under sterile conditions. A spleen cell suspension was prepared using a cell dissociation sieve-tissue grinder (Sigma). Mononuclear cells were separated by gradient centrifugation using Lymphoprep (Nyegaard, Oslo, Norway). Then, a splenocyte layer was removed, resuspended in complete medium, washed 4 times, placed in a 96-well microtiter plate (Flow-Linbro) and incubated for 2 h (37°C, 5% CO_2, 80% humidity).

YAC-1 cells were used as target cells in natural killer cytotoxicity assay. They were incubated in complete medium for 2 h with 200 μCi (^{51}Cr) chromate, and then washed 4 times and mixed with effector cells at different ratios. Natural killer cell activity was measured after 4-h incubation. Experimental ^{51}Cr release (ER) was measured in 100 μl of supernatant. Maximal ^{51}Cr release (MR) was defined as the release after addition of 100 μl 1% sodium dodecyl sulphate. Spontaneous release (SR) was measured in ^{51}Cr-labelled target cells incubated in complete medium alone. Specific lysis was calculated as follows:

$$\text{Specific lysis\%} = \text{ER} - \text{SR}/\text{MR} - \text{SR} \times 100$$

The experiment was performed three times.

2.5. Mitogen-induced proliferative responses

Spleen cells were obtained under sterile conditions as described by Van den Bergh et al. (1991). After centrifugation (10 min, 350 g), they were resuspended in 4 ml of lysis buffer (155 mM ammonium chloride, 10 mM potassium hydrocarbonate, pH 7.0) and kept on ice for 7 min to lyse erythrocytes. Afterwards, 4 ml of fetal calf serum were layered under the cell suspension, which was then centrifuged (20 min, 50 g). The cell pellet was resuspended in culture medium. Cell viability determined with trypan blue was greater than 90%. Spleen cell were cultured in quadruplicate, in a 96-well flat-bottom microtiter culture plate (Beckton Dickinson), 1.0×10^5 cells/0.1 ml/well. Mitogens were added in a volume of 10 microliters per each well. Lipopolysaccharide (Sigma) was added at final concentrations of 0.5 and 5.0 μg/ml, and concanavalin A (Sigma) at 1.0 and 5.0 μg/ml. Since concanavalin A preferentially stimulates T cell proliferation, while lipopolisaccharide stimulates B cell proliferation, splenocytic response to these mitogens was considered either T- or B cell proliferation. After 72-h incubation, the number of cells was assessed by tetrazolium dye (thiazolyl blue) assay, in which the amount of formazan is proportional to the number of metabolically active cells (Hansen et al., 1989). Thiazolyl blue in a volume of 50 μl (5 mg/ml RPMI 1640) was added to each culture. After 3-h incubation (95% air and 5% CO_2 at 37°C), 50 μl of solvent (450 ml of dimethylformamide, 135 g of sodium dodecyl sulphate, and 550 ml of distilled water) was added to each culture. After overnight incubation at room temperature, optical density at 570 nm was measured. The experiment was performed in quadruplicate. The number of splenocytes was calculated using a calibration curve (extinction/splenocyte number). The number of mitogen-stimulated splenocytes obtained from opioid-treated animals was compared to the number of unstimulated splenocytes from the same animals and expressed as a percentage. In addition, the percentage of splenocytes obtained from peptide-treated animals was compared to the percentage of splenocytes from saline-treated animals.

Differences were analyzed with Student's t-test. Results are expressed as the arithmetic mean of three experiments (four measurements in each experiment).

2.6. Animal treatment

In my previous study, opioid peptides given at 1, 10, 20, 100, 200 μg/mouse increased natural killer activity after 1, 7 and 14 days of treatment. The increase was highest after 10 μg/mouse. In all treatment periods, the levels of natural killer activity were similar (Kowalski, 1997). In the present study, I investigated whether [Met^5]enkephalin, des-Met-[Met^5]enkephalin, and Tyr–Gly–Gly given at a dose of 10 μg/mouse for 1, 3 and 7 days affect the proliferation of immune cells. The effects of these opioids on natural killer activity were analyzed for two different opioid doses (1 or 10 μg/mouse) given only once.

The peptides were dissolved in sterile phosphate buffered saline and given i.p. in a volume of 0.1 ml/mouse. In determining the percentages of natural killer, T and B cells, the opioid dose was 10 μg/mouse.

The specificity of opioid response was tested by animal pretreatment with naloxone, an opioid receptor antagonist, give at a dose of 10 μg/mouse.

2.7. Determination of percentages of natural killer, B and T cells

Mononuclear cells were separated by Lymphoprep gradient centrifugation, washed with phosphate buffered saline, and suspended in RPMI 1640 medium supplemented with 5% fetal calf serum and 0.1% sodium azide. Then the cells (1×10^6) were incubated with 10 μl of monoclonal antibodies conjugated with fluorescein isothiocyanate in a total volume of 100 μl for 30 min (4°C). The following antibodies were used: 5E6 for natural killer cells, Thy 1.2 for T cells, and CD45R/B220 for B lymphocytes. After incubation, the cells were washed twice with RPMI medium supplemented with 5% fetal calf serum and 0.1% sodium azide, and then resuspended in 50

Table 1
Effect of fragments of [Met^5]enkephalin on natural killer cell activation in vivo

Agent	Dose (μg/mouse)	% Cytotoxicity ± S.E.		
		100:1	50:1	25:1[c]
Control	–	25 ± 0.6	13 ± 0.2	7 ± 0.2
[Met^5]enkephalin	1	29 ± 0.5[a]	17 ± 0.3[a]	9 ± 0.1
[Met^5]enkephalin	10	32 ± 0.6[a]	18 ± 0.3[a]	12 ± 0.2[a]
Naloxone + [Met^5]enkephalin	10 + 10	23 ± 1.0[b]	11 ± 0.3[b]	6 ± 0.2[b]
Des-Met-[Met^5]enkephalin	1	34 ± 0.5[a]	23 ± 0.2[a]	12 ± 0.2[a]
Des-Met-[Met^5]enkephalin	10	29 ± 0.1[a]	18 ± 0.2[a]	11 ± 0.1[a]
Naloxone + des-Met-[Met^5]enkephalin	10 + 10	25 ± 1.1[b]	14 ± 0.2[b]	10 ± 0.2
Tyr–Gly–Gly	1	30 ± 0.5[a]	18 ± 0.4[a]	10 ± 0.2[a]
Tyr–Gly–Gly	10	40 ± 0.6[a]	27 ± 0.6[a]	16 ± 0.2[a]
Naloxone + Tyr–Gly–Gly	10 + 10	24 ± 0.1[b]	14 ± 0.2[b]	9 ± 0.2[b]
Des-Tyr-[Met^5]enkephalin	1	22 ± 0.6	11 ± 0.1	8 ± 0.2
Des-Tyr-[Met^5]enkephalin	10	23 ± 0.3	12 ± 0.2	8 ± 0.2
Naloxone	10	25 ± 0.8	13 ± 0.3	7 ± 0.2

Mice (3/group) were injected i.p. with different doses of agents dissolved in phosphate buffered saline 1 day before splenic natural killer cell activity was assessed (see Section 2). Results are expressed as the mean ± S.E. of three experiments.
[a]Significant increase in comparison with Control: $P < 0.01$.
[b]Significant decrease by naloxone of the peptide effect in comparison with peptide: $P < 0.01$.
[c]Effector: target cell ratio.

Table 2
The influence of fragments of [Met^5]enkephalin on proliferation of stimulated mouse splenocytes

Agent	Percentage of lymphocytes stimulated of			
	Concanavalin A		Lipopolisaccharide	
	1 μg/ml	5 μg/ml	0.5 μg/ml	5 μg/ml
Control	600 ± 8	1100 ± 22	440 ± 7	784 ± 16
[Met^5]enkephalin	905 ± 8[a]	1662 ± 117[a]	540 ± 11[a]	1064 ± 20[a]
Naloxone + [Met^5]enkephalin	622 ± 10[b]	1072 ± 10[b]	460 ± 5[b]	784 ± 8[b]
Control	605 ± 35	1100 ± 40	440 ± 8	870 ± 6
Tyr–Gly–Gly	846 ± 17[a]	1705 ± 20[a]	760 ± 5[a]	1000 ± 12[a]
Naloxone + Tyr–Gly–Gly	633 ± 10[b]	1175 ± 12[b]	560 ± 7[b]	820 ± 7[b]
Control	580 ± 9	1050 ± 13	460 ± 6	784 ± 8
Des-Met-[Met^5]enkephalin	820 ± 27[a]	1819 ± 48[a]	510 ± 16	1064 ± 25[a]
Naloxone + des-Met-[Met^5]enkephalin	579 ± 11[b]	1130 ± 15[b]	480 ± 6	882 ± 8[b]
Control	600 ± 23	1120 ± 16	480 ± 17	830 ± 47
Des-Tyr-[Met^5]enkephalin	674 ± 24	1277 ± 70	470 ± 11	790 ± 41
Naloxone	561 ± 8	1050 ± 15	450 ± 6	780 ± 7

Mice (3–4/group) were injected i.p. once with studied agent at a dose 10 μg/mouse or phosphate buffered saline 24 h before splenic lymphocytes proliferation was assessed (see Section 2). Naloxone at a dose of 10 μg was injected i.p. 30 min prior to peptide administration. Results are expressed as the mean ± S.E. of three experiments.
[a]Significant change in comparison with control: ($P < 0.01$).
[b]Significant change of peptide effect elicited by naloxone in comparison with peptide, ($P < 0.01$).

Table 3
Effect of fragments of [Met^5]enkephalin on natural killer cells and lymphocytes percentage

Agent	Dose (μg/mouse)	Percentage of		
		Natural killer cells	T lymphocytes	B lymphocytes
Control	–	3 ± 0.2	38 ± 2.9	43 ± 2.7
[Met^5]enkephalin	10	9 ± 0.3[a]	57 ± 2.2[a]	47 ± 2.5
Tyr–Gly–Gly	10	11 ± 0.7[a]	52 ± 1.8[a]	44 ± 1.5
Des-Met-[Met^5]enkephalin	10	8 ± 0.4[a]	46 ± 0.3[b]	45 ± 1.8

Mice (3/group) were injected with agents once. Percentage of natural killer cells and lymphocytes phenotype was determined 1 day after injection (see Section 2). Results are expressed as the mean ± S.E. of three experiments (in each experiment at least 400 cells were counted in 3 samples). Significant increase in comparison with control: [a]($P < 0.01$), [b]($P < 0.05$).

μl of the same medium. At least 400 cells in each smear were counted under a fluorescent microscope and the percentage of cells with green fluorescence was calculated.

3. Results

[Met^5]enkephalin, des-Met-[Met^5]enkephalin and Tyr–Gly–Gly at doses of 1 and 10 μg/mouse increased natural killer activity, while des-Tyr-[Met^5]enkephalin had no effect. Naloxone pretreatment cancelled the stimulatory effects of the peptides (Table 1).

10 μg of [Met^5]enkephalin, des-Met-[Met^5]enkephalin, or Tyr–Gly–Gly increased the proliferation of concanavalin A-stimulated T cells and lipopolisaccharide-stimulated B cells in the suspension of mouse splenocytes. Des-Tyr-[Met^5]enkephalin did not alter the proliferation of mitogen-stimulated splenocytes. In naloxone-pretreated mice, the peptides did not stimulate B and T cell proliferation (Table 2).

Single injections of [Met^5]enkephalin, des-Met-[Met^5]enkephalin, and Tyr–Gly–Gly (each at 10 μg/mouse) increased the percentages of natural killer cells and T cells, but not the percentage of B cells (Table 3).

To evaluate the effects of prolonged administration of [Met^5]enkephalin and its metabolites on cell proliferation, the peptides were injected for 3 or 7 days. [Met^5]enkephalin, des-Met-[Met^5]enkephalin, and Tyr–Gly–Gly (all at 10 μg/mouse) given for 3 or 7 days increased blastogenesis of cells stimulated with lipopolisaccharide (0.5 and 5 μg/ml) or with concanavalin A (1 and 5 μg/ml) (Table 4).

4. Discussion

Although many studies have shown that [Met^5]enkephalin modulates the functions of immune cells, there have been few investigations into the immunomodulatory effects of [Met^5]enkephalin metabolites. Sizemore et al., 1991 have found that Tyr–Gly–Gly and Tyr–Gly increase the in vitro proliferation of T cells stimulated by concanavalin A. Roscetti et al. (1988), have shown that des-Met-[Met^5]enkephalin stimulates the proliferation of human T cells, and have proposed that its N-terminal fragments continue to have opioid activity. However, there are no reports of the in vivo effects [Met^5]enkephalin fragments on the proliferation of lymphocytes and on the activity of natural killer cells.

This study has shown that [Met^5]enkephalin and its N-terminal metabolites increase natural killer activity and stimulate both T and B cells to proliferate. The mechanism of opioid-stimulated proliferation of lymphocytes is not clear. It is most likely that the increased natural killer activity and T-lymphocyte proliferation reflect increased percentages of natural killer and T cells present in the cell populations tested. Since lymphocytes and natural killer cells have been proven to have opioid receptors (Carr, 1992), it cannot be excluded that the opioids studied directly affect the activities of both cell types. Naloxone pretreatment prevented the peptides from increasing T and B cell proliferation and natural killer activating, suggesting that their effects are mediated by opioid receptors.

Opioid peptides may also indirectly alter the activity of immune cells via affecting levels of neurohormones. Such neurohormones as adrenocorticotropin and somatostatin inhibit the activity of natural killer cells and proliferation of lymphocytes; others, such as growth hormone, prolactin, substance P, increase the activity of immune cells. On one

Table 4
Spleens cell response to mitogens in mice administered peptides daily for 3 or 7 days

Agent	Percentage of lymphocytes stimulated of			
	Concanavalin A		Lipopolisaccharide	
	1 μg/ml	5 μg/ml	0.5 μg/ml	5 μg/ml
3 injections				
Control	604 ± 19	1110 ± 16	440 ± 10	780 ± 13
[Met^5]enkephalin	738 ± 21[a]	1469 ± 37[a]	505 ± 10[b]	883 ± 10[b]
Des-Met-[Met^5]enkephalin	681 ± 11[b]	1566 ± 36[a]	544 ± 16[a]	1113 ± 23[a]
Tyr–Gly–Gly	784 ± 11[a]	1749 ± 16[a]	538 ± 20[a]	938 ± 10[a]
7 injections				
Control	636 ± 19	1150 ± 16	460 ± 8	800 ± 10
[Met^5]enkephalin	770 ± 14[a]	1432 ± 37[b]	527 ± 9[b]	905 ± 9
Des-Met-[Met^5]enkephalin	779 ± 13[a]	1454 ± 33[a]	543 ± 6[a]	915 ± 9[b]
Tyr–Gly–Gly	787 ± 11[a]	1750 ± 16[a]	584 ± 4[a]	960 ± 9[a]

Group of six female C57BL/6 mice were administered vehicle or peptides daily for 3 or 7 days by the i.p. route. One day after the last exposure, spleen cells were cultured with mitogens or medium alone as in Section 2. Results are expressed as the mean ± S.E. of two undependent experiment. Significant change in comparison with control: [a]$P < 0.01$, [b]$P < 0.05$.

hand, opioids increase adrenocorticotropin levels in blood (Buckingham, 1982), decreasing the immune cells' activity, and on the other hand, they decrease somatostatin levels (McIntosh et al., 1994) and increase growth hormone (Govaerts et al., 1990) and prolactin levels (Blackford et al., 1992), thus activating lymphocytes. It seems the opioid studied may indirectly increase the activity of immune cells, i.e., via upsetting the balance between the inhibiting and stimulating factors in favour of the latter.

In my study, des-Tyr-[Met^5]enkephalin affected neither natural killer activity nor the proliferation of T and B cells. This finding is in accordance with studies showing that tyrosine is the key amino acid for opioid peptides and also for endorphins, because Heijnen et al. (1986) and Shahabi et al. (1991) have shown that des-Tyr-α-endorphin and des-Tyr-β-endorphin have no biologic activity in immunologic tests.

Animal studies have shown that [Met^5]enkephalin prolongs the lifespan of mice with leukemia L1210 (Plotnikoff et al., 1985), reduces experimental metastasis of B-16 melanoma cells in mice (Faith and Murgo, 1988), inhibits tumor growth in mice with fibroblastoma (Srisuchart et al., 1989), and acts synergistically with azidothymidine in mice infected by Friend virus leukemia (Specter et al., 1994). [Met^5]enkephalin activates natural killer cells, stimulates mitogen-stimulated blastogenesis, and increases the percentages of T cell subset populations in HIV-infected patients (Wybran et al., 1987; Wybran and Plotnikoff, 1991). The rate of lymphocytic proliferation increases not only after single injection of [Met^5]enkephalin or its metabolites, but also after 3 or 7 days of treatment. Similarly, natural killer activity is increased after 7- or 14-day administration of [Met^5]enkephalin (Kowalski, 1997).

The results of this study indicate that [Met^5]enkephalin alone and/or via its N-terminal metabolites may affect the immune cells. Moreover, it seems that the short action of [Met^5]enkephalin may be prolonged by its two active metabolites, Tyr–Gly–Gly and des-Met-[Met^5]enkephalin.

References

Blackford, S.P., Little, P.J., Kuhn, C.M., 1992. Mu- and kappa-opiate receptor control of prolactin secretion in rats: ontogeny and interaction with serotonin. Endocrinology 132, 2891–2897.

Buckingham, J.C., 1982. Secretion of corticotropin and its hypothalamic releasing factor in response to morphine and opioid peptides. Neuroendocrinology 35, 111–116.

Carr, D.J.J., 1992. Neuroendocrine peptide receptors on cells of the immune system. Chem. Immunol. 52, 84–105.

Faith, R.E., Murgo, A.J., 1988. Inhibition of pulmonary metastases and enhancement on natural killer cell activity by methionine-enkephalin. Brain Behav. Immunol. 2, 114–122.

Govaerts, J., Buydens, P., Finne, E., Matton, A., Vanhaelst, L., 1990. The growth hormone secretory response to fentanyl in rat: an involvement of μ type receptors. J. Endocrinol. Invest. 13, 911–915.

Hambrook, J.M., Morgan, B.A., Rance, M.J., Smith, C.F., 1976. Mode of deactivation of the enkephalins by rat and human plasma and rat brain homogenates. Nature 262, 782–783.

Hansen, M.B., Nielsen, S.E., Berg, K., 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119, 203–210.

Heijnen, C.J., Bevers, C., Kavelaars, A., Ballieux, R.E., 1986. Effect of α-endorphin on the antigen-induced primary antibody response of human blood B-cells in vitro. J. Immunol. 136, 213–216.

Kay, N.E., Allen, J., Morley, J.E., 1984. Endorphins stimulate normal human peripheral blood lymphocyte natural killer activity. Life Sci. 35, 53–59.

Kerr, M.A., Kenny, A.J., 1974. The purification and specificity of a neutral endopeptidase from rabbit kidney brush border. Biochem. J. 137, 477–488.

Kowalski, J., 1997. Effect of enkephalins and endorphins on cytotoxic activity of natural killer cells and macrophages/monocytes in mice. Eur. J. Pharmacol. 326, 251–255.

Kraut, R.P., Greenberg, A.H., 1986. Effect of endogenous and exogenous opioids on splenic natural killer cell activity. Nat. Immunol. Cell Growth Regul. 5, 28–40.

Kusnecow, A.W., Husband, A.J., King, M.G., Smith, R., 1989. Modulation of mitogen-induced spleen cell proliferation and the antibody-forming cell response by β-endorphin in vivo. Peptides 10, 473–479.

McIntosh, C., Yago, V., Kwok, Y.N., 1994. Beta-funaltrexamine blockade of opioid-induced inhibition of somatostatin secretion from rat stomach. Eur. J. Pharmacol. 259, 331–334.

Plotnikoff, N., Murgo, A.J., Miller, G.C., Corder, C.N., Faith, R.E., 1985. Enkephalins: immunomodulators. FASEB J. 44, 118–122.

Roscetti, G., Ausiello, C.M., Palma, C., Gulla, P., Roda, L.G., 1988. Enkephalin activity on antigen-induced proliferation of human peripheral blood mononuclear cells. Int. J. Immunopharmacol. 10, 819–823.

Roscetti, G., Bongiorno, L., Urbani, A., Marini, M., Roda, L.G., 1990. Hydrolysis and binding of leucine–enkephalin to lymphomic and erythroleukaemic cell lines. Int. J. Immunopharmacol. 12, 391–396.

Shahabi, N.A., Burtness, M.Z., Sharp, B.M., 1991. *N*-Acetyl-β-endorphin antagonizes the suppressive effect of β-endorphin on murine splenocyte proliferation via a naloxone-resistant receptor. Biochem. Biophys. Res. Commun. 3, 936–942.

Sizemore, R.C., Dienglewicz, R.L., Pecunia, E., Gottlieb, A.A., 1991. Modulation of concanavalin A-induced antigen-nonspecific regulatory cell activity by leu-enkephalin and related peptides and related peptides, 60, pp. 310–318.

Specter, S., Plotnikoff, N., Badley, W.G., Goodfellow, D., 1994. Methionine enkephalin combined with AZT therapy reduce murine retrovirus-induced disease. Int. J. Immunopharmacol. 16, 911–917.

Srisuchart, B., Fuchs, B.A., Sikorski, E.E., Munson, A.E., Loveless, S.E., 1989. Antitumor activity of enkephalin analogues in inhibiting PYB6 tumor growth in mice and immunological effects of methionine-enkephalinamide. Int. J. Immunopharmacol. 11, 487–500.

Van den Bergh, P., Rozing, J., Nagelkerken, L., 1991. Two opposing modes of action of β-endorphin on lymphocyte function. Immunology 72, 537–543.

Wybran, J.N., Plotnikoff, N.F., 1991. Methionine enkephalin, a new lymphokine for the treatment of ARC patients. In: Plotnikoff, N., Murgo, A., Faith, R., Wybran, J. (Eds.), Stress and Immunity, CRC Press, Boca Raton, FL, pp. 417–432.

Wybran, J., Schandene, J., Van Vooren, J.P., Vandermoten, G., Latinne, D., Sonnet, J., De Bruyere, M., Taelman, H., Plotnikoff, N., 1987. Immunologic properties of methionine-enkephalin, and therapeutic implications in AIDS, ARC, and cancer. Ann. N.Y. Acad. Sci. 496, 108–114.