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NOVEL INHIBITORS OF ENKEPHALIN-DEGRADING ENZYMES I: INHIBITORS OF ENKEPHALINASE BY PENICILLINS

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Several penicillins have been found to have pro-antinociceptive properties and also to be enkephalinase (neutral endopeptidase-24.11) inhibitors, carfecillin being the most potent. Carfecillin i.c.v. (but not i.p.) had significant antinociceptive activity in the mouse tail immersion test and completely suppressed abdominal constrictions (acetic acid) in mice (IC_{50} = 23μg/animal). In combination with (D-Ala^2–D-leu^5)-enkephalin (DADL) i.c.v. in the abdominal constriction test the complete protection observed was reversed by the opioid receptor antagonist naltrexone. Carfecillin was a competitive inhibitor of enkephalinase from mouse brain striata (IC_{50} = 207 + 57 nM, cf thiorphan 10.6 ± 1.9 nM) but did not inhibit other known enkephalin- degrading enzymes. Carfecillin provides a new lead structure for the development of more potent enkephalinase inhibitors.

KEY WORDS: Penicillins, Enkephalinase, Enkephalin, Enkephalin-degrading enzymes.

INTRODUCTION

Enkephalins are endogenous pentapeptides serving as neurotransmitters or modulators in the central nervous system by stimulating opioid receptors. Their effects are weak and transient due to termination of the enkephalinergic signal by enzymic degradation as evidenced firstly by extremely rapid deactivation of endogenously administered enkephalins *in vivo*[1,2]; secondly by production of analgesia in rodents[3] and humans[4] with partially enzyme-resistant enkephalin analogues and thirdly, by increased levels of endogenously released enkephalins in the presence of effective inhibitors of the enkephalin-degrading enzymes[5].

A large number of peptidases occur in the CNS, many of which are capable of catalysing the hydrolysis of the enkephalins, e.g. carboxypeptidase[6], acetylcholinesterase[7], peptidyl peptidase A[8] (ACE), dipeptidyl aminopeptidase[9] (enkephalinase B), enkephalinase[10] (neutral endopeptidase-24.11) and a variety of fully or partially characterised aminopeptidases of either a soluble[11–15]or membrane bound form[16–18]. Aminopeptidase and enkephalinase are considered to account for about 90% of the degradation at the synapse and hydrolyse enkephalins at the tyrosinylglycinyl and glycinylphenylalanyl amide bonds respectively. Enkephalinase is considered to be the more important degradation enzyme due to its location at cerebral synaptic membranes[19], its general association with enkephalins in the brain[20–23] and its release characteristics derived from *in vitro* studies using brain tissue slices[24]. In accord with

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this view, inhibitors of enkephalinase increase levels of enkephalins in the brain[25] and produce naloxone-reversible analgesia[26–29]. Thus, enkephalinase inhibitors may have potential as centrally-active analgesics and present an alternative in the treatment of opioid addiction. In this latter context, Livingston *et al.*[30] have recently shown that the precursor of a potent inhibitor of enkephalinase decreased the severity of the precipitated withdrawal syndrome from chronically administered morphine in animals.

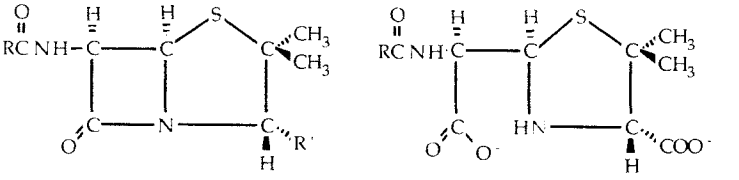
Although several potent inhibitors of enkephalinase are known, eg. thiorphan[26], acetorphan[29] and kelatorphan[31], they are not orally active. A recently described[32] inhibitor SCH 32615, is a selective inhibitor of the enzyme *in vitro*, potentiates *in vivo* exogenously administered enkephalin anlogues and has a direct antinociceptive effect after oral administration with no noted respiratory or gastrointestinal side effects.

This paper describes a new structural type of inhibitor based on the penicillin nucleus as a potential 'lead' compound for the development of a non-toxic analgesic agent.

MATERIALS

The penicilloic acid of ampicillin (PAA) was prepared by incubating a solution of ampicillin (250 mg) with a β-lactamase (TEM-2; 2.5 mg) at pH 7.3, 37° for 2.5 h followed by isolation of the product using a sephadex G-25 column and freeze drying. The product did not exhibit ν(KBr) 1780 cm^{-1} characteristic of the parent penicillin.

(^{3}H-tyrosyl)-[Leu] enkephalin (48.5Ci; mol^{-1}) was obtained from Amersham International, naltrexone from Endo Labs., Try-D-Ala-Gly-Phe-D-Leu (DADL) and [Leu]-enkephalin from Sigma.



PAA

Ampicillin	: R = C_6H_5 CH(NH_2)	; R' = COO^-
Carfecillin	: R = C_6H_5 CH($COOC_6H_5$)	; R' = COO^-
Carbenicillin	: R = C_6H_5 CH(COO^-)	; R' = COO^-
Pivampicillin	: R = C_6H_5 CH(NH_2)	; R' = $COOCH_2$-O-$COC(CH_3)_3$

METHODS

Pharmacology

Nociceptive tests The mouse tail immersion test[33,34] used in this work followed the method described by Sewell and Spencer[35,36]. This procedure employs a nociceptive stimulus temperature of 48° and is sensitive to a wide range of agonist and partial agonist opioid analgestics. "% Antinociceptive effect" was derived from the relation-

ship; area under time-nociceptive latency response curve for drug treated group/area under time-nociceptive latency response curve for control.

The abdominal constriction test[37] possesses a high sensitivity towards opioid analgesics though it is low in specificity. The term "% protection" is used to describe the ability of a drug to reduce the number of abdominal constrictions expressed as a percentage of those in vehicle treated male mice (n = 10) injected intraperitonealy with inducing agent.

In all experiments, means and standard errors (mean ± S.E) were calculated and the level of significance was judged by the "two tailed Student's t-test". The following symbols were used to denote significance levels in all studies: *($p < 0.05$), **($p < 0.01$) ***($p < 0.001$). The "drug alone" group was usually compared statistically with the "vehicle control" group and the "combination" compared with the DADL alone treated animals.

In the evaluation of IC_{50} values and slopes of dose-response relationships, linear regression was used.[38]

Male albino mice of GB1 variants of an ICI strain weighing 18–22 g (Triangle Lab. Newton Abbot, Devon) were fed on a "rat and mouse" breeding diet (Grain Harvesters Wigham, Kent) and allowed tap water ad libitum. Food and water supply were withdrawn two hours prior to experimentation.

Animal house and laboratory conditions were maintained on a 12 h–12 h light-dark cycle, the temperature was maintained at 20 ± 1° and all experiments were carried out between 11.00 and 17.00 h. The test compounds were dissolved in normal saline. Intracerebroventiricular injections (icv) in conscious mice followed the method of Haley and McCormick[39] as modified by Brittain and Handley[40].

Antinociceptive activity in combination with DADL In the mouse tail immersion test the compounds were administered at respective doses of 250 μg/animal (i.c.v.) or 250 mg/Kg (i.p) either concurrently or 1 h before DADL, 1 μg/animal (i.c.v). In the abdominal constriction test, DADL 1 μg/animal (i.c.v) and the test inhibitor compound 250 μg animal (i.c.v) or 250 mg/Kg (i.p) were injected 15 min and 1 h respectively prior to injection of the inducing agent (3% acetic acid).

Naltrexone-reversible analgesia. Naltrexone 1 mg/Kg (i.p) wa used in all experiments since this dose-level completely reversed the antinociceptive activity of DADL in the tail immersion test and abdominal constriction test and almost totally reversed the antinociceptive activity of centrally administered DADL, 1 μg/animal, only a residual antinociceptive effect (20.6%) being recorded.

Biochemistry

Preparation of a 'particulate fraction' A procedure based on the technique used by Hudgin *et al.*[17], as adapted from the original method of Malfroy *et al.*[20], was employed here.

3 male Swiss mice (20 ± 2 g) were decapitated and their brains removed. The striatal tissue was immediately transferred to 10 mls of ice cold 50 mM Tris HCl buffer (pH = 7.4) and homogenised using a tight fitting Tri-stir glass-teflon homogeniser (3 × 5 s at 1000 rpm). After centrifugation at 1000 g for 1 min in a Chillspin (4°C) the resulting pellet was discarded and the supernatant fraction was recentrifuged at 120,000 g for 15 min (4°C). The pellet produced by the second centrifugation

was then resuspended in 10 mls of ice cold buffer (3 × 5 s at 10000 rpm). The resuspended pellet was then centrifuged at 120000 g for 15 min (4°C) and a final pellet obtained. The final pellet was washed without resuspension with 3 × 5 mls of ice cold buffer. This pellet was then resuspended into 8 mls of ice cold buffer (3 × 5 s at 10000 rpm) and was the 'particulate fraction' used in these studies. The particulate fraction was stored on ice until required, its protein concentration being determined by the method of Lowry *et al.*[41] The mean value obtained was found to be 80 ± 10 μg protein/100 μl.

In the studies on enkephalinase B the final pellet was washed with only 1 × 5 ml of ice-cold buffer; this was found to give more reproducible results.

Enkephalinase 50 μl of 'particulate fraction' was preincubated in a shaking water bath for 15 min at 25°C with puromycin (0.1 mM), captopril (1 μM) and putative enkephalinase inhibitors at appropriate concentrations each added in 10 μl volumes of 50 mM Tris HCl buffer (pH = 7.4).

Incubations were started by the addition of ^{3}H-[Leu]-enkephalin (40 nM final concentration) added in 10 μl of buffer and a suitable concentration of unlabelled [Leu]-enkephalin in 10 μl of buffer (K_m determinations) and 10 μl of untreated buffer (for K_m and IC_{50} determinations). This gave a final incubation volume of 100 μl.

Incubations lasted 15 min at 25°C and were terminated by the addition of 10 μl of 3N hydrochloric acid. Suitable blanks were obtained by adding the hydrochloric acid prior to incubation.

After termination of incubations 10 μl of a mixture of unlabelled [Leu]-enkephalin and its metabolites, Tyr, Tyr-Gly, Try-Gly-Gly was added (final concentration of each in 120 μl 0.1–0.5 mg/ml) to the incubation mixture.

50 μl of incubation mixture and suitable reference compounds were then applied in 10 μl aliquots to a TLC plate (plastic, silica gel 60 Merck 5748) and thoroughly dried.

Plates were developed in ethyl acetate: propan-2-ol:water:acetic acid (40:40:19:1), full development taking approximately 2.5 h. After development the plates were removed and allowed to dry, sprayed with ninhydrin reagent (0.5% in acetone) and heated at 55°C for 15 min.

Spots corresponding to [Leu]-enkephalin (rf = 0.86), Tyr (Rf = 0.71), Tyr-Gly (Rf = 0.62) and Tyr-Gly-Gly (Rf = 0.51) were cut out and placed in plastic scintillation vials. To each vial 1 ml of water was added followed by 15 ml of Cocktail T (BDH). The vials were sealed, well agitated, left to stand for 2 h and then placed in LKB 1217 Rackbeta liquid scintillation counter and the amount of tritiated compound associated with each vial calculated.

Enkephalinase B 50 μl of particulate fraction was preincubated in a shaking water bath for 15 min at 25°C with puromycin (0.1 mM), captopril (1 μM), thiorphan (100 nM) and putative enkephalinase B inhibitors at appropriate concentrations, each added in 10 μl volumes of 50 mM Tris HCl buffer (pH = 7.4).

Incubations were started by the addition of ^{3}H-[Leu]-enkephalin (40 nm final concentration) added in 10 μl of buffer, and a suitable concentration of unlabelled [Leu]-enkephalin in 10 μl of buffer (for K_m determinations) which gave a final incubation volume of 100 μl.

Incubations lasted 15 min at 25°c and were terminated by the addition of 10 μl of 3N hydrochloric acid. Suitable blanks were obtained by adding the hydrochloric acid prior to incubation.

After termination of incubations, 10 μl of a mixture of unlabelled [Leu]-enkephalin and its metabolites, Tyr, Tyr-Gly and Try-Gly-Gly were added (final concentration of each in 120 μl 0.1–0.5 mg/ml) to the incubation mixture.

50 μl of incubation mixture and suitable reference spots were then applied in 10 μl aliquots to TLC plate (plastic, silica gel 60 Merck 5748) and thoroughly dried. The plates were processed and tyrosine and tyrosine-containing metabolites estimated as described above for the enkephalinase A assay.

Aminopeptidase The assay of aminopeptidase activity in the particulate fraction is described in the following paper.

Angiotensin I — converting enzyme (ACE) The inhibitory activity of the compounds towards ACE was determined by Dr J Nixon at Roche Products Ltd., Welwyn using guinea-pig lung.

RESULTS

Pharmacology

Antinociceptive activity In the tail immersion test, carbenicillin, ampicillin, pivampicillin and PAA when administered i.c.v. or i.p. alone showed no significant activity. Carfecillin i.c.v., but not i.p. showed significant activity (Figure 1).

In the abdominal constriction test, when administred i.c.v., carbenicillin, ampicillin and PAA exhibited mild significant protective activity pivampicillin moderate significant activity whilst carfecillin yielded complete suppression of constrictions (Figure 2) which conformed to a sigmoidal dose relationship (not shown) (IC_{50} of carfecillin = 23 μg/animal). When administered i.p., pivampicillin and carfecillin produced significant activity whereas the remainder showed no appreciable protective activity.

Antinociceptive activity in combination with DADL In the tail immersion test, central injection of all five compounds significantly prolonged the antinociceptive effects of

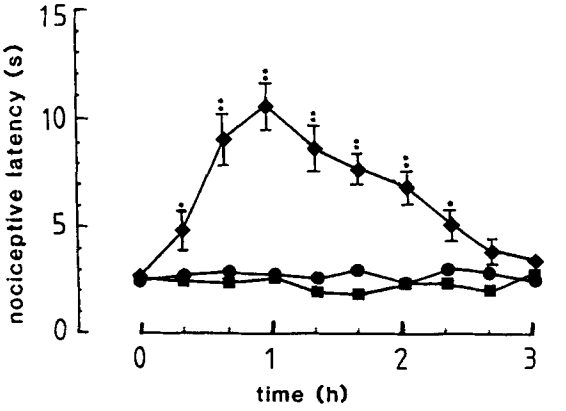


FIGURE 1 Antinociceptive activity of carfecillin (250 μg/aminal icv — ◆) and (250 mg/Kg ip — ●) compared with saline vehicle (■) in the tail immersion test in mice.

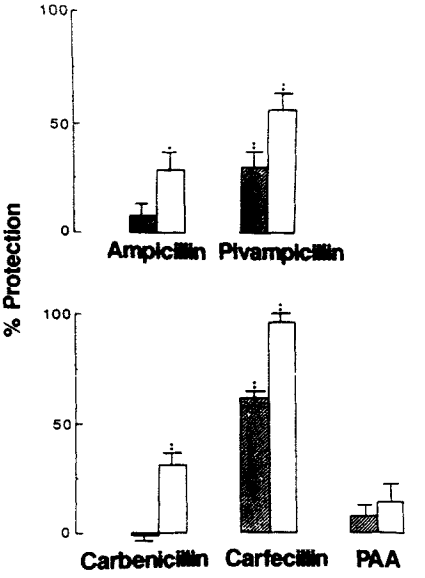


FIGURE 2 Comparison of the % protective activities of penicillins and PAA 250 mg/Kg ip) (▨) and 250 μg/animal (icv) (□) in the mouse abdominal constriction test.

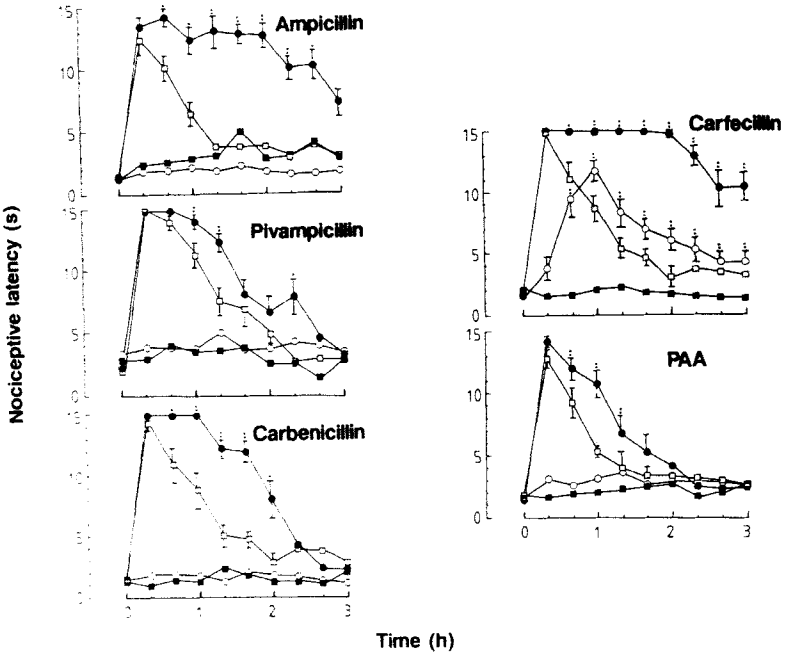


FIGURE 3 Antinociceptive activity of test penicillins and PAA (icv) in combination with DADL (icv) in the tail immersion test — (■) — saline; — ● — test compound 250 μg/animal (icv) plus DADL 1 μg/animal (icv); □ - DADL 1 μg/animal (icv); - ○ - test compound 250 μg/animal (icv).

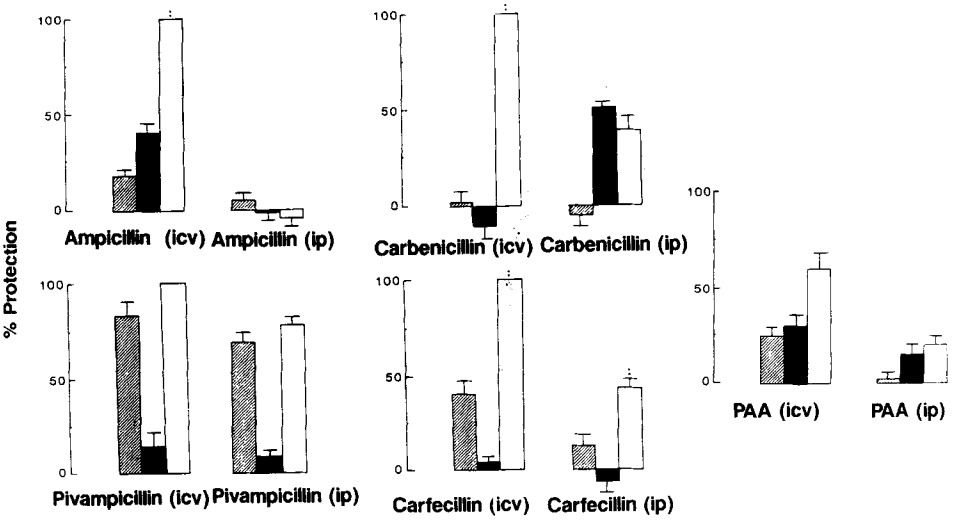


FIGURE 4 Comparison of the protective activity of penicillins or PAA alone (▩ 250 μg/animal icv or 250 mg/Kg ip), DADL alone (■ 1 μg/aminal icv), or DADL (icv) following penicillin or PAA (icv or ip) (□ administered at the above doses) in the abdominal constriction test.

DADL but none had significant actions alone following i.p. administration (Figure 3).

In the abdominal constriction test all four penicillins co-administered intracerebrally with DADL gave complete protection (Figure 4). The protection was greater than would be expected from the net effects of DADL by itself and the penicillin administered alone, particularly in the case of ampicillin, carbenicillin and carfecillin. PAA in combination with DADL gave a moderately significant effect which may have reflected the product of additive activity. When administered intraperitoneally with DADL (i.c.v.) only pivampicillin manifested a highly significant effect whereas carbenicillin and carfecillin were moderately active whilst ampicillin and PAA were devoid of efficacy.

Naltrexone reversible antinociceptive activity In the tail immersion test intraperitoneal injection of naltrexone (1 mg/kg) significantly reversed the antinociceptive effect induced by DADL (1 μg/animal) concurrently injected intracerebrally with one of the penicillins (ampicillin, pivampicillin and carbenicillin at doses of 250 μg/animal or carfecillin at a dose of 125 μg/animal) (Figure 5).

Similarly naltrexone also reversed the protective effects of DADL (1 μg/animal) co-administered (i.c.v.) with all of the compounds (250 μg/animal) in the abdominal constriction test (Figure 6). This naltrexone reversal was also noted in animals treated with DADL (1 μg/animal) following peripheral (i.p.) pretreatment with pivampicillin or carfecillin (250 mg/kg) in the abdominal constriction test (Figure 6).

Biochemistry

The K_m and V_{max} values for enkephalinase activity in the particulate fraction with

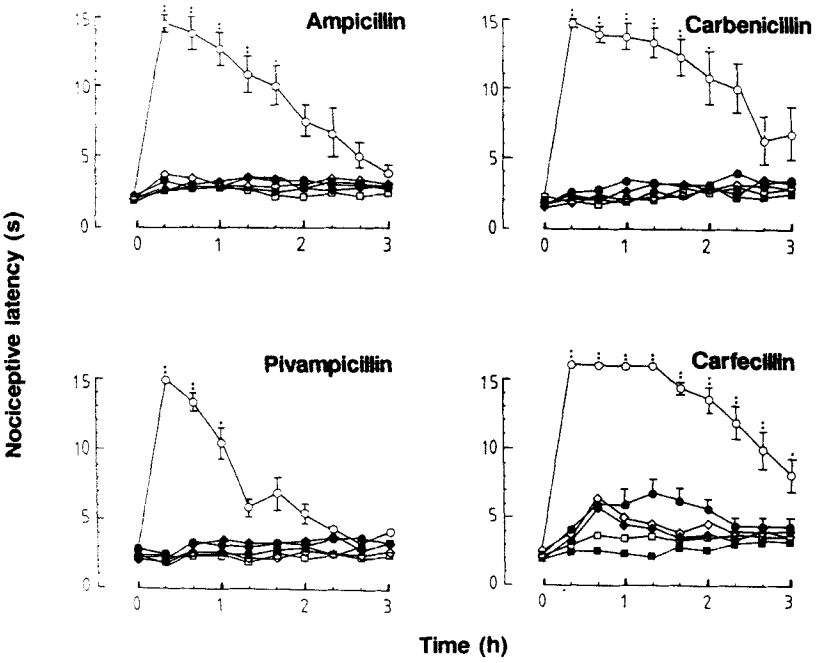


FIGURE 5 Effect of nalterexone (ip) on the antinociceptive activity of penicillins (icv) alone and in combination with DADL (icv) in the tail immersion test. – ○ –, penicillin 250 μg/animal (except carfecillin 125 μg/animal) (icv) plus DADL 1 μg/animal (icv); — ● — penicillin 250 μg/animal (except carfecillin 125 μg/animal) (icv) plus DADL 1 μg/animal (icv) plus naltrexone 1 mg/Kg (ip); — ■ — naltrexone 1 mg/Kg (ip); – ■ – saline; – ◆ – penicillin 250 μg/animal (except carfecillin 125 μg/animal) (icv) plus naltrexone 1 mg/Kg (ip); — ◇ — penicillin 250 μg/animal (except carfecillin 125 μg/animal) (icv).

[Leu]-enkephalin (5–80 μM) as substrate were 26.1 ± 2.5 μM and 473 ± 31 p mol/min/mg protein respectively as determined graphically by the Lineweaver-Burke method.[42]

Amplicillin, carbenicillin and carfecillin were inhibitors of enkephalinase with IC_{50} values of 45.7 ± 3.9 μM, 435 ± 46 μM and 207 ± 57 nM respectively (see Figure 7). For comparative purposes, thiorphan a potent inhibitor of the enzyme was studied and had IC_{50} value of 10.6 ± 1.9 nM (cf.[26] IC_{50} = 4.7 ± 1.2 nM).

The compounds were competitive reversible inhibitors of the enzyme as evidenced by the method of Dixon[43] (not shown) Pivampicillin had only a weak inhibitory effect (34%) at 1 mM concentration whereas PAA was inactive. All the compounds were inactive as inhibitors of ACE, aminopeptidase and enkephalinase B at 1 mM concentration. The K_m and V_{max} values for enkephalinase B in the particulate fraction with [Leu]-enkephalin as substrate were 14.0 ± 5.1 μM and 193 ± 30 p mol/min/mg protein respectively. The K_m and V_{max} values for the aminopeptidase activity with [Leu]-enkephalin as substrate were 33.4 ± 4.3 μM and 497 ± 57 p mol/min/mg protein respectively (cf.[17], K_m = 14.86 ± 0.95 μM and V_{max} = 592 ± 12 p mol/min/mg protein). The IC_{50} value for puromycin, an established aminopeptidase inhibitor was 8.4 ± 0.9 μM (cf.[21] 1 μM).

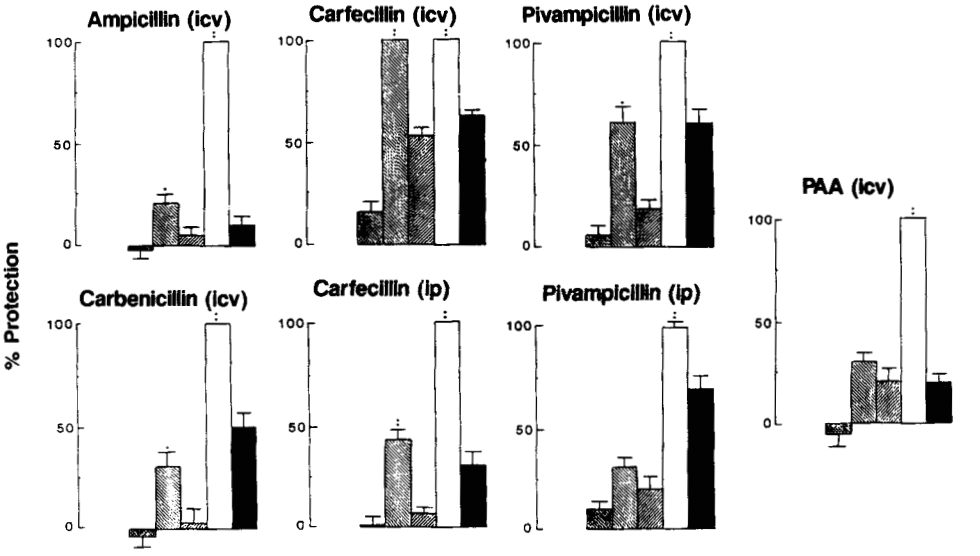


FIGURE 6 The effect of naltrexone (ip) on the antinociceptive activity of penicillins or PAA (icv) alone or in combination with DADL (icv) in the abdominal constriction test. – ▩ – naltrexone 1 mg/Kg (ip); – ▧ – penicillin or PAA 250 μg/animal (icv) or 250 mg/Kg (ip); – ▨ –penicillin or PAA 250 μg/animal (icv) or 250 mg/Kg (ip) plus naltrexone 1 mg/Kg (ip); – □ – penicillin or PAA 250 μg/animal (icv) or 250 mg/Kg (ip) plus DADL 1 μg/animal (icv); – ■ – penicillin or PAA 250 μg/animal (icv) plus DADL 1 μg/animal (icv) plus naltrexone 1 mg/Kg (ip).

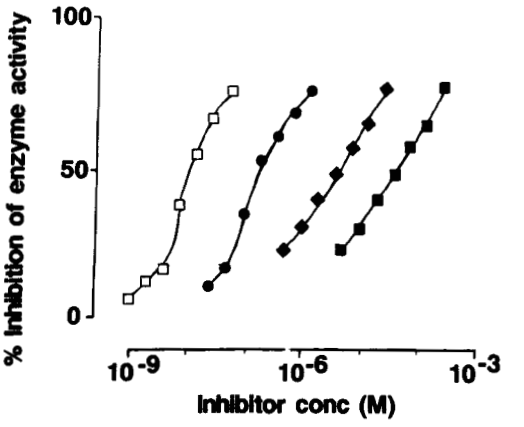


FIGURE 7 Comparison of the IC_{50} values of thiorphan (□), ampicillin (◆), carbenicillin (■) and carfecillin (●) against enkephalinase.

DISCUSSION

The four penicillins and PAA, when administered centrally by themselves, exhibit significant inhibitory activity in the abdominal constriction test but only carfecillin shows a significant effect in the tail immersion test. Schwartz *et al.*[21] have observed that enkephalinase inhibitors show a similar differentiation in activity in the two tests. All five compounds when administered intracerebrally potentiated the antinociceptive action of the aminopeptidase-resistant enkephalin analogue DADL, providing further support for an enkephalinergic action by the penicillins. Confirmation of an enkephalinergic role came from the observation that the antinociceptive action in combination with DADL was significantly decreased in both tests by naltrexone, a specific opioid receptor anatagonist.

However, the presence of naltrexone did not completely remove the antinociceptive effects of a combination of pivampicillin (i.c.v. and i.p.) and carfecillin (i.c.v. and i.p.) separately with DADL in the writhing test which might suggest the presence of a non-opioid component in the antinociceptive action of these compounds. The non-opioid component could be attributed to a common hydrolysis product, phenol, released from the penicillin, the opioid component being due to the ampicillin or carbenicillin derived from pivampicillin or carfecillin respectively.

Enkephalins are targets for several degrading enzymes present in the brain which hydrolyse specific amide bonds; aminopeptidase (Tyr-Gly), enkephalinase B (Gly-Gly), enkephalinase (Gly-Phe), ACE (Gly-Phe) and carboxypeptidases (Phe-Leu (Met)). Enkephalinase and aminopeptidase are considered the main degrading agents for the enkephalins due to their location near the synapses in high concentration. *In vitro* studies using the particulate fraction from rat brain striatia showed that ampicillin and carbenicillin were weak inhibitors (IC_{50} = 45.7 ± 3.9 μM, 435 ± 46 μM respectively) whereas carfecillin was a stronger inhibitor (IC_{50} = 207 ± 57 nM) of enkephalinse. These inhibitors had no noted effect on ACE, enkephalinase B and aminopeptidase. Pivampicillin did not significantly inhibit enkephalinase, probably resulting from an absence of a free carboxyl group for effective interaction with the cationic binding site on the enzyme.[44,45] PAA was similarly inactive as an inhibitor of the enzyme which would indicate the requirement for an intact β-lactam ring in effective inhibitors.

Taken together the pharmacological and biochemical data indicate that the penicillins exhibit some antinociceptive activity as a consequence of build up of brain enkephalins resulting from inhibition of enkephalinase.

Although not highly potent inhibitors of enkephalinase, the penicillins studied here provide a new lead structure for the development, by suitable chemical modification, of more potent inhibitors as potential antinociceptive agents after removal of the undesired antibiotic activity.

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