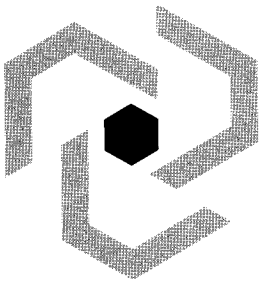

# Hormone and Metabolic Research




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# Serum Pyrrolidone Carboxypeptidase Activity in N-methyl Nitrosourea Induced Rat Breast Cancer

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## Abstract

Pyrrolidone carboxypeptidase (Pcp) (E.C. 3.4.19.3) is an omega peptidase widely distributed in animal fluids and tissues and hydrolyses N-terminal pyroglutamic residues from biologically active peptides such as gonadotropin releasing hormone (GnRH). Previous results obtained by us showed a decrease in human breast cancer Pcp activity, suggesting that this enzyme activity or its putative substrates may play a mayor role in breast cancer pathogenesis. The aim of the present work is to analyse serum Pcp activity in N-methyl-nitrosourea (NMU) induced rat mammary tumours using pyroglutamyl-β-naphthylamide as substrate. Serum Pcp activity was significantly lower in NMU-treated rats than in controls. Moreover, multiple regression analysis showed a significant correlation between Pcp activity and the number and size of tumours and the body weight of the animals. Since NMU-induced carcinomas are mainly oestrogen-dependent, the decrease observed in Pcp activity may reflect an increase in circulating levels of GnRH that lead to an increase in gonadal steroid hormones production responsible, at least in part, for the initiation and promotion of the disease.

**Key words**
Pyroglutamyl aminopeptidase · NMU · Mammary gland · GnRH · Tumour growth parameters



## Introduction

Hormonal regulation mechanisms of tumour growth [1] have been described for human mammary tumours [2], cell lines [3] and experimentally induced tumours [4]. Tumours induced in rats by administration of chemical carcinogens such as 7,12 dimethylbenz[a]anthracene (DMBA) and N-methyl-nitrosourea (NMU) constitute useful tools for dissecting the multi-step process of carcinogenesis, which involves initiation, promotion and progression [5]. NMU-induced mammary tumours appear to be more oestrogen-dependent than those induced by DMBA [6]; furthermore, they are capable of forming metastases [7]. One of the major attributes of NMU model is that the proportion of ovarian hormone-dependent mammary carcinomas is similar to that observed in human disease; other attributes are the aggressive and locally invasive nature as well as the clear operational distinction between the initiation and promotion stages of the disease process based on the action of NMU as a direct methylating agent [6,8–10].

Pyrrolidone carboxypeptidase (Pcp), also called pyroglutamyl aminopeptidase (E.C. 3.4.19.3), is an omega peptidase widely distributed in animal fluids and tissues [11] that hydrolyses N-terminal pyroglutamyl residues from biologically active peptides such as gonadotropin-releasing hormone (GnRH) and arylamide derivatives in a highly selective manner [12]. In a previous work, we described changes in Pcp activity in human breast cancer. This enzymatic activity was modified in tumours and surrounding

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tissues, suggesting that changes in this enzyme or its putative substrates may play a major role in breast cancer pathogenesis [13]. The aim of the present work was to analyse Pcp activity in serum from NMU-induced rat mammary tumours and to evaluate the putative value of this activity as a biological marker of the disease.

## Materials and Methods

### Animals and treatment

Forty female virgin Wistar rats (164.7 ± 4.7 g body weight) were used in this study. The animals were provided by the animal house-care of the University of Jaén and maintained in an environment controlled under constant temperature (25 °C) with a 12 h light/dark cycle. All animals were allowed access to water and food *ad libitum*. The experimental procedures for animals use and care were in accordance with the 86/609/EEC European Community Council directive. The rats were randomly divided into two groups. One group was injected intraperitoneally with three doses of 50 mg/kg body weight of NMU dissolved in distilled water (10 mg/ml) at 50, 80 and 110 days after birth, as described by Rivera et al. [14]. All rats were in estrus at the first NMU injection as verified by daily vaginal smears. Control group received the vehicle only. For tumour detection and growth control, rats were examined by palpation 2 days each week after the second NMU injection. The number of tumours in the cervicothoracic and abdominal-inguinal regions were recorded, and the major and minor diameters of each tumour was measured using callipers. Body weight was determined every week. The following tumour growth parameters were also determined: latency period (LP) – the number of days between the first NMU injection and the appearance of the first tumour; tumour incidence (TI) – percentage of the rats that developed at least one tumour; and mean tumour number per rat (n/t) – the number of tumours per rat in animals developing at least one tumour. After 122 days of first NMU injection, animals were sacrificed under equithensin anaesthesia (2 ml/kg body weight). Blood samples were obtained through the left cardiac ventricle and centrifuged for ten minutes at 3,000 g to obtain serum. These samples were frozen and stored at – 80 °C until use.

### Pyrrolidone carboxypeptidase assay

Serum Pcp activity were measured fluorimetrically using pyroglutamyl-β-naphthylamide (pGLUNNap) as the substrate according to the method previously described by us [15]. Briefly, 10 µl of each sample were incubated in triplicate for 30 minutes at 37 °C with 100 µl of the substrate solution: 100 mM of pGLUNNap, 0.65 mM dithiothreitol (DTT) and 1.3mM ethylenediaminetetraacetic acid (EDTA) in 50 mM of phosphate buffer at pH 7.4. All the reactions were stopped by adding 100µl of 0.1 M acetate buffer at pH 4.2. The amount of β-naphthylamine released as the result of the enzymatic activity was measured fluorimetrically at 412 nm emission wavelength with and excitation wavelength of 345 nm. Proteins were quantified also in triplicate using bovine serum albumin (BSA) as standard.

### Statistical analysis

To analyse the differences between control group and the animals with mammary tumours due to NMU injections, we used Student's *t*-test for unpaired variables. All comparisons with p-values below 0.05 were considered significant.

The number of tumours in the cervicothoracic and abdominal-inguinal regions, the major and minor diameter of each tumour and the body weight were used to explain the behaviour of the dependent variable, Pcp activity. A stepwise regression was carried out to select the best independent variables following the largest adjusted R-squared value as a criterion. Then, with the 9R program in the BMDP statistical package, the best regression was selected. The data and statistics obtained are given in Tables **1 – 3**.

Table **1** Values of experimental and calculated Pcp activity (pmol/min/mg protein) from the proposed model

| *Rat #* | *Pcp (experimental values)* | *Pcp (calculated values)* | *Increase (%)*[a] |
|---|---|---|---|
| 1 | 50.59 | 56.59 | – 15.24 |
| 2 | 46.72 | 32.03 | 45.86 |
| 3 | 53.51 | 61.19 | – 12.55 |
| 4 | 79.18 | 62.32 | 27.05 |
| 5 | 33.21 | 45.64 | – 27.23 |
| 6 | 52.17 | 32.21 | 61.97 |
| 7 | 25.82 | 49.55 | – 47.89 |
| 8 | 41.98 | 55.10 | – 23.81 |
| 9 | 84.38 | 63.76 | 32.34 |

[a] Increase (%) $= \frac{\text{Pcp experimental} - \text{Pcp calculated}}{\text{Pcp calculated}} \times 100$

Table **2** Regression model for Pcp activity[a]

| *Variable* | *Regression coefficient* | *Standard error* | *Contribution to R-SQ* |
|---|---|---|---|
| Number of tumours in the cervicothoracic region | 19.52 | 13.23 | 0.047 |
| Number of tumours in the abdominal-inguinal region | – 7.87 | 8.8 | 0.0172 |
| Major diameter | – 46.39 | 39.63 | 0.0296 |
| Minor diameter | 57.63 | 44.49 | 0.0362 |
| Body weight | 0.11 | 0.12 | 0.0202 |

[a] n = 9; Mallows $Cp = 6.0$; Mean absolute error: 15.35; $R = 0.9558$; $R^2 = 0.9136$; $F(5,4) = 8.46$; $p = 0.0299$.

Table **3** Correlation Matrix for the Variables Used

| | | | | | |
|---|---|---|---|---|---|
| Number of tumours in the cervicothoracic region | 1 | | | | |
| Number of tumours in the abdominal-inguinal region | – 0.473 | 1 | | | |
| Major diameter | – 0.43 | – 0.137 | 1 | | |
| Minor diameter | 0.5567 | 0.051 | – 0.962 | 1 | |
| Body weight | – 0.568 | 0.025 | 0.1055 | – 0.349 | 1 |

## Results

Tumour growth parameters in rats after 122 days of the first NMU injection showed a LP (Mean ± SEM) of 113.0 ± 4.2 days between the first NMU injection and the appearance of the first tumour, with a 60% of TI. The mean tumour number per rat (Mean ± SEM) was 1.93 ± 0.4 tumours. Specific Pcp activity in serum of controls and NMU-treated rats is shown in Fig. **1**. Serum Pcp activity decreased significantly ($p < 0.05$) by 28% in NMU-treated rats when compared with control group.

Multiple regression analysis statistics are given in Tables **1** to 3. Table **1** shows the observed values versus the predicted values for the dependent variable Pcp activity, which can be used to detect cases in which the variance is not constant. Table **2** shows the results of fitting a multiple linear regression model to describe the relationship between Pcp activity and 5 independent variables. The equation of the fitted model is Pcp activity = 19.5188 × number of tumours in the cervicothoracic region − 7.86782 x number of tumours in the abdominal-inguinal region − 46.3921 x major diameter + 57.6298 × minor diameter + 0.11343 × body weight. Since the p-value in the ANOVA table is less than 0.05, there is a statistically significant relationship between the variables at the 95% confidence level. The R-Squared statistic indicates that the model as fitted explains 91.3568% of the variability in Pcp activity. The mean absolute error of 15.35 is the average value of the residuals. Table **3** shows estimated correlations between the coefficients in the fitted model. These correlations can be used to detect the presence of serious multicollinearity, that is, correlation amongst the predictor variables. A plot of the experimental against expected values for the regression equation obtained is depicted in Fig. **2**. Residuals *vs.* experimental Pcp activity values are plotted in Fig. **3**.

## Discussion

The present report notices that serum Pcp activity is decreased in a well-established model on NMU-induced rat mammary breast cancer. Since one of the susceptible substrates of Pcp is GnRH, this decrease indicates the existence of high circulating levels of this peptide hormone. In a previous report, we described a significant decrease in Pcp activity in neoplasic and adjacent tissues in human breast cancer when compared with unaffected tissue, indicating that local factors may be selectively modified by the tu-

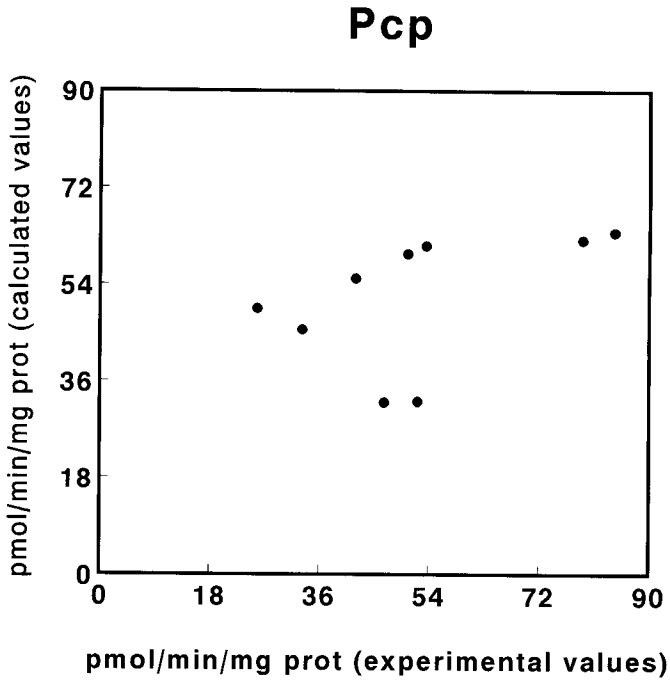


Fig. **2** Plot of experimental *vs.* expected values of Pcp activity.

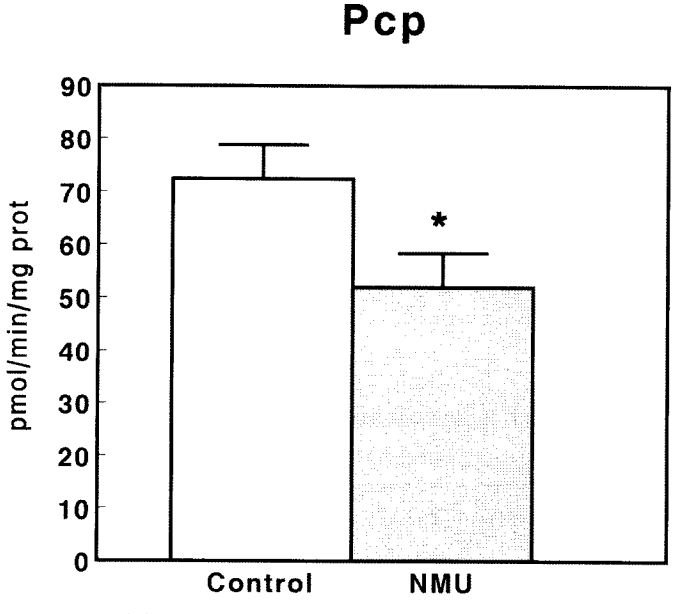


Fig. **1** Pyrrolidone carboxypeptidase (Pcp) activity in serum of control and NMU-treated rats. Result are expressed in picomoles of pyroglutamyl-β-naphthylamide hydrolysed per min and per mg of protein (Mean ± SEM; n = 9; *$p < 0.05$).

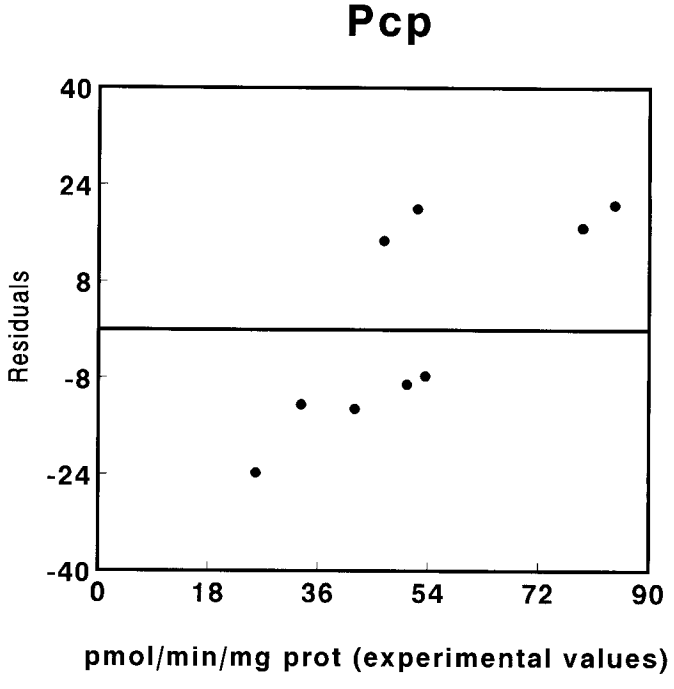


Fig. **3** Plot of residuals *vs.* experimental values of Pcp activity.

mour process in the affected tissue [13]. In this connection, GnRH receptors and GnRH mRNA have been found in breast tissue, raising the possibility of a local role for GnRH in the human mammary gland [16]. In fact, GnRH receptor (GnRH-R) had been inmunolocalised in the cytoplasm of carcinoma cells, and was also detected focally in the cytoplasm of morphologically normal glandular epithelial adjacent to the carcinoma. This study indicated that GnRH-R is widely distributed in human breast carcinoma cells and regulates GnRH actions locally [17] although GnRH-R has been found in cancer and normal tissues, but the prevalence for GnRH-R was higher in tumour than in not-tumour tissue [18]. These indicate that GnRH may be an important local intracrine, autocrine and/or paracrine hormonal factor in the pathogenesis of breast cancer and suggests a role for GnRH in the tumour process. However, increased levels of GnRH also lead to increased levels of gonadal steroid hormones [19]. Estradiol stimulates both mammary epithelial tissue growth and mitotic activity, thus rendering the host more sensitive to the carcinogen [20]. It has been documented that high doses of oestrogens shorten the cellular cycle and lengthen the G0G1 phase in B5ER cells [3]; oestrogen receptors are present in some NMU-induced carcinomas [9], which could help to explain the increase of carcinogen-induced damage. To conclude, due to NMU-induced carcinomas are mainly oestrogen-dependent, the decrease observed in Pcp activity may reflect an increase in circulating levels of GnRH, which lead to an increase on gonadal steroid hormone production responsible, at least in part, for the initiation and promotion of the disease. Therefore, Pcp activity represents a tumour marker for detection of cancer and can be assayed by simple, reproducible and cheap techniques. Although further studies are needed to establish its sensitivity and specificity (predictive values), we propose Pcp activity as a serum marker to rapidly predict the sensitivity of a tumour to therapy, the maintenance of remission or an eventual occult disease, which might permit a better monitoring of cancer patients and a rapid selection of more effective therapeutic means.

Moreover, the multiple regression analysis showed that residuals are normally distributed and independent, and there is no autocorrelation between them. In the same way, the Mahalanobis distance shows that extremely high values do not exist at a confidence level of 95%. If we consider leverage values about the influence of a sample value, there are no sample values with leverage values greater than three times that of an average data point for Pcp activity in the model. The largest studentized residuals in absolute value among cases are 2.13. In conclusion, serum Pcp activity reflects its importance in breast cancer pathogenesis.

## Acknowledgments

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