Vol. 110, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1983 Pages 235-242

GROWTH INHIBITION OF HUMAN BREAST CANCER CELLS INDUCED BY CALCITONIN

Y. Iwasaki, J. Iwasaki and H.C. Freake

Endocrine Unit, Royal Postgraduate Medical School,
Du Cane Road, London, W12 OHS, U.K.

Received November 10, 1982

SUMMARY: The human breast cancer cell line (T47D) has specific, high affinity calcitonin receptors and calcitonin-responsive adenylate cyclase. Human, salmon and [Asu^{1,7}]eel calcitonin inhibited cell growth in a dose-related manner with almost equipotency. Analogues of human calcitonin demonstrated slight cell growth inhibition. We found extreme growth inhibition with daily treatment with dibutyryl cyclic AMP (10^{-4} M). In contrast to calcitonin 1,25-$(OH)_2D_3$ had a biphasic effect on cell growth. Physiological doses (5×10^{-10} M) of 1,25-$(OH)_2D_3$ stimulated growth of T47D, whereas treatment by supraphysiological amounts (2.5×10^{-7} M) caused significant inhibition of growth. Calcitonin and 1,25-$(OH)_2D_3$ appeared to have additive effects.

INTRODUCTION: CT receptors are found in bone cells (1) and renal plasma membranes (1,2), sites where functions for CT have been established. But there have been several reports that CT receptors are also found in tissues where the significance of CT receptors is not clear, such as breast cancer cell lines (3,4), bronchial carcinoma cells (5), lymphocytes (6) and testicular cell membrane (7). Human breast cancer cell lines (T47D, MCF 7) contain specific high affinity CT receptors and CT responsive adenylate cyclase (3,4). In many cells including MCF 7 it has been reported that growth is negatively correlated with the levels of intracellular cyclic AMP (8,9,10). We have used T47D cells to investigate whether CT can affect the growth of human breast cancer cells in culture. In addition, we

ABBREVIATIONS: CT, calcitonin; hCT, human calcitonin; sCT, salmon calcitonin; eCT, eel calcitonin; E_2, β-oestradiol; dibutyryl cyclic AMP, N^6, $O^{2'}$-dibutyryl adenosine 3', 5'-monophosphate; 1,25-$(OH)_2D_3$, 1,25-dihydroxycholecalciferol.

0006-291X/83/010235-08$01.50/0

extended a previous finding showing a stimulatory effect of 1,25-$(OH)_2D_3$ on T47D cell growth (11) to higher doses of the hormone, alone and in combination with CTs.

MATERIALS AND METHODS:

Materials: [Asu^{1,7}]eCT was a gift from Toyo Jozo Co., Ltd., Shizuoka, Japan; synthetic hCT, Lys^{11} hCT and Asn^{26}Thr^{27} hCT were provided by Ciba-Geigy, Basel, Switzerland; sCT (Batch CH 2244) was given by the Armour Pharmaceutical Co., Ltd., Eastbourne, U.K. and 1,25-$(OH)_2D_3$ (Ro 21-5535) was provided by Roche Products Ltd., Welwyn Garden City, U.K. Dibutyryl cyclic AMP, dibutyryl cyclic GMP and L-arginine HCl were purchased from Sigma Chemical Co., Ltd., Poole, Dorset, U.K.

Growth Experiments: T47D cells, a human breast cancer cell line, were routinely grown and subcultured as previously described (11). Cells were distributed to each well of Linbro multiwell dishes (well diameter 3.5 cm, Flow Laboratories, Irvine, Ayrshire, Scotland) in a volume of 2 ml (1∿2 x 10^5 cells/well). The medium was aspirated after 24∿28 h incubation. The wells were rinsed with phosphate buffered saline and the cells incubated in medium containing 5% charcoal-stripped foetal calf serum (12) and various concentrations of additives. Fresh calcitonin (in 20 µl phosphate buffered saline) was added daily and the medium was changed every 3∿4 days. Wells were extracted at different times for DNA measurement.

Extraction and Measurement of DNA: The procedure was modified from the extraction method of Patterson (13) and the Burton assay (14). Cell monolayers were washed 3 times with cold phosphate buffered saline (2 ml/well) and rinsed with chilled 0.2 M $HClO_4$ (1 ml/well). Cold 0.2 M $HClO_4$ (1 ml/well) was added again, left to stand for 10 min at 4°C, and then aspirated. Cells were incubated in 0.3 M NaOH for 1 h at 37°C and transferred to polypropylene tubes cooled on ice. Cold 1.0 M $HClO_4$ (1 ml/tube) was added, the tubes were left on ice for 10 min, vortexed, and centrifuged for 10 min at 9500 x g at 4°C. The pellet was washed with cold 0.2 M $HClO_4$ (2 ml/tube), centrifuged for 10 min at 9500 x g at 4°C and resuspended in 1.5 M $HClO_4$ (1 ml/tube). These samples were kept at -20°C until assayed. DNA standards (0∿80 µg/ml) were prepared in triplicate by serial dilutions of highly polymerized calf thymus DNA in 1.5 M $HClO_4$. Samples and standards were heated at 70°C in a shaking water bath for 30 min. They were cooled on ice and 0.6 ml Burton reagent (14) was added. The tubes were left for 14 h at room temperature in the dark and centrifuged for 10 min at 9500 x g at 4°C. Absorbance (600 nM) was measured using a Unicam SP 1800 spectrophotometer. DNA from six wells was assayed for each treatment condition and the data analysed by Student's t test.

RESULTS: Daily addition of [Asu^{1,7}]eCT produced a significant dose-related inhibition of T47D cell growth, as assessed by DNA content, after both 6 and 13 days of treatment (Fig. 1). In contrast E_2 stimulated cell growth at both time points. This experiment has been repeated with the same results on two additional occasions. In further experiments, after

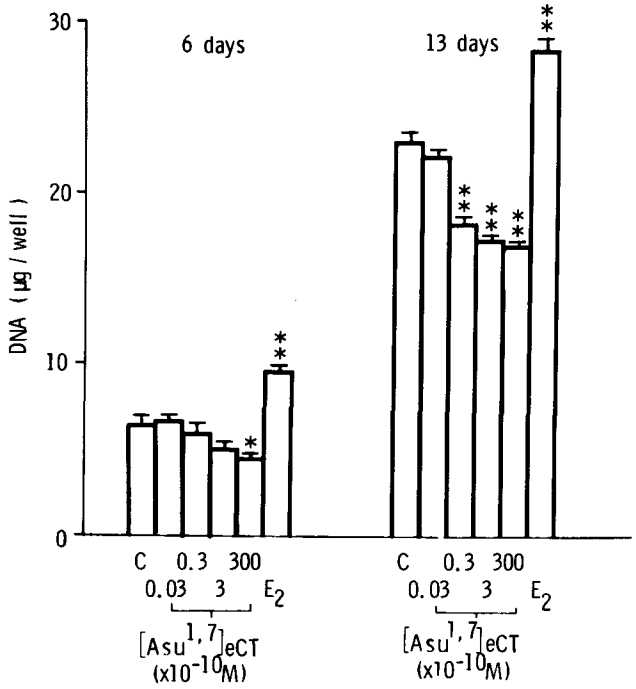
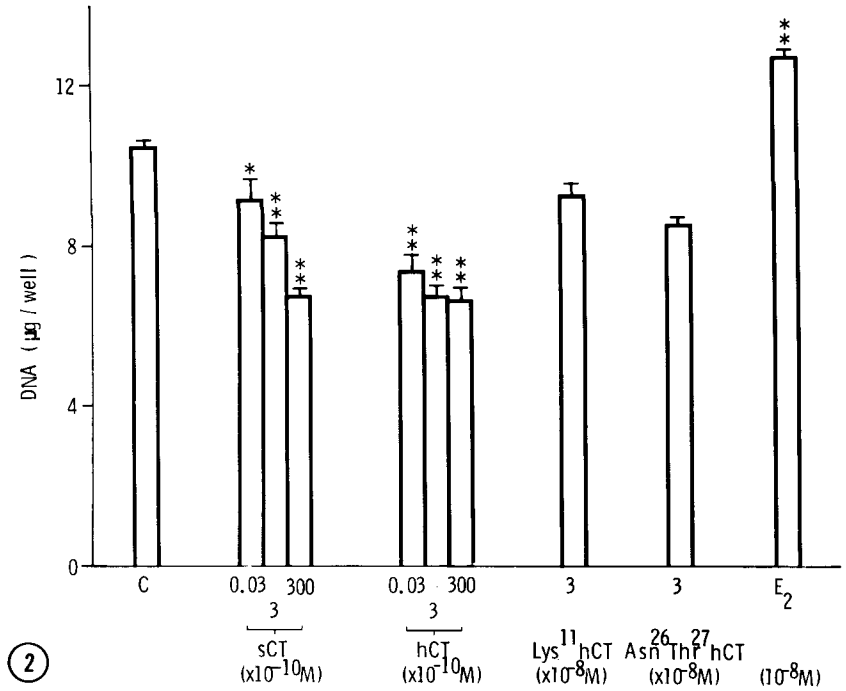


Fig. 1 Effects of [Asu1,7]eCT on the growth of T47D cells. Cells were grown in multiwell dishes as described in METHODS. [Asu1,7]eCT, dissolved in phosphate buffered saline, or vehicle alone, was added daily (20 µl/well) to give the final concentrations shown. Oestradiol was used as a positive control. After 6 and 13 days of treatment cells were extracted in situ for DNA assay. Each bar represents the mean ± SE of six wells. *P < 0.05, **P < 0.005 versus control cultures.

6 days both hCT and sCT had similar inhibitory effects on DNA synthesis as [Asu1,7]eCT, whereas analogues of hCT, Lys^{11} hCT and $Asn^{26}Thr^{27}$ hCT, produced less marked inhibition of cell growth (Fig. 2). Though 10^{-6} M dibutyryl cyclic AMP was without effect, a higher concentration (10^{-4} M) produced even greater suppression (reduced to 42% of control) and similar inhibition was seen with L-arginine (1 mg/ml) (Fig. 3). Growth arrest was also observed with these treatments after 13 days (data not shown).

Subsequently the time course of the effect of CTs and dibutyryl cyclic AMP was studied: consistent inhibition of cell growth was seen by hCT, sCT and 10^{-4} M dibutyryl cyclic AMP (Fig. 4). The most prominent suppression of DNA synthesis by CTs and dibutyryl cyclic AMP was observed from day 4



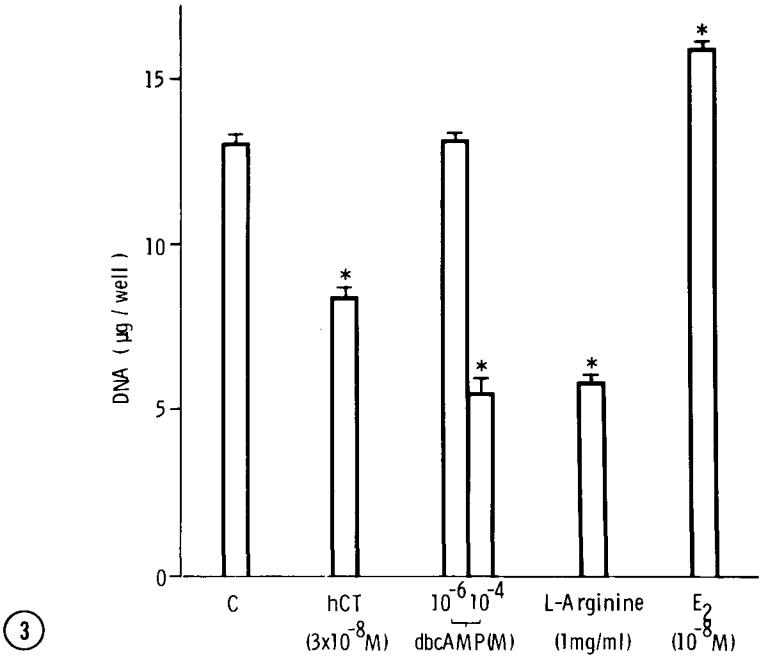


Fig. 2 Effects of sCT, hCT and analogues of hCT on growth of T47D cells after 6 days treatment. Each DNA value represents the mean ± SE of 6 replicates. *P < 0.005, **P < 0.001 versus control value.

Fig. 3 Effects of dibutyryl cyclic AMP and L-arginine on growth of T47D cells after 6 days treatment. Each bar is shown as mean ± SE of 6 replicates. *P < 0.001 versus control value.

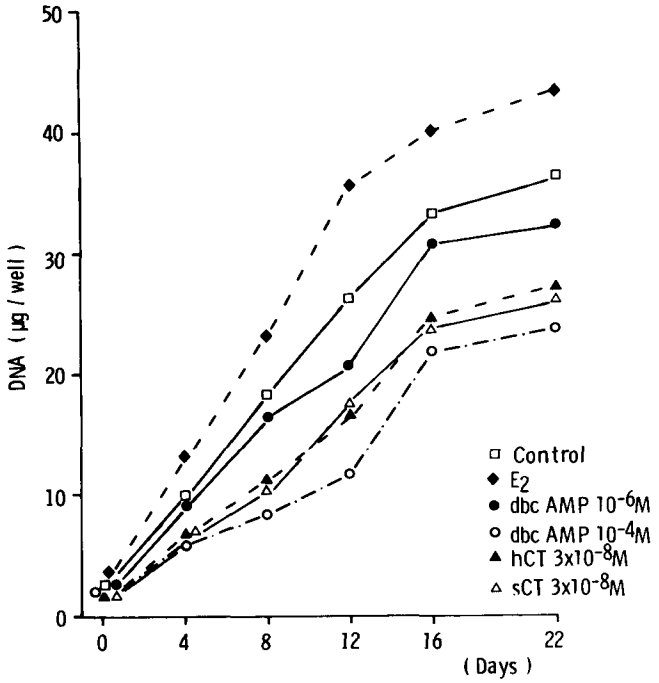


Fig. 4 Time course of the effects of sCT, hCT, dibutyryl cyclic AMP and oestradiol on growth of T47D cells. Each point shows the mean value of DNA content of 6 replicates.

to day 12 of treatment when cells were considered to be in the logarithmic phase of growth. 10^{-8} M E_2 significantly stimulated cell growth at each time point.

In contrast to CTs, near physiological concentrations (5×10^{-10} M) of 1,25-$(OH)_2D_3$ had stimulatory effects on the growth of T47D human breast cancer cells as previously reported (11). The effects of concomitant incubation with sCT (3×10^{-8} M) were additive (Fig. 5). Supraphysiological doses (2.5×10^{-7} M) of 1,25-$(OH)_2D_3$ caused a profound inhibition of cell growth (51% of control), and in combination with sCT (3×10^{-8} M) almost complete suppression of the DNA synthesis was observed.

DISCUSSION: The effects of CT in breast cancer cells are presumed to be accomplished via its receptors, through increased cyclic AMP which triggers the activation of protein kinase and phosphorylation of specific proteins,

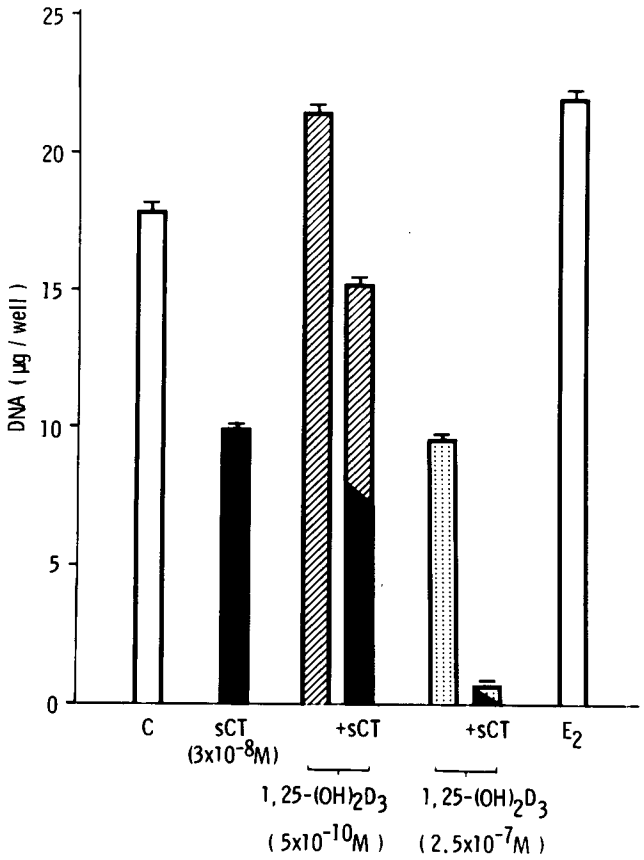


Fig. 5 Effects of physiological and supraphysiological concentrations of 1,25-$(OH)_2D_3$ on DNA contents of T47D cells with or without addition of sCT. Each bar represents the mean ± SE of 6 replicates. Oestradiol was used as positive control.

thereby modifying their biological activity. Therefore this mechanism may play a part in the inhibitory effects of CTs on T47D cell growth (3,4).

This suggestion is supported by the inhibitory effects observed with 10^{-4} M dibutyryl cylic AMP. Since a similar concentration of dibutyryl cyclic GMP was without effect (data not shown), the inhibition produced by dibutyryl cyclic AMP was not due to the butyric acid produced from these dibutyric compounds. L-arginine also inhibits cell growth of T47D. Cho-Chung et al (15) reported a similar growth arrest with L-arginine in MCF 7 cells and found that it was accompanied by a sharp increase in NAD-dependent ADP-ribosylation of the membrane proteins and NAD-dependent

activation of adenylate cyclase. Thus the growth inhibition of T47D cells produced by CTs, dibutyryl cyclic AMP and L-arginine may all be mediated via increased cyclic AMP.

The T47D cells were not synchronized in our experiments but the most prominent inhibitory effect on cell growth with CTs was observed in the logarithmic phase when the cell population in G_1 phase is increased (16). In contrast insulin and EGF, cell growth stimulants, have been suggested to enhance the traverse of cells through G_2 phase and mitosis (17).

HCT, sCT and [Asu^{1,7}]eCT were all almost equipotent in their suppression of T47D cell growth. This contrasts with the findings that hCT competes less effectively for ^{125}I-sCT binding in breast cancer cells and also is a less potent stimulator of adenylate cyclase activity in this system (4). The discrepancy with the binding studies may be explained by the heterologous label employed. In the adenylate cyclase experiments a broken cell preparation was used and it is likely that the less stable human analogue would be more quickly broken down than its eel and salmon counterparts. The lower biological activity of mammalian CTs is usually attributed to faster breakdown (18).

1,25-$(OH)_2D_3$ has specific receptors in T47D cells and causes stimulation of cell growth at near physiological concentrations (11). However, higher concentrations have a markedly inhibitory effect. This biphasic response is similar to that reported for oestrogen in MCF 7 cells (12). Cho-Chung has shown antagonistic interactions between cyclic AMP and oestrogen in the growth control of hormone dependent mammary tumours (19). In his experiments, the nuclear translocation of the cyclic AMP binding protein, the regulatory subunit of protein kinase, type II, was inversely related to the nuclear translocation of oestrogen receptors, during growth and regression of the tumours. He postulated that the interaction between cyclic AMP and oestrogen may occur at a nuclear level via specific actions on nuclear protein phosphorylation. It is of interest

to speculate that similar interactions may occur between CT and 1,25-$(OH)_2D_3$ in the growth control of T47D cells. However, it should be noted that our experiments show that either hormone is capable of producing its effects in the absence of the other.

The *in vivo* consequences of these results are unknown and need to be investigated in a CT and 1,25-$(OH)_2D_3$ receptor positive animal tumour model. One possibility is that the transient hypocalcaemic action of CT in breast cancer with bone metastases (20) may be due in part to a direct action on the tumour cells themselves.

ACKNOWLEDGEMENT: We wish to thank Professor Iain MacIntyre for helpful discussions during the course of this work.

REFERENCES:

1. Marx, S.J., Woodard, C.J. and Aurbach, G.D. (1972) Science 178, 999-1001.
2. Marx, S.J., Woodard, C.J. and Aurbach, G.D. (1973) J. Biol. Chem. 248, 4797-4802.
3. Findlay, D.M., Michelangeli, V.P., Eisman, J.A., Frampton, R.J., Moseley, J.M., MacIntyre, I., Whitehead, R. and Martin, T.J. (1980) Cancer Res. 40, 4764-4767.
4. Martin, T.J., Findlay, D.M., MacIntyre, I., Eisman, J.A., Michelangeli, V.P., Moseley, J.M. and Partridge, N.C. (1980) Biochem. Biophys. Res. Commun. 96, 150-156.
5. Hunt, N.H., Ellison, M., Underwood, J.C.E. and Martin, T.J. (1977) Br. J. Cancer 35, 777-784.
6. Marx, S.J., Aurbach, G.D., Gavin, J.R.III and Buel, D.W. (1974) J. Biol. Chem. 249, 6812-6816.
7. Chausmer, A., Stuart, C. and Stevens, M. (1980) J. Lab. Clin. Med. 96, 933-938.
8. Cho-Chung, Y.S., Clair, T., Bodwin, J.S. and Berghoffer, B. (1981) Science 214, 77-79.
9. Friedman, D.L. (1976) Physiol. Rev. 56, 652-708.
10. Pastan, I.H., Johnson, G.S. and Anderson, W.B. (1975) Annu. Rev. Biochem. 44, 491-522.
11. Freake, H.C., Marcocci, C., Iwasaki, J. and MacIntyre, I. (1981) Biochem. Biophys. Res. Commun. 101, 1131-1138.
12. Lippman, M.E. and Bolan, G. (1975) Nature 256, 592-593.
13. Patterson, M.K.Jr. (1979) In: Jacoby, W.B. and Paston, I.H. (eds.) Methods in Enzymology, vol. 58, pp. 141-152. Academic Press, New York.
14. Burton, K. (1956) Biochem. J. 62, 315-323.
15. Cho-Chung, Y.S., Clair, T., Bodwin, J.S. and Hill, D.M. (1980) Biochem. Biophys. Res. Commun. 95, 1306-1313.
16. Kuroda, Y. and Furuyama, J. (1963) Cancer Res. 23, 682-687.
17. Richman, R.A., Johnson, R.A. and Friedman, D.L. (1981) Proc. Soc. Exp. Biol. Med. 168, 196-203.
18. Habener, J.F., Singer, F.R., Deftos, L.J., Neer, R.M. and Potts, J.T. (1971) Nat. New Biol. 232, 91-92.
19. Cho-Chung, Y.S. (1979) Life Sci. 24, 1231-1240.
20. Nilsson, O., Almquist, S. and Karlberg, B.E. (1978) Acta Med. Scand. 204, 249-252.