with untreated small cell lung cancer (SCLC). *Eur J Cancer Clin Oncol* 1988, **24**, 1791–1794.
5. Wampler GL, Carter WH, Campbell ED, Goldman ID. Demonstration of a schedule-dependent therapeutic synergism utilizing the interacting drugs methotrexate and teniposide in L1210 leukemia. *Cancer Treat Rep* 1987, **71**, 581–591.
6. Chello PL, Sirotnak FM, Dorick DM, Moccio DM. Schedule-dependent synergism of methotrexate and vincristine against murine L1210 leukemia. *Cancer Treat Rep* 1979, **63**, 1889–1984.
7. Houghton JA, Meyer WH, Houghton PJ. Scheduling of vincristine: drug accumulation and response of xenografts of childhood rhabdomyosarcoma determined by frequency of administration. *Cancer Treat Rep* 1987, **71**, 717–721.
8. Minna JD, Pass H, Glatstein E, Ihde DC. Cancer of the lung. In: De Vita VT, Hellman S, Rosenberg SA, eds. *Cancer Principles and Practice of Oncology* 3rd ed. Philadelphia, Lippincott, 1989, 591–705.

Eur J Cancer, Vol. 27, No. 2, pp. 143–146, 1991.
Printed in Great Britain

0277–5379/91 $3.00 + 0.00

Modulation by Oestrogen and Progestins/Antiprogestins of Alpha Interferon Receptor Expression in Human Breast Cancer Cells

Jan H.J. Martin, Bronac M. McKibben, Maria Lynch and Hendrik W. van den Berg

Human breast cancer ZR-75-1 cells expressed 1516 (105) (mean [S.D.]) interferon (IFN) receptors (IFNR) per cell with K_d of 0.61 (0.15) nmol/l. Oestrogen independent ZR-PR-LT and tamoxifen resistant ZR-75-9a1 8 μmol/l cells expressed similar numbers of IFNR. ZR-75-9a1 cells, which had been maintained in the absence of tamoxifen or known oestrogenic activity for 46 weeks, expressed a significantly higher number of IFNR (3170 [315]). Exposure of ZR-75-1 cells to 10^{-9} mol/l 17β-oestradiol (E2) led to a consistent reduction in IFNR numbers whilst 10^{-6} mol/l tamoxifen slightly increased IFNR expression. Since IFN increases oestrogen receptors in this cell line, IFN and E2 appear to have opposite effects on expression of each others' receptor. 10^{-9} mol/l medroxy progesterone acetate and mifepristone significantly increased IFNR numbers whilst ORG 2058 decreased IFNR expression and ZK 98.299 had no effect. Progestin/antiprogestin induced IFNR increase in this cell line correlated with down-regulation of progesterone receptor (PR). Thus an IFN/ER/PR axis may exist in ZR-75-1 cells and variants.
Eur J Cancer, Vol. 27, No. 2, pp. 143–146, 1991.

INTRODUCTION

ALTHOUGH INTERFERONS (IFNs) have significant antiproliferative effects on human breast cancer cells *in vitro* and *in vivo* [1, 2] results with IFNs in the treatment of patients with breast cancer have been disappointing [3, 4]. However, the combination of IFN and tamoxifen in the treatment of breast cancer may be of benefit since IFNs increase oestrogen receptor [ER] expression *in vivo* [5] and *in vitro* [6, 7] and increase the sensitivity of breast cancer cells *in vitro* to the growth inhibitory activity of anti-oestrogens [7, 8]. In a pilot study complete remission of lymph-node metastases was achieved in one patient with IFN-α_{2c} and tamoxifen [9].

IFN binds to high-affinity cell surface receptors [10]. We have investigated IFN receptor (IFNR) expression in ZR-75-1 human breast cancer cells and tamoxifen resistant and oestrogen independent variants and correlated IFNR content with ER and progesterone receptor (PR) expression. To establish whether heterospecific receptor modulation occurs we have also investigated the effect of oestradiol and progestins/antiprogestins on IFNR number.

Correspondence to H.W. van den Berg.
J.H.J. Martin, M. Lynch and H.W. van den Berg are at the Department of Therapeutics and Pharmacology, The Whitla Medical Building, and B.M. McKibben is at the Department of Medicine, The Royal Victoria Hospital, The Queen's University of Belfast, 97 Lisburn Rd, Belfast BT9 7BL, Northern Ireland, U.K.
Received 26 Jul. 1990; accepted 23 Nov. 1990.

MATERIALS AND METHODS

Cells and culture conditions

ZR-75-1 human breast cancer cells were obtained from Flow Laboratories. Cells were routinely maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were grown in 5% CO_2 at 37°C. ZR-PR-LT cells were selected for their ability to grow in the absence of known oestrogenic activity [11] and were routinely maintained in RPMI 1640 lacking phenol red and supplemented with heat treated (53°C for 1 h) 5% FCS stripped by dextran-coated charcoal.

A tamoxifen resistant variant of ZR-75-1 cells, ZR-75-9a1 8 μmol/l, was routinely maintained in RPMI 1640 supplemented with 5% FCS and 8 μmol/l tamoxifen [12]. ZR-75-9a1 8 μmol/l cells which had been maintained for 46 weeks at the time of this

study in RPMI 1640 lacking phenol red and tamoxifen and supplemented with 5% FCS stripped by dextran-coated charcoal are referred to as ZR-75-9a1 cells.

Drugs

Medroxyprogesterone acetate (MPA) and 17β-oestradiol (E2) were obtained from Sigma. Mifepristone and ZK 98.299 were gifts from Dr H. Michna, Schering AG. ORG 2058 and ^{125}I-NaI (37 GBq/ml) were obtained from Amersham International. Human recombinant IFN-α_{2c} (2.3×10^8 IU/mg) was a gift from Dr G.R. Adolf, Bender, Vienna.

Radioiodination of IFN-α_{2c}

IFN-α_{2c} was radioiodinated with the Iodogen method [13]. Iodogen was obtained from the Pierce Chemical Company, Illinois. 25 μg IFN-α_{2c} in 100 μl 0.2 mol/l phosphate-buffered saline (PBS) pH 7.4 was added to 5 μg iodogen followed by 37 MBq ^{125}I-NaI. The mixture was fractionated by reverse-phase high-performance liquid chromatography in a Waters Associates (Milford) gradient system fitted with an analytical C8 column. The gradient was trifluoroacetic acid (TFA)/water (0.05/99.95) and TFA/water/acetonitrile (0.05/29.95/70.0) was used for elution. The flow rate was 1.5 ml/min and 0.75 ml fractions were collected every 30 s. The column effluent was monitored at 214 nm (Absorbance Units Full Scale 0.2) and the radioactivity in each fraction was counted. The peak fractions were pooled and stored at −20°C in aliquots containing 4% FCS. Labelled IFN had a specific activity of 11.0-16.7 TBq/mmol.

A "cold" iodination of IFN-α_{2c} was done by substituting an equimolar concentration of KI for ^{125}I-NaI.

Interferon binding to whole cells

IFNR expression was measured in a whole-cell binding assay similar to that described [7]. Cells (5×10^4 or 2×10^5) were plated into 24-place multiwell dishes and allowed to attach for 48 h. In certain experiments cells were allowed to attach for 24 h and medium was then replaced with medium with or without drug and receptor assays were done 6 days later. All further steps were done on ice. The medium was removed and IFN binding assessed with either a single concentration of radioactive ligand (1000 or 4000 IU/ml) or a range of concentrations (100–5000 IU/ml) in the absence or presence of a 100 fold excess of cold IFN-α_{2c} to assess non-specific binding. After exposure of cells to radioactive ligand each well was washed three times with ice-cold PBS and cell layers were solubilised with 500 μl 1 mol/l NaOH for 1 h at 37°C. Contents were removed and wells were washed with a further 500 μl 1 mol/l NaOH. Washings were added to the appropriate tubes and radioactivity counted. The exact concentration of free ^{125}I-IFN was measured by counting a 100 μl sample of each concentration.

Maximum binding capacity (*B*max) and affinity (*K*d) were calculated after linearisation of specific binding data [14]. Lines were fitted by linear regression analysis. Data from each control and treated pair were analysed by deriving *t* values from standard deviations associated with the two regression lines [15]. Mean basal IFNR values for each cell line were compared using Student's *t* test.

Table 1. ER and PR status of ZR-75-1 cells and variants

Cell line	ER	PR basal	PR induced*
ZR-75-1	214 (10)	81 (11)	1237 (133)
ZR-PR-LT	ND	1675 (134)	1443 (215)
ZR-75-9a1 8μmol	ND	ND	ND
ZR-75-9a1	ND	ND	ND

*5 day exposure to E2 (10^{-9} mol/l).
All values are fmol/mg protein [mean (S.E.)], $n = 3$. ND = not detectable.

Binding competition studies

The relative affinities of unlabelled and "cold" iodinated IFN-α_{2c} for binding sites were measured by incubating ZR-75-1 cells with 1000 IU/ml ^{125}I-IFN-α_{2c} in the presence of increasing concentrations of cold ligand.

RESULTS

Table 1 summarises the ER and PR status of the cell lines used. ZR-75-1 cells are ER positive and express low basal levels of PR that are inducible by E2. ZR-PR-LT cells [11] fail to express specific binding sites characteristic of type 1 ER but possess PR at a concentration similar to induced values in the parent line. The tamoxifen resistant lines, ZR-75-9a1 8 μmol/l ZR-75-9a1 are ER and PR negative [12].

The time course of specific binding of ^{125}I-IFN-α_{2c} to ZR-75-1 cells at 4°C and 37°C is shown in Fig. 1. At 4°C specific binding

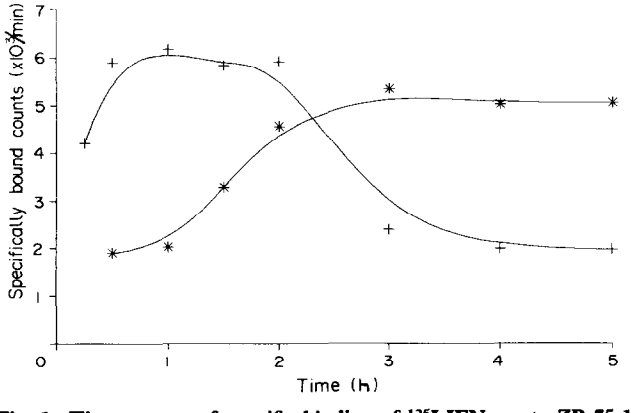

Fig. 1. Time course of specific binding of ^{125}I-IFN-α_{2c} to ZR-75-1 cells at 4°C(*) and 37°C(+). Free ^{125}I-IFN-α_{2c} concentration was 4000 IU/ml.

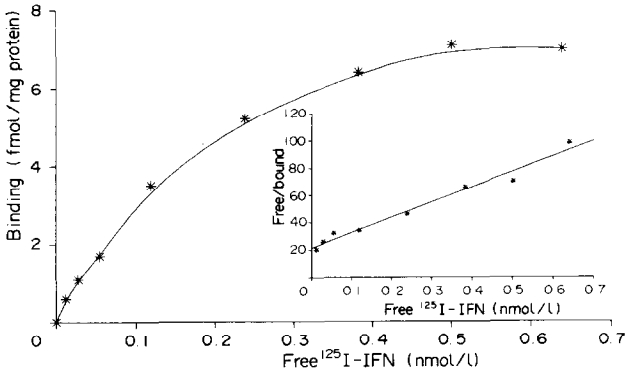

Fig. 2. Specific binding of ^{125}I-IFN-α_{2c} to ZR-75-1 cells. Inset = Woolf transformation.

Table 2. IFNR expression by ZR-75-1 cells and variants

Cell line	Receptors per cell	Kd (nmol/l)
ZR-75-1	1516 (105)	0.61 (0.15)
ZR-PR-LT	1443 (147)	0.53 (0.10)
ZR-75-9a1 8μmol/l	1292 (87)	0.55 (0.10)
ZR-75-9a1	3170 (315)*	0.67 (0.14)

*P = 0.005 vs. ZR-75-1.

increased during the first 3 h of incubation and thereafter stabilised. At 37°C specific binding stabilised after 30 min and then decreased after 2 h. This may be due to internalisation and degradation of the IFNR complex [16]. An incubation of 4 h at 4°C was selected for all further experiments.

Figure 2 shows a typical saturation binding experiment. Specific binding of ^{125}I-IFN-α_{2c} to ZR-75-1 cells was saturable over the concentration range used and was linear on Woolf transformation.

Table 2 shows IFNR expression by ZR-75-1 cells and variants. Both ZR-PR-LT and ZR-75-9a1 8 μmol/l contained similar numbers of IFNR to the parent ZR-75-1 cells. However, ZR-75-9a1, maintained in the absence of tamoxifen or oestrogens, expressed a significantly higher number of IFN receptors per cell (3170 [315]) compared with ZR-75-1 cells. There was no significant difference between the cell lines in the apparent affinity of IFN for specific binding sites.

A 6 day exposure of ZR-75-1 cells to 10^{-9} mol/l E2 consistently led to a 20-40% reduction in IFNR expression (Table 3). This effect was significant at the 10 or 5% level in two out of three experiments. In contrast, 10^{-6} mol/l tamoxifen slightly increased IFNR levels, although in a single experiment this effect did not reach significance.

Table 4 shows the effect of a 6 day exposure to progestins or antiprogestins on IFNR expression in ZR-PR-LT cells. Both MPA and mifepristone which effectively down-regulate PR in these cells over 6 days (data not shown), significantly increased IFNR levels. However, ZK 98.299 and ORG 2058, which do not significantly alter PR expression in these cells, either decreased IFNR numbers (ORG 2058) or had no significant effect (ZK 98.299).

The results of a competition binding assay are shown in Fig. 3. "Cold" iodinated IFN-α_{2c} showed an approximate 5 fold reduction in its ability to displace ^{125}I-IFN compared with non-iodinated IFN-α_{2c}.

Table 3. Effect of 6 day exposure of ZR-75-1 cells to 10^{-9} mol/l E2 or 10^{6} mol/l tamoxifen on IFNR expression

Receptors per cell		K_d (nmol/l)	
−E2	+E2	−E2	+E2
906	486*	0.25	0.31
1403	1118†	0.36	0.47
1419	1125	0.5	0.45
−Tam	+Tam	−Tam	+Tam
1162	1452	0.54	0.58

Tam = tamoxifen.
*P < 0.1 vs control.
†P < 0.05 vs. control.

Table 4. Effect on IFNR expression of 6 day exposure of ZR-PR-LT cells to 10^{-9} mol/l progestins or antiprogestins

	Receptors per cell		K_d (nmol/l)	
Drug	Control	Treated	Control	Treated
MPA	1011	1354*	0.29	0.26
Mifepristone	2289	3226*	0.50	0.43
ORG 2058	1736	1351*	0.35	0.22
ZK 98.299	2445	2347	0.50	0.50

*P < 0.05 vs. control.

DISCUSSION

We have demonstrated the presence of specific IFNR sites on ZR-75-1 cells and variants. IFNRs are expressed by several human tumour cell lines, with most cell types expressing between 1000 and 2000 receptor sites per cell [10]. Our values were consistent with these.

Anti-oestrogen resistance in ZR-75-1 cells routinely maintained in the presence of 8 μmol/l tamoxifen was associated with loss of detectable ER and PR (ref. 12 and Table 1). However, IFNR levels were unchanged compared with the parent cell line. Similarly oestrogen independence, which was associated with a loss of detectable type 1 ER and elevated levels of PR (ref. 11 and Table 1), was not accompanied by a significant change in IFNR levels compared with the ZR-75-1 parent cell line. The tamoxifen resistant line maintained in the absence of tamoxifen or known oestrogens (ZR-75-9a1) expressed a significantly higher number of IFNR compared with the other lines. Thus, whilst short or chronic exposure to tamoxifen did not significantly affect IFNR expression, removal of the drug allowed up-regulation of IFNR numbers in the anti-oestrogen resistant line.

Exposure of ZR-75-1 cells to E2 for 6 days resulted in a reduction in IFNR number. Since we have shown that IFN-α_{2c} increases ER expression in these cells [17], IFN and E2 appear to have opposite effects on expression of each other's receptor.

Progestins and antiprogestins down-regulate PR levels in human breast cancer cells and generally oppose the effects of oestrogens [17]. We have found that the down-regulation of PR by MPA and mifepristone in ZR-PR-LT cells is accompanied

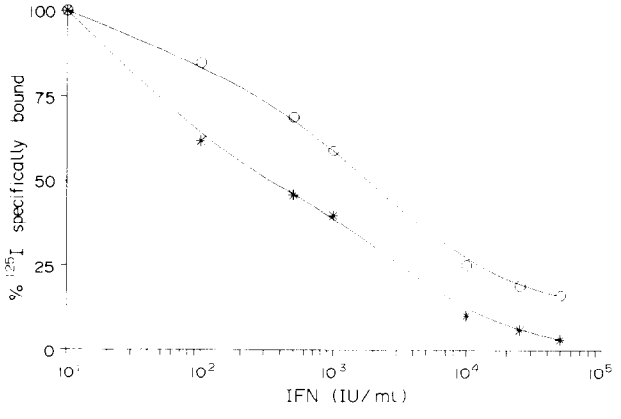

Fig. 3. Ability of IFN-α_{2c} (*) and "cold" iodinated IFN-α_{2c} (○) to displace ^{125}I-IFN-α_{2c} from specific binding sites on ZR-75-1 cells. Free ^{125}I-IFN-α_{2c} concentration was 1000 IU/ml.

by an increase in IFNR expression (Table 4). Neither ORG 2058 or ZK 98.299 down-regulated PR at the concentration used or up-regulated IFNR. The reason for these observations is not known, but it is of interest that the latter two compounds lack the potent glucocorticoid/antiglucocorticoid activity of MPA and mifepristone [18]. Further studies are required to establish the dose-dependency of IFNR modulation in relation to the affinity of progestins or antiprogestins for the PR and the glucocorticoid receptor.

Reduction induced by progestins or antiprogestins in PR expression in ZR-PR-LT cells resulted in an opposite effect on IFNR number to that observed in E2 treated ZR-75-1 cells, which also responded to E2 exposure with a 6 fold increase in PR levels. IFNR expression is, therefore, inversely related to PR expression. This suggestion is supported by the observation that ZR-75-9a1 cells, which lack PR, express a significantly higher number of IFNR than ZR-75-1 cells. However, elevated PR expression in ZR-PR-LT cells was not accompanied by a significant reduction in IFNR.

The observation that "cold" iodinated IFN-α_{2c} has approximately one fifth of the affinity of the natural peptide for binding sites on ZR-75-1 cells suggests that the affinity of native IFN-α_{2c} for its receptor, assessed by saturation binding analysis of ^{125}I-IFN-α_{2c}, may be underestimated by a similar factor. This would result in a K_d for the natural peptide of approximately 0.1 nmol/l or 460 IU/ml. This value is in good agreement with the antiproliferative effects of IFN-α_{2c} towards ZR-75-1 cells, which are maximal at 500–1000 IU/ml [7].

Our results suggest that an ER/PR/IFNR axis exists in human breast cancer cells in culture. This may have important consequences for combined modality therapy *in vivo*. IFNR may be depressed by circulating oestrogens, thus compromising the ability of IFN to modulate ER expression. However, our results do indicate that co-administration of tamoxifen may prevent IFNR down-regulation and possibly increase IFNR levels. Progestins or antiprogestins may also facilitate IFN action by increasing IFNR numbers, and additional studies of this combination therapy in breast cancer are warranted.

1. Gastl G, Marth C, Leiter E, *et al.* Effects of recombinant alpha arg-interferon and gamma interferon on human breast cancer cell lines: dissociation of antiproliferative activity and induction of HLA-DR antigen expression. *Cancer Res* 1985, **45**, 2957–2961.
2. Taylor-Papadimitriou J, Shearer M, Balkwill FR, Fantes KH. Effects of HuIFN alpha 2 and HuIFN gamma (Namalwa) on breast cancer cells grown in culture and as xenografts in the nude mouse. *J Interferon Res* 1982, **2**, 479–491.
3. Sherwin SA, Mayer D, Ochs JJ, *et al.* Recombinant leucocyte A interferon in advanced breast cancer. *Ann Int Med* 1983, **98**, 598–602.
4. Nethersell A, Smedley H, Katrak M, Wheeler T, Sikora K. Recombinant interferon in advanced breast cancer. *Br J Cancer* 1984, **49**, 615–620.
5. Pouillart P, Palangie P, Jouve M, *et al.* Administration of fibroblast interferon to patients with advanced breast cancer: Possible effects on skin metastasis and on hormone receptors. *Eur J Cancer Clin Oncol* 1982, **18**, 929–935.
6. Sica G, Natoli V. The mechanism of action of antiestrogens. In: Pannuti F, ed. *Antiestrogens in Oncology: Past, Present and Prospects*. Amsterdam, Excerpta Medica, 1985, 54–61.
7. van den Berg HW, Leahey WJ, Lynch M, Clarke R, Nelson J. Recombinant human interferon alpha increases oestrogen receptor expression in human breast cancer cells (ZR-75-1) and sensitises them to the anti-proliferative effects of tamoxifen. *Br J Cancer* 1987, **55**, 255–257.
8. Kangas L, Nieminen A-L, Cantell K. Additive and synergistic effects of a novel antiestrogen, toremifene (Fc-1157a), and human interferons on estrogen responsive MCF-7 cells *in vitro*. *Med Biol* 1985, **63**, 187–190.
9. Porzsolt F, Otto AM, Trauschel B, Buck C, Wawer AW, Schonenberger H. Rationale for combining tamoxifen and interferon in the treatment of advanced breast cancer. *J Res Clin Oncol* 1989, **115**, 465–469.
10. Branca AA. Interferon receptors. *In Vitro Cell Devel Biol* 1988, **24**, 155–165.
11. van den Berg HW, Martin J, Lynch M. High progesterone receptor concentration in a variant of the ZR-75-1 human breast cancer cell line adapted to growth in oestrogen free conditions. *Br J Cancer* 1990, **61**, 504–507.
12. van den Berg HW, Lynch M, Martin J, Nelson J, Dickson GR, Crockard AD. Characterisation of a tamoxifen-resistant variant of the ZR-75-1 human breast cancer cell (ZR-75-9a1) and stability of the resistant phenotype. *Br J Cancer* 1989, **59**, 522–526.
13. Fraker PJ, Speck JC. Protein and cell membrane iodinations with a sparingly soluble chloroamide, 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril. *Biochem Biophys Res Commun* 1978, **80**, 849–857.
14. Keightly DD, Cressie NAC. The Woolf plot is more reliable than the Scatchard plot in analysing data from hormone receptor assays. *J Steroid Biochem* 1980, **13**, 1317–1323.
15. Davies OL, Goldsmith PL. Linear relationships between two variables. In: *Statistical Methods in Research and Production*. Edinburgh, Oliver and Boyd, 1972, 193–194.
16. Branca AA, Baglioni C. Down-regulation of the interferon receptor. *J Biol Chem* 1982, **257**, 13197–13200.
17. Horwitz KB, Wei LL, Sedlacek SM, D'Arville CN. Progestin action and progesterone receptor structure in human breast cancer: a review. *Recent Progr Hormone Res* 1985, **41**, 249–316.
18. Schneider MR, Michna H, Nishino Y, El Etraby MF. Antitumor activity of the progesterone antagonists ZK 98.299 and RU 38.486 in the hormone dependent MXT mammary tumor model of the mouse and the DMBA- and the MNU-induced mammary tumor models of the rat. *Eur J Cancer Clin Oncol* 1989, **25**, 691–701.

Acknowledgement—This study was supported by the UK Cancer Research Campaign.