Down-Regulation of Nitric Oxide Production by Droloxfene and Toremifene in Human Breast Cancer Cells: Interferon Alpha has No Further Effect

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Citation

Abstract
We investigated the effect of tamoxifen, 4-OH tamoxifen, toremifene droloxfene, interferon-Ñ2a, interferon-Ñ2b and interferon-Ñ2c, singly and in combination, for their effect on nitric oxide production by MCF-7 and ZR-75-1 human breast cancer cells. Tamoxifen and 4-OH tamoxifen singly had no effect on nitric oxide production by both cell lines. However treatment with droloxfene or toremifene significantly reduced nitric oxide production by both MCF-7 and ZR-75-1 human breast cancer cell lines. Combination treatment with anti-estrogens and interferon-*2a interferon-*2b or interferon-*2c had no synergistic or additive effect compared to each drug singly.

INTRODUCTION
Nitric oxide (NO), is a product of the nitric oxide synthase (NOS) family of isoenzymes, which convert L-arginine to L-citrulline and nitric oxide (NO). The exact role of nitric oxide NO in tumour biology has not yet been fully elucidated as it exhibits both pro- and anti- tumour functions. We have previously shown the presence of the L-arginine/NO pathway in ZR-75-1 human breast cancer cells and have demonstrated reduced expression of iNOS and eNOS in a variant cell line which has acquired estrogen independence. We have also shown a negative correlation between eNOS expression and histologic grade in primary invasive ductal breast carcinoma.

Interferons (IFN) are cytokines that regulate various cellular responses. They have been shown to have anti-proliferative activities towards several human cancer cell lines including breast, melanoma and colon; and are also effective against many malignancies particularly, bladder, renal, multiple myeloma, hairy cell leukaemia and metastatic melanoma.

Tamoxifen is still the treatment of choice for patients with advanced breast cancer. Although tamoxifen acts primarily as an anti-estrogen, there is evidence that some of its anti-tumour activity may be unrelated to estrogen antagonism. In particular, it may have an effect on the nitric oxide pathway as it has been shown that tamoxifen causes an increase in nitric oxide synthase activity in C3H10T1/2 fibroblasts and induces apoptosis in K562 erythroleukaemia cells by activating nitric oxide synthase.

IFN and tamoxifen can augment each others anti-tumour activities. On the one hand, it has been demonstrated that IFN increases ER expression which then subsequently sensitisises the cells to the anti-proliferative effects of tamoxifen whereas on the other hand, it has also been shown that tamoxifen enhances IFN-regulated gene expression in breast cancer cells.

In addition to tamoxifen, other triphenylethylene compounds, such as droloxfene and toremifene have been shown to be effective in the treatment of breast cancer and are currently undergoing further investigation for their potential beneficial effects on the cardiovascular system and in osteoporosis.

Although several studies, have previously investigated the effectiveness of a combination of tamoxifen and IFN in breast cancer the available data is somewhat conflicting and no firm conclusion has been drawn. Our previous investigations had shown that tamoxifen could down-
regulate PMA-induced NO production in ZR-75-1 cells (2) and that combination treatment with toremifene plus interferon-2a or -2b could result in a synergistic anti-proliferative response (14). We have chosen, in this study, to further investigate the effects of different anti-estrogens, both singly and in combination with different IFN-α subtypes, on the nitric oxide pathway of human breast cancer cells. Our results demonstrate a significant down-regulation of nitric oxide production by droloxifene and toremifene in both MCF-7 and ZR-75-1 human breast cancer cells with no further additive or synergistic effects of different interferon alpha subtypes.

MATERIALS AND METHODS

MATERIALS

IFN-α2a (Roferon-A) was a gift from Roche Products Ltd., Herts, UK. IFN-α2b (Intron A) was a gift from Schering-Plough Ltd., Herts, UK. IFN-α2c was from Biowhittaker U.K. Ltd., Berks, England. Droloxifene, toremifene and 4-OH tamoxifen were gifts from Dr. M. Rowlands, CRC, Surrey. All other chemicals were from Sigma Chemical Company (Poole, U.K.).

CULTURE OF BREAST CANCER CELLS

The MCF-7 and ZR-75-1 cell lines (ECACC, Porton Down, Salisbury, England) were routinely maintained at 37°C in RPMI 1640 medium supplemented with HEPES (20mM), FCS (10%), penicillin (50 IU/ml), streptomycin (50 μg/ml) and glutamine (300 μg).

DETERMINATION OF NO PRODUCTION

Cells (5 x 10^4) were plated into 24-well plates and allowed to attach for 24 h. Medium was then replaced with medium with or without drug and nitrite assay was performed 5 days later. Nitric oxide was measured as the amount of nitrite (NO2-) which is a stable end-product of NO metabolism. Cell free supernatants were collected and stored at -20°C until analysis. Nitrite concentration in the medium was quantitated by a colourimetric assay based on the Greiss reaction (25). Briefly, samples (100 μl) were mixed with an equal volume of Greiss reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2.5% H3PO4) and incubated at room temperature for 10 min. The absorbance was measured at 550nm in a microplate reader. Sodium nitrite was used as a standard

STATISTICAL ANALYSIS

Statistical significance was determined using the student’s t-test and the differences were regarded as significant for values of p<0.05.

RESULTS

Effect of IFN-α2a, α2b and α2c on nitric oxide production by ZR-75-1 human breast cancer cells.

During a 5 day culture period untreated ZR-75-1 cells secreted 3.1 nmol/ml NO2- (5 x 104 cells) into the culture medium. A 5 day treatment of ZR-75-1 cells with IFN-α2a, α2b or α2c (0.1-1000 IU/ml) did not result in a significant change in nitrite production (Figure 1).
Down-Regulation of Nitric Oxide Production by Droloxifene and Toremifene in Human Breast Cancer Cells: Interferon Alpha has No Further Effect

Cells (5 x 104) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without IFN- 2a (white), IFN- 2b (pale grey) or IFN- 2c (dark grey) at 0.1-1000 IU/ml and cultured for 5 days. After 5 days the supernatant was removed and aliquots (100μl) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

Effect of tamoxifen, 4-OH tamoxifen, droloxifene and toremifene on nitric oxide production by ZR-75-1 human breast cancer cells.

During a 5 day culture period untreated ZR-75-1 cells secreted 3.1 mmol/ml NO2- (5x104 cells) into the culture medium. A 5 day treatment of ZR-75-1 cells with tamoxifen (results previously reported in ref 2) or 4-OH tamoxifen (10⁻¹⁰-10⁻⁹M) did not result in a significant change in nitrite production (Figure 3). Treatment of the same cells with droloxifene (10⁻¹⁰-10⁻⁹M) and toremifene (10⁻⁸-10⁻⁶M) resulted in a significant (p<0.001) reduction in nitrite production for all concentrations.

Figure 3
Figure 3: Effect of anti-estrogens on nitrite production by ZR-75-1 cells.

Cells (5 x 104) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without tamoxifen (white), 4-OH tamoxifen (pale grey), droloxifene (dark grey) or toremifene (very dark grey) at 10⁻¹⁰-10⁻⁸M and cultured for 5 days. After 5 days the supernatant was removed and aliquots (100μl) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

Effect of tamoxifen, 4-OH tamoxifen, droloxifene and toremifene on nitric oxide production by MCF-7 human breast cancer cells.

During a 5 day culture period untreated MCF-7 cells secreted 2.6 mmol/ml NO2- (5x104 cells) into the culture medium. A 5 day treatment of MCF-7 cells with tamoxifen (10⁻⁹-10⁻⁶M) or 4-OH tamoxifen (10⁻¹⁰-10⁻⁸M) did not result in a significant change in nitrite production (Figure 4). Treatment of the same cells with droloxifene (10⁻¹⁰-10⁻⁹M) and toremifene (10⁻⁸-10⁻⁶M) resulted in a significant
Down-Regulation of Nitric Oxide Production by Droloxifene and Toremifene in Human Breast Cancer Cells: Interferon Alpha has No Further Effect

(p<0.001) reduction in nitrite production for all concentrations.

**Figure 4**
Figure 4: Effect of anti-estrogens on nitrite production by MCF-7 cells.

Cells (5 x 10⁴) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without tamoxifen (white), 4-OH tamoxifen (pale grey), droloxifene (dark grey) or toremifene (very dark grey) at 10⁻¹⁰⁻¹⁰⁻⁶ M and cultured for 5 days. After 5 days the supernatant was removed and aliquots (100*l) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

**Effect of combinations of IFN- 2a, 2b or 2c with anti-estrogens on nitric oxide production by ZR-75-1 human breast cancer cells.**

Pretreatment of ZR-75-1 cells with IFN- 2a, IFN- 2b or IFN- 2c, followed by treatment with tamoxifen, did not show any significant difference in NO production compared to either, control cells, IFN only treated cells or tamoxifen only treated cells (Figure 5). Similar results were demonstrated for pretreatment with IFN- 2a, IFN- 2b or IFN- 2c, followed by treatment with 4-OH tamoxifen (Figure 6), droloxifene (Figure 7) or toremifene (Figure 8).

**Figure 5**: Effect of tamoxifen in combination with IFN- 2a, 2b and 2c on nitrite production by ZR-75-1 cells.

Cells (5 x 10⁴) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without IFN- 2a (white), IFN- 2b (pale grey), IFN- 2c (dark grey), at 10 IU/ml and cultured for 2 days. After 2 days in culture, medium was removed and replaced with fresh IFN-containing medium, with or without tamoxifen (2 M). After 5 days the supernatant was removed and aliquots (100*l) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

**Figure 6**: Effect of 4-OH tamoxifen in combination with IFN- 2a, 2b and 2c on nitrite production by ZR-75-1 cells.

Cells (5 x 10⁴) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without IFN- 2a (white), IFN- 2b (pale grey), IFN- 2c (dark grey), at 10 IU/ml and cultured for 2 days. After 2 days in culture, medium was removed and replaced with fresh IFN-containing medium, with or without 4-OH tamoxifen (2 M). After 5 days the supernatant was removed and aliquots (100*l) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

**Figure 7**: Effect of droloxifene in combination with IFN- 2a, 2b and 2c on nitrite production by ZR-75-1 cells.

Cells (5 x 10⁴) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without IFN- 2a (white), IFN- 2b (pale grey), IFN- 2c (dark grey), at 10
IU/ml and cultured for 2 days. After 2 days in culture, medium was removed and replaced with fresh IFN-containing medium, with or without droloxifene (2 M). After 5 days the supernatant was removed and aliquots (100 μl) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

Figure 8: Effect of toremifene in combination with IFN-2a, 2b and 2c on nitrite production by ZR-75-1 cells. Cells (5 x 10^4) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without IFN-2a (white), IFN-2b (pale grey), IFN-2c (dark grey), at 10 IU/ml and cultured for 2 days. After 2 days in culture, medium was removed and replaced with fresh IFN-containing medium, with or without toremifene (2 M). After 5 days the supernatant was removed and aliquots (100 μl) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

Pretreatment of MCF-7 cells with IFN-2a, IFN-2b or IFN-2c, followed by treatment with tamoxifen, did not show any significant difference in NO production compared to either, control cells, IFN only treated cells or tamoxifen only treated cells (Figure 9). Similar results were demonstrated for pretreatment with IFN-2a, IFN-2b or IFN-2c, followed by treatment with 4-OH tamoxifen (Figure 10), droloxifene (Figure 11) or toremifene (Figure 12).

Figure 9: Effect of tamoxifen in combination with IFN-2a, 2b and 2c on nitrite production by MCF-7 cells. Cells (5 x 10^4) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without IFN-2a (white), IFN-2b (pale grey), IFN-2c (dark grey), at 10 IU/ml and cultured for 2 days. After 2 days in culture, medium was removed and replaced with fresh IFN-containing medium, with or without tamoxifen (2 M). After 5 days the supernatant was removed and aliquots (100 μl) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).
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Figure 10: Effect of 4-OH tamoxifen in combination with IFN-2a, 2b and 2c on nitrite production by MCF-7 cells. Cells (5 x 10⁴) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without IFN-2a (white), IFN-2b (pale grey), IFN-2c (dark grey), at 10 IU/ml and cultured for 2 days. After 2 days in culture, medium was removed and replaced with fresh IFN-containing medium, with or without 4-OH tamoxifen (2 M). After 5 days the supernatant was removed and aliquots (100 µl) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

Figure 11: Effect of droloxifene in combination with IFN-2a, 2b and 2c on nitrite production by MCF-7 cells. Cells (5 x 10⁴) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without IFN-2a (white), IFN-2b (pale grey), IFN-2c (dark grey), at 10 IU/ml and cultured for 2 days. After 2 days in culture, medium was removed and replaced with fresh IFN-containing medium, with or without droloxifene (2 M). After 5 days the supernatant was removed and aliquots (100 µl) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

Figure 12: Effect of toremifene in combination with IFN-2a, 2b and 2c on nitrite production by MCF-7 cells. Cells (5 x 10⁴) were seeded into wells of a culture plate in 1ml of RPMI 1640 medium and allowed to attach for 24 h. Medium was then replaced with medium with or without IFN-2a (white), IFN-2b (pale grey), IFN-2c (dark grey), at 10 IU/ml and cultured for 2 days. After 2 days in culture, medium was removed and replaced with fresh IFN-containing medium, with or without toremifene (2 M). After 5 days the supernatant was removed and aliquots (100 µl) were analysed for nitrite concentration. This was determined by reacting with an equal volume of Greiss reagent and measuring the absorbance at 540nm in a Bio-Rad plate reader. The results of a typical experiment which has been repeated 6 times are presented. Values presented are means SE of experimental replicates (n=3).

DISCUSSION
Tamoxifen is still the treatment of choice for hormone-dependent breast cancer patients. In addition to its anti-estrogenic properties, tamoxifen has been shown to have many other mechanisms of action including modulation of the multi-drug resistance pump, p-glycoprotein (26), formation of DNA adducts (27) and effects on calcium metabolism (28). Furthermore, tamoxifen has been demonstrated to have a role in the NO pathway as it increases NO production in K562 erythroleukaemia cells (29) and C3H 10T1/2 fibroblasts (30). Conversely, tamoxifen inhibits oestrogen and progesterone induced NO production by T47D human breast cancer cells (31) and we have previously shown that tamoxifen can down-regulate PMA induced nitrite production by ZR-75-1 human breast cancer cells (2).

Despite its widely accepted role in the treatment of breast cancer, tamoxifen has been associated with an increased risk of endometrial cancer (32) and therefore alternatives to tamoxifen such as droloxifene and toremifene have been investigated. However, their effects on nitric oxide...
Down-Regulation of Nitric Oxide Production by Droloxifene and Toremifene in Human Breast Cancer Cells: Interferon Alpha Has No Further Effect

metabolism is, at present, poorly understood. The results of the current study show that in addition to their known anti-estrogenic properties, both of these tamoxifen analogues have a further mechanism of action, namely down regulation of NO production. These results are in accordance with a study in which toremifene was shown to shift the balance between nitric oxide and endothelin-1 in breast cancer patients (3).

As NO and IFN-alpha play opposing roles in angiogenesis, with NO promoting angiogenesis (3) and IFN- inhibiting angiogenesis (4), we chose to investigate the possibility that IFN-alpha inhibition of angiogenesis may be via down-regulation of NO production. Our results show however, that all three IFN- subtypes have no significant effect on NO production and suggest therefore IFN- inhibited angiogenesis is unlikely to be mediated via down regulation of NO production.

Much research has been directed at investigating combinations of interferons and anti-estrogens in breast cancer (5). Our previous work investigating combinations of different IFN- subtypes with toremifene in the ZR-75-1 human breast cancer cell line, demonstrated a synergistic or additive anti-proliferative effect which was shown to be dependent upon the IFN- subtype used (24). The results in the current investigation demonstrate that there is no significant change in NO production following combination treatment with different IFN- subtypes and anti-estrogens.

The role of NO in tumour biology is not fully understood at present and NO is known to have both pro- and anti-tumour functions. In conclusion, we have demonstrated a significant down-regulation of NO production by droloxifene and toremifene in ZR-75-1 and MCF-7 human breast cancer cells. This novel mechanism of action for these two anti-estrogens will require further investigation but their inhibition of NO synthesis may provide a novel therapeutic approach for reducing angiogenesis.

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