cancer, combination therapies using two or more antitumour drugs with differing mechanisms of action have generally proved more effective than single-drug therapies. In this basis, combinations of FTIs with a variety of commonly used anticancer agents have been tested on human tumours [1]. The FTI R115,777 represents one of the first-in-class inhibitors to enter the clinic [2]. Clinical trials have shown that this compound has an acceptable safety profile, allowing the administration of biologically active doses. Phase II clinical trials in patients with metastatic breast carcinoma have shown that R115,777 has reproducible single-agent activity [3]. In this present study, the activity of combined treatment with Tam and R115,777 was investigated using a mammary cancer cell line (MCF-7), and the results are compared with those from our previous studies using FTI-277 (an experimental FTI not in clinical usage) to evaluate the potential value of this combined therapy in the treatment of advanced breast cancer.

The antitumour effect of Tam is believed to be due to a combination of hormone receptor dependent (estrogen receptor (ER)-mediated with two receptor subtypes, ERα and ERβ) and independent (non-ER-mediated) mechanisms [4]. The signalling proteins in the non-ER-mediated pathways [5] include interactions with microsomal anti-estrogen-binding sites (AEBS) that are high affinity binding sites for Tam [6,7]. AEBS are different from ERs because they have no affinity for 17β-estradiol or steroidal anti-estrogens, and we recently reported that these sites consist of several proteins involved in cholesterol metabolism [8]. Previous reports have suggested that Tam inhibits MCF-7 cell proliferation [9] by binding to both ERs and AEBS [7,10] and can induce apoptotic cell death, both *in vitro* [11] and *in vivo* [12].

In parallel, FTIs inhibit the growth of a broad variety of human tumour cells *in vitro* and studies to date have not identified any cellular characteristics that predict the antitumour efficacy of this class of agent. *In vitro*, however, FTI treatment of tumour cells has been associated with activation of apoptotic pathways [13].

We have previously shown the involvement of pocket proteins [14] and genomic ER effects [15] in the additive efficacy of a combination of Tam and FTI277. To dissect out that portion of the Tam effect associated with the 'AEBS pathway', we compare here the effects of combining R115,777 with a selective AEBS ligand (N-pyrrolidine(-phenylmethyl-phenoxy)-ethanamine-HCl (PBPE)) [10] or with a selective estrogen receptor ligand (ICI182,780) on MCF-7 cell proliferation. The effects of such treatments on the induction of cellular apoptosis have been evaluated by monitoring cell cycle alterations and caspase involvement. The contribution of ERs to apoptosis induction has also been determined by comparing the effects of combined treatments with either the pure anti-estrogen ICI182,780 or the AEBS selective ligand PBPE.

Materials and methods

Cell lines

The human adenocarcinoma breast cell line MCF-7 was obtained from the American Tissue Culture Collection (Manassas, VA, USA). MCF-7 cells were grown routinely in RPMI 1640 medium supplemented with 5% fetal bovine serum (Gibco BRL, Life Technologies, Cergy Pontoise, France) and 2 mM L-glutamine. The cells were incubated at 37°C in a humidified 5% CO_2 incubator.

For all experiments, the cells were treated with R115,777 (Janssen Research Foundation, Janssen Pharmaceutica, L.P., Titusville, NJ 08560, USA), Tam (Sigma-Aldrich S.a.r.l. St Quentin Fallavier, France), ICI182,780 (Tocris Bioscience, Ellisville, Missouri 63021 USA), PBPE (synthesized in our laboratory) [16] or vehicle, and the medium was changed every 2 days. R115,777, Tam, ICI182,780 and PBPE were all dissolved in ethanol and then diluted 10^3-fold directly into the culture medium.

Sulphorhodamine B assay for proliferation

The quantitative sulphorhodamine B (SRB) colorimetric assay [17] was used to determine the growth inhibitory effect of drugs on MCF-7 cells. Cells were seeded at 3,500 per well in 96-well plates and grown for 24 h. The cells were then treated with increasing concentrations of compounds and grown for a further 5 days. The medium was changed after 2 days. At the end of the incubation, cells were fixed with 50% trichloracetic acid (1 h at 4°C), stained for 30 minutes at room temperature with 50 µl of a 0.4% w/v SRB solution (Sigma) in 1% acetic acid. SRB was then removed and cells were quickly rinsed four times with 1% acetic acid. After air-drying, protein-bound dye was dissolved in 150 µl of 10 mM unbuffered Tris base (pH 10.5) for 5 minutes on a gyratory shaker. The pink SRB was quantified by measuring the optical density at 540 nm. For each condition, average cell density and the standard deviation were calculated from the data of six wells.

Isobologram analysis

Dose response interactions between the following combinations: R115,777-Tam, R115,777-ICI182,780, R115,777-PBPE at the IC_{50} (inhibitory concentration 50%) point were evaluated by the isobologram method of Steel and Peckham [18]. When the data points of the drug combination fall within the area surrounded by two lines (envelope of additivity), the combination is regarded as additive. When the data points of the drug combination fall to the left of the envelope (i.e., the combined effect is caused by lower doses of the two agents than predicted), the combination is regarded as having a supra-additive effect (synergism). Finally, when the data points fall to the right of the envelope, the combination is regarded as having a protective effect.

To determine the envelope of additivity, the two dose-response curves of R115,777 with either Tam, ICI182,780, or