PBPE alone were plotted according to bi-exponential equations. Based on these data, two isoeffect curves were constructed for each association. In all cases, an incremental effect was produced by R115,777 for any selected dose of Tam, ICI182,780, or PBPE.

Mode I line

The addition was calculated by taking the increment in dose starting from 0 that produced a log cell density that summated to the IC_{50} (hetero-addition where there would be no influence of drug treatment on R115,777 action). If the agents are acting additively through independent mechanisms, the combined data points would lie near this mode I line.

Mode II line

The addition was calculated by taking the increment in dose starting from the point on the dose-response curve of Tam, ICI182,780 or PBPE that produces a log cell density that summated to the IC_{50} (iso-addition where drug treatment would modify the basal state of the cells before R115,777 action). If the agents are acting additively through a similar mechanism, the combined data points would lie near this mode II line.

Cell cycle analysis

For each condition, 1.5×10^5 cells were seeded into 60 mm diameter dishes and treated as described above. Following treatment, cells were collected by trypsinization, washed twice and resuspended in 500 µl ice cold PBS. The cells were then fixed in 1.5 ml ice cold absolute ethanol for 30 minutes at 4°C, washed twice in PBS and stained with propidium iodide (100 µg/ml RNase A in PBS, 0.25% Tween 20 and 50 µg/ml propidium iodide) for 1 h at 37°C. DNA content was determined by flow cytometry on a FACS Calibur (Beckton Dickinson and Co., Meglan, France). Data were collected from 10,000 cells. The percentage of apoptotic cells was calculated by dividing the number of cells displaying red fluorescence lower than the G0–G1 diploid peak by the total number of cells collected times 100.

DAPI staining

Cells were grown on glass coverslips in 60 mm Petri dishes, washed once with PBS, fixed in PBS/3.7% formaldehyde for 15 minutes and washed twice with PBS. The coverslips were then mounted on glass slides with Vectashield mounting medium with 4'-6-diamidino-2-phenylindole (DAPI; AbCys 75010 Paris, France). In each experiment, a minimum of 200 nuclei were quantified using the ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA, USA).

Detection of caspase cleavage by flow cytometry

Floating and adherent cells were combined and fixed in methanol at -20°C. After 30 minutes, the cells were washed twice with PBS containing 0.1% Tween 20. Non-specific binding was blocked with PBS containing 1% bovine serum albumin and 0.1% Tween 20 at room temperature. After 10 minutes, the blocking buffer was removed and the cells were incubated in 100 µl M30 CytoDeath antibody (1:100; Boehringer Ingelheim, France) at room temperature for 60 minutes. To visualize M30 CytoDeath antibody, a FITC conjugated second antibody was used (Boehringer) and the FITC signal was evaluated by flow cytometry. The results are expressed as an index of specific fluorescence (median fluorescence intensity - median fluorescence intensity of untreated cells/median fluorescence intensity of untreated cells × 100).

Detection of caspase cleavage by immunocytochemistry

Floating and adherent cells are represented in cytospin preparations. Cells were fixed in absolute ethanol/acetic acid (99:1) for 1 minute. The staining was performed by a Techmate Horizon™ slide processor using a two-step peroxidase-conjugated polymer backbone visualisation system (EnVision™, DAKO, Glostrup, Denmark) according to the manufacturer's protocol. The chromogenic substrate was DAB (3,3'-diaminobenzidine). The primary antibody used was the M30 CytoDeath antibody (Boehringer). Negative controls were performed by omission of the primary antibody. All determinations were performed with at least 400 cells being quantified with the ImageQuant software for each experimental condition.

Western-blot analyses

At the completion of the experiments, MCF-7 cell monolayers were washed with ice-cold PBS (Biowittaker Walkersville, MD, USA) and were then scraped into 100 µl of ice-cold lysis buffer: 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol and protease cocktail inhibitor (Sigma). The lysates were then placed on ice, vortexed vigorously at intervals over 10 minutes, centrifuged at 15,000 g for 10 minutes at 4°C and the supernatants stored at -80°C.

Equal amounts of total protein (5 µg) were submitted to 12.5% SDS-PAGE and then transferred to PVDF membranes. Proteins were visualized using the ECL+ detection system (Amersham Biosciences Europe GmbH,(Succursale France, F-91898 Orsay Cedex, France)) after incubation (overnight at 4°C for primary and 1 h at room temperature for secondary antibodies) using the primary antibody HDJ2 from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and the secondary antibody anti-mouse horseradish peroxidase.

Protein abundance was quantified by analysis of autoradiographs. Relative band intensities were quantified by densitometric analysis (Molecular Dynamics, Sunnyvale, CA, USA). Quantification of protein levels by this method was linear over the range of protein concentrations analysed and exposure times employed in these studies.

Cellular levels of [^{3}H]tamoxifen

Cells were seeded at 180,000 per dish in 60 mm dishes and incubated with Tam and/or R115,777 as described above in