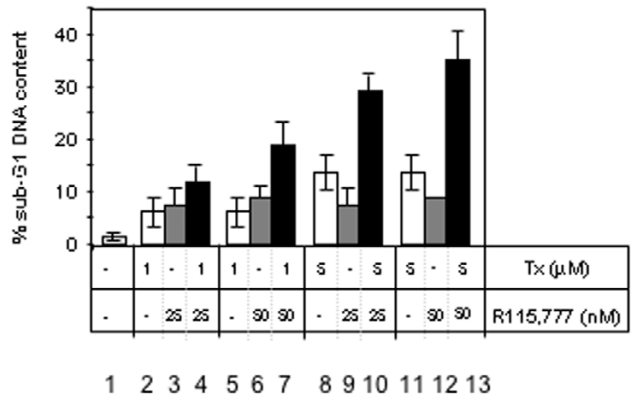
Figure 2



DNA fragmentation was determined using flow cytometric analysis. MCF-7 cells were incubated for 5 days with tamoxifen (Tx) and R115,777. Floating and adherent cells were harvested, fixed and their DNA was stained using propidium iodide. Data are shown as the percentage of cells with a sub-G1 DNA content over the total number of cells. Each point is the mean value from 6 × 10^4 cells, and data are expressed as the mean of three independent experiments. Error bars indicate the standard error of the mean.

worked synergistically with FTI-277 in the induction of apoptotic cell death in ER positive breast tumour lines.

R115,777 enhances the apoptotic effect of tamoxifen

We examined the effects of a combination of Tam and R115,777 on the DNA content of MCF-7 cells using flow cytometry of propidium iodide-stained cells (Fig. 2). The compounds were added for 5 days either individually or in combination and in both cases the appropriate carrier was added as a control. The appearance of a significant fraction of cells with a DNA content less than 2 N would be indicative of apoptotic cells. Such a cell population was not detected among untreated MCF-7 cells. Using these cells we confirmed that Tam treatment induced an increase of sub-G1 DNA content at 1 and 5 μM (Fig. 2, lanes 2, 5, 8 and 11). We also showed that cells incubated with high concentrations of R115,777 (25 or 50 nM) showed an increase of the sub-G1 DNA content equivalent to that obtained with Tam 1 μM. Interestingly, the combination of R115,777 and Tam produced a large increase in the sub-G1 population: 35 ± 5% in the presence of both agents (Fig. 2, lane 13) compared to only 9 ± 3% (Fig. 2, lane 12) or 14 ± 4% (Fig. 2, lane 11) with each agent alone. Cell death was assayed by two other methods to confirm that death detected by flow cytometry of propidium iodide-stained cells was related to apoptosis. First, we used DAPI staining, which determines nuclear morphology (Fig. 3A, left) and counted normal and condensed nuclei (Fig. 3A, right). The number of condensed and fragmented nuclei increased with 5 μM Tam (3.5 ± 0.1%, Fig 3A right lane 2) or with R115,777 (up to 5.9 ± 0.3%, Fig 3A right lanes 3–6) treatment, compared to untreated cells. Once again, the association of Tam (5 μM) with R115,777 (from 5 to 25 nM) significantly enhanced the number of cells with condensed nuclei (13.1 ± 0.6 to 22 ± 1%, Fig 3A right lanes 7–9).

Apoptosis was further assessed using the monoclonal antibody M30 CytoDeath (M30), which is specific for the neoepitope in cytokeratin 18 that becomes available after an early caspase cleavage during apoptosis. The specific caspase cleavage site within cytokeratin 18 was assessed either immuncytochemically (Fig. 3B) or was analysed by flow cytomoetry (Fig. 3C). We used M30 CytoDeath to selectively stain apoptotic cells, and M30 positive cells were scored (Fig. 3B). Of the cells treated by Tam alone, 19.6% were positive (Fig. 3B, lane 2). This result confirmed that Tam induced cleavage of the specific caspase cleavage site within cytokeratin 18 in breast cancer cells [11,22]. R115,777 treatment induced 4.1% to 8.4% M30 positive cells (Fig 3B, lanes 3–6), as previously reported for other FTIs with mammalian cells [23]. Once again, the combined effects of R115,777 and Tam were higher (up to 40.4%, Fig 3B, lanes 7–10) than that expected from the sum of the individual effects. Flow cytometry analysis confirmed the immunocytochemical counts. R115,777 plus Tam significantly increased the M30-positive cell population (up to 74.2%; Fig. 3C, lanes 7–10). The M30-positive cell population was much smaller following treatment with either R115,777 (10.4% to 22.1%; Fig. 3C, lanes 3–6) or Tam (22.5%; Fig. 3C, lane 2) alone. These results were obtained in three independent experiments with little intra-assay variations; however, the difficulty of cell counting (from 200 to 400 cells per assay) has prevented our carrying out the extensive series of experiments required for isobologram constructions.

Overall, these data show that R115,777, which induces negligible apoptosis by itself, when combined with Tam results in significant apoptosis induction in MCF-7 cells. To differentiate between the involvement of the ER and AEBS pathways in this Tam effect, we compared the apoptosis-inducing activities of ER or AEBS selective ligands

Effects of R115,777 and different anti-estrogens on caspase cleavage

To define any possible involvement of the ER or AEBS pathways in apoptosis induction, a set of experiments similar to those described in Fig. 3c were performed using R115,777 in association with three different 'anti-estrogens', Tam, ICI182,780 or PBPE. Flow cytometric analyses were performed following M30 staining of MCF-7 cells treated for 5 days with these three different combinations of agents. As shown in Fig. 4, 10 μM PBPE was able to increase the number of M30 positive cells (lane 12) to the same extent as 5 μM Tam (lane 4). In contrast, 5 nM ICI182,780 treatment resulted in only modest cytokeratin 18 cleavage (Fig. 4, lane 8). The combination of R115,777 with ICI182,780 resulted in slightly increased M30 staining (Fig. 4, lanes 9–11) compared with R115,777 treatment alone (Fig. 4, lanes 1–3), suggesting that ER is only minimally involved in the synergistic induction of