Cancer Chemother Pharmacol (1998) 41: 339–342 © Springer-Verlag 1998

SHORT COMMUNICATION

Lu Jin · Nicole Legros · Guy Leclercq
Ian R. Hardcastle · Michael Jarman

Length increase of the side chain of idoxifene does not improve its antagonistic potency in breast-cancer cell lines

Received: 20 April 1997 / Accepted: 28 August 1997

Abstract Linkage of specific residues onto steroidal estrogens through a long aliphatic side chain leads to "pure antiestrogens" devoid of residual estrogenic activity. Therefore, we assessed whether an increase in the length of the side chain of the triphenylethylenic antiestrogen idoxifene might increase its antagonistic potency. Culture of MCF-7 and tamoxifen-resistant variant RTX6 cells in the presence of CB 7675, a $(CH_2)_8$ derivative of idoxifene $[(CH_2)_2]$, ruled out this possibility. This compound partly blocked MCF-7 cell growth only at 10^{-6} *M* to almost the same extent as tamoxifen and failed to inhibit the growth of RTX6 cells, whereas the pure antiestrogen RU 58 668 was effective on both cell lines at much lower concentration. This absence of improvement was reflected in the observation of an efficiency for down-regulating progesterone receptor no better than that of tamoxifen. Pure antiestrogens are known to down-regulate the estrogen receptor, whereas triphenylethylenic antiestrogens up-regulate the receptor; CB 7675 behaves as the latter in agreement with its lack of strong antagonistic activity.

Key words Antiestrogen · Idoxifene analogue · Mammary tumor · MCF-7 cells · RTX6 cells

Introduction

Triphenylethylenic antiestrogens, of which tamoxifen is the compound of reference, are known to antagonize the development of breast cancers. Although the mode of action of these drugs is not established, it seems quite clear that they act through binding to the estrogen receptor (ER). Various other targets have been described and may be involved in their antitumor potency [22]. Of such additional targets, calmodulin (CaM) seems to be important in view of its participation in many biological processes related to signal transduction [9].

The prominent role played by the basic side chain of triphenylethylenic antiestrogens as well as their steroidal counterparts is now well recognized: it is absolutely required for antiuterotrophic activity [12]; compounds without such a chain also fail to inhibit the growth of mammary tumor cells in culture. However, such drugs almost invariably maintain some residual estrogenic activity, which in mammary cell culture maintains a significant growth rate and confers an ability to induce progesterone receptor (PgR) [6]. On the other hand, it has been demonstrated that the linkage of specific residues onto steroidal estrogens through a long aliphatic (polymethylene) side chain leads to strong antiestrogens devoid of residual estrogenic activity ("pure antiestrogens") [4, 21]. It was therefore of interest to assess whether an increase in the length of the side chain of triphenylethylenic antiestrogens might also improve their antagonistic potency. Data reported herein concerning CB 7675, a $[(CH_2)_8]$ homologue of idoxifene $[(CH_2)_2]$ that has about the same binding affinity for ER as tamoxifen [9] but a higher level of anti-CaM activity [10], appear to refute this possibility.

Materials and methods

Compounds

CB 7675 was synthesized as previously described [10]. Tamoxifen was obtained from Zeneca (Macclesfield, UK) and RU 58 668, from Roussel Uclaf (Romainville, France).

Cells and growth media

MCF-7 cells (origin: Michigan Cancer Foundation, Detroit, Mich.) are maintained in our laboratory at 37 °C in Earle's base MEM

L. Jin · N. Legros · G. Leclercq (✉)
Laboratoire J.-C. Heuson de Cancérologie Mammaire,
Institut Jules Bordet – Service de Médecine,
Rue Héger-Bordet 1, B-1000 Brussels, Belgium
Tel.: + 32.2.535.34.91; Fax: 32.2.534.73.28
e-mail: lcanmamm@resulb.ulb.ac.be

I.R. Hardcastle · M. Jarman
Cancer Research Campaign Center for Cancer Therapeutics,
Institute of Cancer Research – CRC Laboratory,
Sutton, Surrey, SM2 5NG, UK

containing 10% heat-inactivated fetal calf serum (56 °C, 1 h) as well as L-glutamine, penicillin, streptomycin, and gentamicin at the usual concentrations (all materials from Gibco, Gent, Belgium). Tamoxifen-resistant RTX6 cells [8] (an MCF-7 variant obtained from Dr. J.C. Faye, INSERM U 168, Toulouse, France) are maintained under the same conditions in RPMI in the presence of 10^{-6} *M* tamoxifen, which is removed before each experiment.

Growth experiments

The effect of CB 7675, tamoxifen and RU 58 668 (control compounds) on cell growth was assessed after 120 h of culture according to a previously described protocol [13]. In brief, MCF-7 and RTX6 cells were plated in 35-mm petri dishes in DCC-FCS MEM and RPMI, respectively (MCF-7 2×10^4 cells/ml, RTX6 4×10^4 cells/ml). Compounds were added to the medium after 24 h and replaced 48 h later by fresh medium containing the compounds. Cells were harvested 72 h later and their growth was evaluated by measurement of their DNA content by the diphenylamine method [3]. The phenol red content of the growth media was maintained during all experiments to insure a significant basal growth rate (and PgR level) that could be subjected to inhibition by antiestrogens. Each culture was performed in quadruplicate.

Receptor assays

ER and PgR levels were measured after 3 days of culture in the presence or absence of CB 7675. ER levels were measured by multipoint DCC assay using [^{3}H]-E_2 as the labeling agent [7] and by Abbott enzyme immunoassay (ER-EIA) on both cytosol and nuclear extracts [14]; PgR levels were assessed solely on the cytosol by multipoint DCC assay using [^{3}H]-ORG 2058 as the labeling agent [7]. The effect of tamoxifen and RU 58 668 was similarly established.

Results and discussion

The antitumor activity of CB 7675 was tested on the breast-cancer cell line MCF-7 as well as on its tamoxifen-resistant clone RTX6 [8]. The ability of the drug to inhibit growth and decrease the PgR level was compared with that of tamoxifen. The total lack of residual estrogenic activity of the estradiol derivative RU 58 668 [11, 17, 20] justifies the choice of this compound as an additional control for pure antiestrogenicity. The potency of the latter in arresting the growth of RTX6 cells at low concentration (10^{-8} *M*) was another criterion for its selection (~75% inhibition in two experiments).

Figure 1 (upper part) shows that CB 7675 at 10^{-6} *M* blocked MCF-7 cell growth to almost the same extent as tamoxifen whilst producing no significant inhibition of the tamoxifen variant clone RTX6. Inhibition of MCF-7 cells did not exceed 50% of the control value, sharply differing from that always offered by RU 58 338 (80% inhibition at 10^{-9} *M* [11]). This partial inhibition, observed with all triphenylenic antiestrogens, could be ascribed to a residual estrogenic activity capable of partially antagonizing their inhibition potency at high concentration. Hence, an increase in the length of the side chain carrying the aminoalkyl substituent required for antagonism [12, 19] fails to increase antiestrogenicity. This lack of improvement was reflected in both cell lines in an efficiency for down-regulating basal PgR concentration no better than that of tamoxifen (Fig. 1, lower part). Thus, CB 7675 as well as tamoxifen decreased PgR levels by 50% at 10^{-6} *M* (see box), whereas RU 58 668 is known to produce such an effect at concentrations as low as between 10^{-9} and 10^{-8} *M* [11]; at 10^{-7} *M* this pure antiestrogen totally suppresses PgR synthesis. Similar behavior was obtained under 10^{-10} *M* E_2 stimulation; at 10^{-6} *M*, CB 7675 partly impeded PgR induction (control 81 fmol/mg protein, E_2 516 fmol/mg protein, E_2 + CB 7675 268 fmol/mg protein), whereas RU 58 668 abrogated it totally at 10^{-8} *M* (experiment performed solely in MCF-7 cells). Confirming the analogy in behavior between CB7675 and tamoxifen, a tendency to enhance PgR synthesis (residual estrogenic activity) was recorded at 10^{-8} *M* in MCF-7 cells (Fig. 1, lower part).

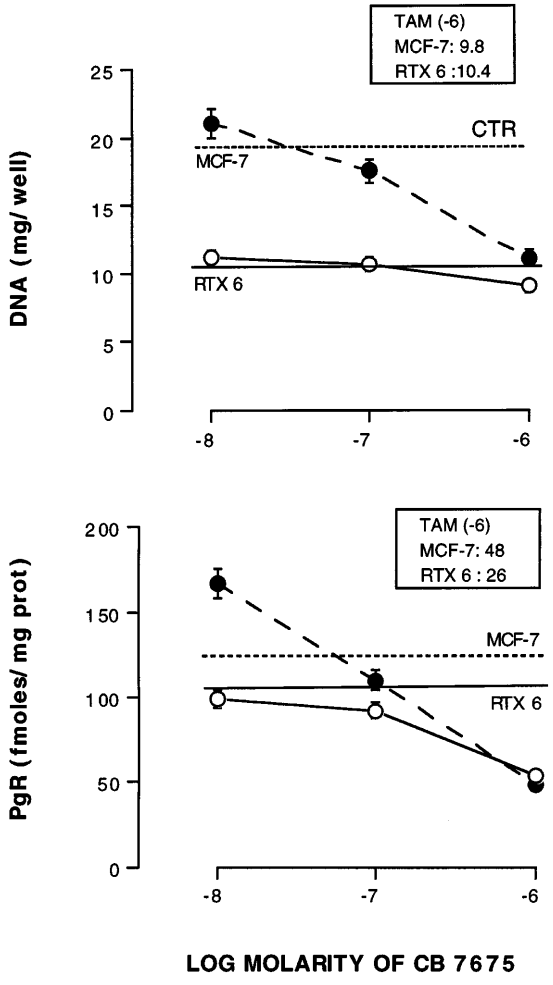

Fig. 1 Effect of CB 7675 on the growth and PgR levels of MCF-7 and RTX6 cells. On both graphs the *horizontal lines* refer to untreated control (*CTR*) cells. The *black symbols* (*dotted lines*) refer to the data established with MCF-7 cells and the *white symbols* (*full lines*), to those established with RTX6 cells. The *boxes* provide comparative data for 10^{-6} *M* tamoxifen (*TAM*)

Triphenylethylenic antiestrogens up-regulate ER in the cell nucleus in a form incapable of binding [^{3}H]-E_2 under exchange condition (EIA+, DCC−) [14, 15],

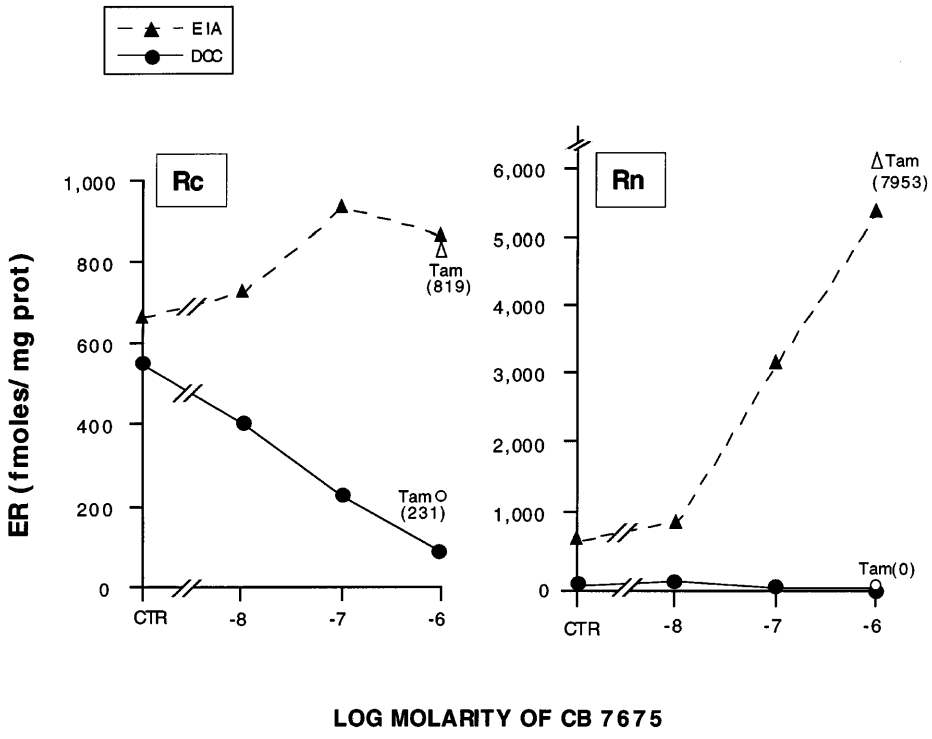

Fig. 2 Effect of CB 7675 on ER levels of MCF-7 cells (*Rc* Cytosol, *Rn* nuclear extract). On the graph the *full lines* refer to [^{3}H]-E_2-binding assays and the *dotted lines*, to enzyme immunoassays. The additional *white symbols* (△, ○) refer to the ER levels measured under tamoxifen (*Tam*) treatment at 10^{-6} *M* (control compound for up-regulation efficiency)

whereas pure antiestrogens such as RU 58 668 down-regulate the receptor [2, 11]. Data reported in Fig. 2 clearly show that treatment of MCF-7 cells with CB7675 led to an accumulation of the receptor in the cell nucleus. At 10^{-6} *M* the drug was almost as effective as tamoxifen, suggesting similar degrees of efficiency. Up-regulation also occurred in RTX6 cells, although to a lower extent (1.5-fold increase at 10^{-6} *M*), as previously reported for tamoxifen [15]. Hence, according to this criterion, CB 7675 also fails to behave as a pure anti-estrogen.

Altogether, our data clearly indicate that increasing the length of the side chain of a triphenylethylenic antiestrogen does not improve its antagonistic potency. Hence, "pure antiestrogenicity" of compounds such as RU 58 668 is not caused by a steric hindrance provoked by their long side chain. The chemical structure of the residue located at the end of the long side chain of pure antiestrogens therefore seems of major importance for strong antagonism. One may speculate that this residue recognizes a specific site on the ER and/or an associated protein. The hypothesis of such a specific interaction between the receptor and the aminoalkyl residue located at the end of the side chain of triphenylethylenic antiestrogens is not supported by the present data. On the other hand, the requirement for an absence of planarity of such molecules [16] led us to consider that an interaction of the phenyl residue bearing this side chain with the receptor may be of importance. One may speculate that linkage of the side chain, irrespective of its length, onto this phenyl ring may modify the interaction of the latter, with a specific hydrophobic center of the receptor playing a role in its activation. Indeed, estrogen derivatives bearing a hydrophobic residue in position 11β (a center corresponding to the aforementioned phenyl ring of triphenylethylenic antiestrogens according to crystallographic data [5]) are usually characterized by a strong degree of estrogenicity. Supporting this view is the additional observation of a lack of antiestrogenicity in 11β-aminoalkyl-substituted derivatives of estradiol devoid of such a phenyl group [11, 18]. This hypothesis may also explain the antiestrogenicity of triphenylethylenic derivatives without aminoalkyl-bearing side chains [1].

Whatever might be the mechanisms of action of pure and conventional triphenylethylenic antiestrogens, in view of the present data it appears that a side chain length increase in the latter compounds does not improve their in vitro antiestrogenicity or their antimammary tumor activity.

Acknowledgements This work was funded by a grant from the "Foundation Medic". This collaboration was also supported by the CGRI of "communauté Française de Belgique" and by the British Council. Lu Jin is the recipient of a grant from the "Fonds Jean-Claude Heuson de Cancérologie Mammaire". Ian Hardcastle and Michael Jarman are supported by the Cancer Research Campaign.

References

1. Borgna JL, Coezy E, Rochefort H (1982) Mode of action of LN 1643 (a triphenylbromoethylene antiestrogen): probable mediation by the estrogen receptor and high affinity metabolite. Biochem Pharmacol 31: 3187
2. Borras M, Laios I, EI Khissiin A, Seo H-S, Lempereur F, Legros N, Leclercq G (1996) Estrogenic and antiestrogenic

regulation of half-life of covalently labeled estrogen receptor in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 57: 203
3. Burton KA (1956) A study of the conditions and mechanism of the diphenylamine reaction for colorimetric estimation of deoxyribonucleic acid. Biochem J 62: 315
4. Claussner A, Nédélec L, Nique F, Philibert D, Teutsch G, Van de Velde P (1992) 11β-Aminoalkyl estradiols, a new series of pure antiestrogens. J Steroid Biochem Mol Biol 41: 609
5. Duax WL (1981) Molecular details of receptor binding and hormonal action of steroid derived from X-ray crystallographic investigations. J Steroid Biochem 15: 41
6. Eckert RL, Katzenellenbogen BS (1992) Effects of estrogens and antiestrogens on estrogen receptor dynamics and the induction of progesterone receptor in MCF-7 human breast cancer cells. Cancer Res 42: 139
7. EORTC Breast Cancer Cooperative Group (1980) Revision of the standard for the assessment of hormone receptors in human breast cancer. Eur J Cancer 16: 1513
8. Faye JC, Jozan S, Redeuilh G, Baulieu EE, Bayard F (1983) Physicochemical and genetic evidence for specific antiestrogen binding sites. Proc Natl Acad Sci USA 80: 3158
9. Hardcastle IR, Rowlands MG, Jarman M (1996) Anti-oestrogens as calmodulin antagonists. Curr Med Chem 3: 211
10. Hardcastle IR, Rowlands MG, Martin G, Grimshaw RM, Houghton J, Jarman M, Sharff A, Neidle S (1996) Homologues of idoxifene: variation of estrogen receptor binding and calmodulin antagonism with chain length. J Med Chem 39: 999
11. Jin L, Borras M, Lacroix M, Legros N, Leclercq G (1995) Antiestrogenic activity of two 11β-estradiol derivatives on MCF-7 breast cancer cells. Steroids 60: 512
12. Jordan VC, Gosden B (1982) importance of the alkylaminoethoxy side chain for estrogenic and antiestrogenic actions of trioxifene in the mature rat uterus. Mol Cell Endocrinol 27: 291
13. Leclercq G, Devleeschouwer N, Heuson JC (1983) Guide-lines in the design of new antiestrogens and cytotoxic-linked estrogens for treatment of breast cancer. J Steroid Biochem 19: 73
14. Leclercq G, Legros N, Piccart MJ (1992) Accumulation of a non-binding form of estrogen receptor in MCF-7 cells under hydroxytamoxifen treatment. J Steroid Biochem Mol Biol 41: 545
15. Legros N, Jin L, Leclercq G (1997) Tamoxifen-induced estrogen receptor up-regulation in mammary tumor cells is not related to growth inhibition. Cancer Chemother Pharmacol 39: 380
16. Murphy CS, Jordan VC (1989) Structural components necessary for the antiestrogenic activity of tamoxifen. J Steroid Biochem 34: 407
17. Nique F, Van De Velde P (1995) RU 58668. Drugs Fut 20: 362
18. Quian X, Adul-Haji YJ (1990) Synthesis and biological activities of 11β-substituted estradiol as potential antiestrogens. Steroids 55: 238
19. Robertson DW, Katzenellenbogen JA, Hayes JR, Katzenellenbogen BS (1982) Antiestrogen basicity-activity relationship: a comparison of the estrogen receptor binding and antiuterotrophic potencies of several analogues of (*z*)-1,2-diphenyl-1-{4-[2-(dimethyl-amino)ethoxy]phenyl}-1-butene (tamoxifen, Nolvadex) having altered basicity. J Med Chem 25: 167
20. Van De Velde P, Nique F, Bouchoux F, Brémaud J, Hameau MC, Lucas D, Moratille C, Viet S, Philibert D, Teusch C (1994) RU 58 668, a new pure antiestrogen inducing a regression of human mammary carcinoma implanted in nude mice. J Steroid Biochem Mol Biol 48: 187
21. Wakeling AE, Bowler J (1988) Novel antiestrogens without partial agonist activity. J Steroid Biochem 31: 645
22. Wolf DM, Fuqua SAW (1995) Mechanisms of action of antiestrogens. Cancer Treat Rev 21: 247