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Effects on Mouse Uterus of Three Antitumoral Drugs Acting upon Estrogen and Progesterone Receptors Directly and Through Other Transduction Pathways

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ABSTRACT. Three antitumoral drugs, tamoxifen (Tam), medroxyprogesterone acetate (MPA), and 8-Cl-cyclicAMP (8Cl), were administered separately and in combination to normal adult mice in order to record their effects on uterus weight, on estrous cycle, and on two estrogen receptor (ER) and progesterone receptor (PgR) parameters, namely content and nucleo-cytoplasm distribution. Tam decreased uterus weight (49%) and total ER content (118 ± 6 vs 328 ± 20 fmol/mg protein in controls) but increased total PgR (1183 ± 230 vs 743 ± 52 fmol/mg protein in controls) and nuclear retention of ER and PgR. MPA down-regulated PgR content and increased uterus weight (36%), but failed to modify ER and PgR nuclear retention. The only parameter changed by 8Cl was nucleo-cytoplasm PgR distribution. Tam + MPA association produced the same results as Tam alone for ER and PgR nuclear retention, but receptor content was not significantly different from that of controls. Both drugs, administered separately, had opposite effects on PgR content; when both were acting concurrently, an algebraic addition of effects was observed, as if both transcription circuits were triggered independently. Remaining Tam effects, not modified by a combination with MPA, indicated the predominance of Tam on the corresponding parameters. When Tam and 8Cl were administered together, 8Cl counteracted the effect of Tam only on PgR content. When associated with MPA, 8Cl changed the effects of MPA on ER and PgR nuclear retention, whereas on receptor content, only that of ER was increased (502 ± 47 vs 328 ± 20 fmol/mg protein in controls). These crossed effects indicate that interrelations between different transduction pathways can affect certain functional circuits while sparing others. The possibility of acting pharmacologically upon different transcription pathways represents a novel approach to modify drug effects directed to specific transduction targets through cross-talk between their components. BIOCHEM PHARMACOL **55**;3:273–278, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. uterine steroid receptors; transduction pathway pharmacology; combined drugs on uterus; uterine pharmacology; antitumor drugs on uterus; cross-talk pharmacology

The purpose of this study was to analyze the effects of three drugs used as antitumoral agents in experimental and human mammary tumors upon the normal mouse uterus as a target tissue of steroid hormones. The drugs chosen for this study were Tam†, MPA, and 8Cl. Tam is widely used in mammary neoplasias and was regarded initially as an antiestrogen, although it also presents estrogenic action depending on dose, cell type, and species [1, 2]. It binds to ERs and by this mechanism competes with the natural ligand (estradiol) for the same receptor molecule. MPA is a progesterone analog and, as such, it binds to PgRs. It is used in mammary cancer to counteract estrogen action [3]. 8Cl is a cAMP analog showing preferential binding to the RII regulatory subunit of PKA [4]. Its antitumoral action is attributed to such binding and subsequent stimulation of cellular differentiation and also to cell cycle progression blockade by 8-Cl-adenosine, one of its metabolites [5]. Cross-talk between proteins of cAMP and steroid receptor pathways is one of the regulatory mechanisms underlying cellular multiplication in hormone-sensitive tissues [6].

As regards murine mammary tumors previously induced by MPA [7] and studied in our laboratory, MPA and 8Cl showed diverse effects on tumor growth and on ER and PgR content according to the type of tumoral hormone dependence [6, 8]. MPA accelerated tumor growth in the hormone-dependent subline but failed to modify the rate of tumor development of hormone-autonomous tumors. Concurrently, MPA produced changes in ER isoform profiles in such hormone-autonomous tumors but not in hormone-dependent ones; ER content was not affected in the autonomous subline but was raised markedly in the hormone-dependent tumors [8]. 8Cl decreased ER and PgR

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† *Abbreviations:* Tam, tamoxifen; MPA, medroxyprogesterone acetate; 8Cl, 8-chloro-cyclic adenosine monophosphate; ER, estrogen receptor; PgR, progesterone receptor; and PKA, protein kinase A.

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content in the autonomous subline but increased the ER level without modifying PgR content in the dependent one [6]. In addition, 8Cl showed an opposite effect on tumor growth, which was inhibited in the hormone-autonomous subline but stimulated in hormone-dependent tumors. Simultaneous administration of MPA and 8Cl resulted in suppression of the inhibitory 8Cl effect on tumor growth in the autonomous tumor subline, whereas in the hormone-dependent tumors, growth was additive, as if each of the involved transduction pathways were acting separately [6]. These contrasting effects were attributed to the different molecular configuration of each signaling circuit member according to the tumor hormone dependence. Therefore, cross-talk between these components was affected diversely by the corresponding drugs.

It was of interest to record the effects of these drugs, used separately and in combination, on steroid receptor content and nucleo-cytoplasm location in the uterus of the same strain of mice devoid of tumors. Tam was included as a drug acting competitively with estrogens. Effects on uterus weight as well as on estrous cycle were also recorded.

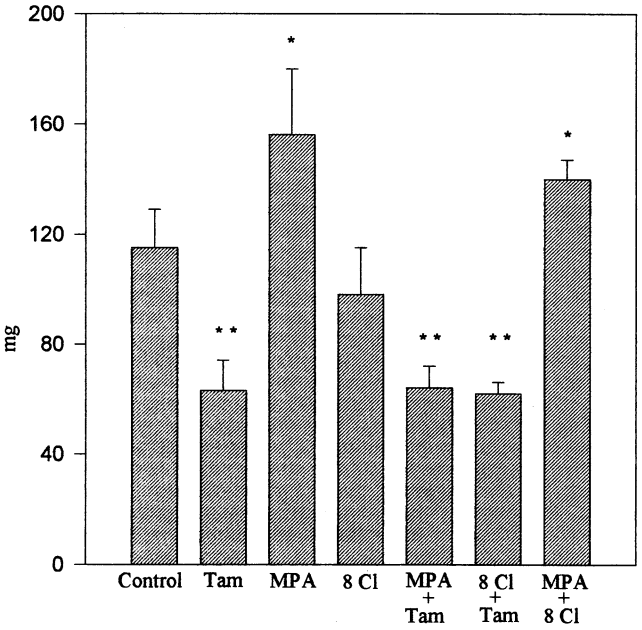

FIG. 1. Effects of Tam, MPA, and 8Cl, used separately and in combination, upon mouse uterus weight. Values are means ± SD (N = 6). Key: (*)$P < 0.05$; and (**)$P < 0.01$ (vs control).

MATERIALS AND METHODS

Adult syngeneic female BALB/c mice, 50- to 60-days-old, were used, and animal care followed institutional guidelines. The control group (N = 6 animals) was without treatment, and six experimental groups (N = 6 for each group) were subjected to the following protocols: (1) Tam administered s.c., 7 mg/kg in olive oil, once weekly; (2) MPA, in a 20 mg depot, administered s.c. for slow release during the experimental period; (3) 8Cl, in a slow release 3 mg s.c. pellet, every 10 days; (4) Tam + MPA; (5) Tam + 8Cl; and (6) MPA + 8Cl. For the latter three combined treatments, each compound was given at the same dosage and vehicle as for its separate administration. Tam and MPA were supplied by Gador Laboratories; 8Cl was provided by Dr. Y. S. Cho-Chung, National Cancer Institute. Estrous cycle was controlled daily by vaginal smears. After 30 days of treatment, uteri were removed, cleaned, weighed, and processed at 0–4°. Animals with cycling ovaries were killed at diestrus. Tissue homogenization was performed with a buffer containing Tris, 10 mM; EDTA, 1.5 mM; β-mercaptoethanol, 2 mM; sodium molybdate, 10 mM; glycerol, 10%, w/v; pH 7.4. ER and PgR were determined in the cytosol and in the nuclear fraction as previously described [8]. Briefly, cytosol aliquots obtained by high speed centrifugation of the homogenate were incubated for 18–20 hr at 4° with 10 nM [^{3}H]17β-estradiol or with [^{3}H]R5020 for total binding and with a 2 μM concentration of unlabeled ligand for nonspecific binding. Free ligand was adsorbed with a dextran-coated charcoal suspension. Nuclear fraction, obtained after a 10-min centrifugation of the homogenate at 900 × *g*, was washed three times with molybdate buffer, and the residue was incubated as stated for cytosol. Aliquots of the adsorbed cytosol and suspension of the washed nuclear pellet were used for radioactivity counting. For the purpose of receptor subcellular distribution, the term cytosol expresses their cytoplasmic localization.

Statistical analysis

Data are given as means ± SD. Significance was calculated by one-way ANOVA and by the Neuman-Keuls test.

RESULTS

Weight

The drugs employed in this study acted diversely on uterus weight (Fig. 1). Tam decreased uterus weight from 115 ± 14 mg in controls to 63 ± 11 mg. The latter value apparently represents the minimal weight also reached when Tam was associated with the other drugs. MPA administered separately increased uterus weight 36% ($P < 0.05$), but 8Cl *per se* failed to affect this parameter. When MPA and 8Cl were combined, the effect of MPA was dominant.

Ovarian Cycle

Administration of Tam induced permanent diestrus in the animals and MPA, permanent metaestrus; 8Cl altered periodicity in the estrous cycle. The effects of Tam and MPA were dominant when associated with 8Cl. A combination of Tam and MPA resulted in permanent diestrus, as with Tam alone.

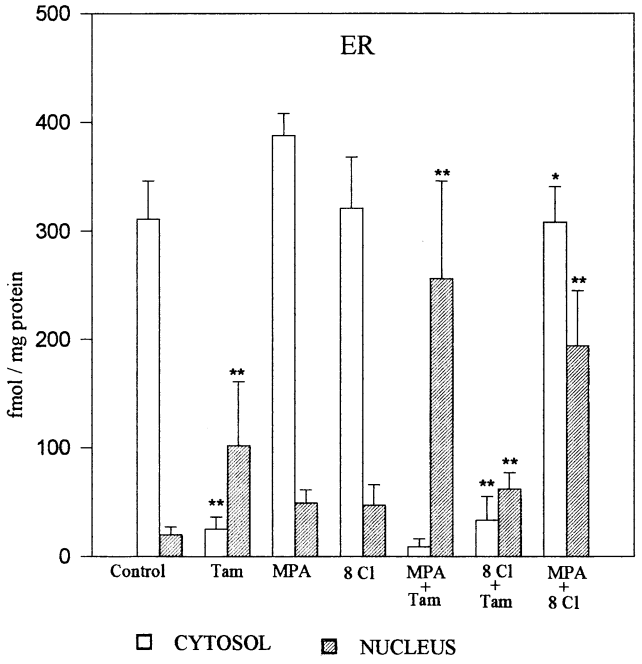

FIG. 2. Effects of Tam, MPA, and 8Cl, used separately and in combination, upon content and subcellular distribution of mouse uterine ER. Values are means ± SD (N = 6). Key: (*)$P < 0.05$; and (**)$P < 0.01$ (vs control).

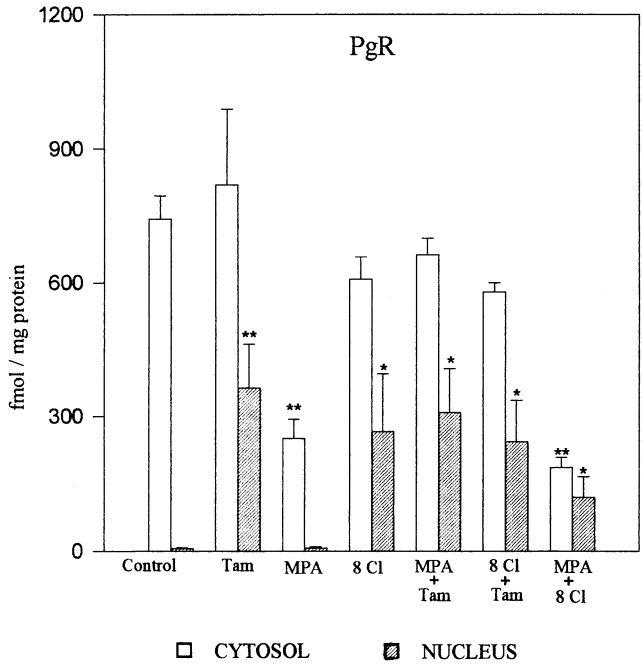

FIG. 3. Effects of Tam, MPA, and 8Cl, used separately and in combination, upon content and subcellular distribution of mouse uterine PgR. Values are means ± SD (N = 6). Key: (*)$P < 0.05$; and (**)$P < 0.01$ (vs control).

ER

With respect to ER content, the mean control value in untreated animals was 328 ± 20 fmol/mg cytosol protein, with minimal presence (20 ± 7 fmol/mg protein) in the nuclear fraction. Tam markedly reduced ER content to 118 ± 6 fmol/mg protein and induced its nuclear retention (95% of total content, Fig. 2). MPA and 8Cl, which separately failed to modify ER content, acted differently when given together with Tam. MPA counteracted the inhibitory effect of Tam, and ER content was kept at control values. 8Cl associated with Tam failed to change the inhibitory action of the latter on ER content. Nuclear retention of ER by Tam alone remained unchanged by simultaneous administration of MPA or 8Cl. By combining MPA and 8Cl, an increase in ER content (502 ± 47 fmol/mg protein) was observed, and a moderate nuclear retention of the receptor molecule was recorded. However, no such nuclear retention was observed with either drug (Fig. 2).

PgR

The PgR presence in control mouse uterus was found in the cytosolic fraction alone (743 ± 52 fmol/mg protein). MPA down-regulated PgR expression (284 ± 80 fmol/mg protein) without modifying the cytosolic receptor location (Fig. 3). On the contrary, Tam, showing an estrogen-like effect [9], increased PgR content (1183 ± 230 fmol/mg protein) and 31% of the receptor was retained in the nucleus ($P < 0.01$). 8Cl failed to modify PgR content but induced 34% of nuclear retention ($P < 0.05$), as that produced by Tam. When both drugs, Tam and 8Cl, were combined, PgR content remained at control levels and nuclear retention was similar to that produced by each drug separately. A combination of MPA with 8Cl showed that the effects of MPA were dominant with respect to down-regulation of PgR content, but not to nuclear retention (39%), which was not significantly different from that produced by 8Cl alone.

Table 1 summarizes data in a semiquantitative way, to provide a global profile of the observed drug effects upon uterine parameters related to their steroid hormone sensitivity.

DISCUSSION

Interrelations between transcriptional circuits acting upon the expression of a given protein are affected by the use of drugs directed to one of those transduction routes [10]. As a result, unexpected effects on the expression of genes foreign to the pharmacological target may be observed. Cross-talk between transcription factors of different biochemical pathways [11, 12], phosphorylation/dephosphorylation of these proteins [13], changes in their isoform profile [8, 14], and binding to the corresponding DNA response elements [15] are some of the mechanisms underlying the affected transcriptional interrelations. The use of a single drug shows its effects on the expression of the selected parameters, while the addition of a second agent acting preferentially on a different transduction pathway should indicate which of those parameters are affected, as an expression of interactions between both routes. If the

TABLE 1. Effects of Tam, MPA, and 8Cl, used separately and in combination, upon several mouse uterine parameters: Semiquantitative notation

Treatments	Weight	ER		PgR	
		Content	Nuc ret	Content	Nuc ret
Tam	↓	↓	↑	↑	↑
MPA	↑	NS	NS	↓	NS
8Cl	NS	NS	NS	NS	↑
Tam + MPA	↓	NS	↑	NS	↑
Tam + 8Cl	↓	↓	↑	NS	↑
MPA + 8Cl	↑	↑	↑	↓	↑

Key: nuc ret: nuclear retention; ↑: significantly higher than controls; ↓: significantly lower than controls; and NS: not significantly different from controls.

effects of the first drug are not modified by the second compound, it can be taken as a relative dominance of the first one or, alternatively, that the circuits affected by each agent are not closely interrelated in the expression of the recorded parameters.

From the drugs used in the present study, the cAMP analog 8Cl, binds preferentially to one of the regulatory PKA subunits [16], triggering several biochemical effects such as: phosphorylation of cAMP proteins, including CREB, CREM and associated molecules [17]; phosphorylation of nuclear receptors such as ER [18] and PgR [19], besides other PKA target transcription factors [20]; and shift in the differentiation/proliferation equilibrium, favoring differentiation, in several cell lines [21]. When cAMP analogs were associated with compounds acting via the PgR and ER, marked changes in pharmacological effects were apparent [22]. 8-Br-cAMP, when administered with RU486, an antiprogestinic agent, was able to change antagonist to agonist progestinic effects of the latter compound, and this was attributed to modifications in cross-talk between proteins of the cAMP and PgR transcriptional pathways [23]. Likewise, an opposite effect of 8Cl on the growth of murine mammary tumors according to the different progesterone hormone dependence of these tumors has been reported [6]. Conformational changes in PgR molecules for the hormone-autonomous and for the hormone-dependent tumor sublines, reflected in cross-talk between members of both transduction pathways, have been proposed as a possible mechanism responsible for the contrasting response. In the same tumoral line, MPA diversely influenced the expression of ER isoforms according to the tumor hormone dependence [8]. In the hormone-autonomous subline, administration of MPA induced the appearance of an ER isoform not present in controls, whereas in the hormone-dependent subline this effect was not observed. Interrelations between PgR and ER pathways were probably influenced by the different configuration of the PgR protein in the two tumor sublines.

Focusing on the behavior of Tam, widely used as antitumoral agent in breast cancer, possible interrelations between different transductional pathways may be illustrated when the drug is administered alone or in combination with compounds acting on other biochemical circuits. Binding of estradiol and Tam to ER induces conformational changes in the receptor molecule, reflected in the subsequent binding of the complex to its cognate estrogen response elements (ERE) and in the expression of their corresponding genes [14]. Cross-talk with proteins of other transduction pathways is also altered [24], as well as some dynamic parameters such as nucleo-cytoplasmic receptor translocation [25]. Dynamic phosphorylation changes may be part of the mechanisms involved. We have observed nuclear retention of ER and PgR in mouse uterus after Tam treatment, more pronounced for ER and unchanged by combined treatment with MPA or 8Cl. Similarly, the effects of Tam on uterus weight (down-regulation) and on estrous cycle (diestrus) were not modified by association with MPA or 8Cl. However, it has been reported that cAMP is involved in the growth modulation of mouse uterus by Tam, through regulation of phosphodiesterase and calmodulin activities [26]. PgR content, which is increased by Tam administration, is counteracted when Tam is combined with MPA or 8Cl. Because MPA alone was found to down-regulate PgR expression, the results of MPA-Tam association correspond to the algebraic addition of both effects, as if the two pathways were acting independently on PgR levels. When Tam was associated with 8Cl, all parameters remained as with Tam alone, except for increased PgR content, which by the addition of 8Cl was maintained at control values. Given that Tam is acting via the ER, the effect of 8Cl through the cAMP pathway reflects an interaction between both circuits on PgR content, probably through changes in the phosphorylation pattern [10]. Other mechanisms apart from Tam binding to ER have been proposed to explain its antitumoral action [27–29]. With the scope of this report, our aim is to stress the significance of conformational changes induced by Tam in the ER protein, further reflected in the interrelations with components of other transductional circuits.

Intracellular distribution of steroid receptors has been a matter of controversy [30]. In the initial "two-step" model, the role of the cytoplasmic receptor molecule was to transport the cognate hormone by hormone-receptor complex translocation to the nucleus. There, by assembling as a homodimer, the active form reaches DNA-binding sites, triggering its transcriptional message [1]. Later, with the advent of steroid receptor antibodies applied to immunocytochemistry [31] and with the use of enucleated cell

techniques [32], the exclusive nuclear presence of ER and PgR was postulated. However, by the biochemical binding method, cytoplasmic and nuclear ERs have been found *in vivo* in the rat uterus, whereas *in vitro* the receptor molecule was present only in the cytosol [30]. Currently, the existence of basic amino acid sequences (nuclear localization signals) necessary for the entry of protein molecules into the nucleus has been demonstrated in all members of the steroid receptor family [33, 34]. The concept of a dynamic traffic between cytoplasm and nucleus as a modulatory transcriptional mechanism is emerging. The receptor molecule, as an active transcription factor, shuttles into and out of the nucleus according to genomic physiological requirements. Thus, the cytoplasm can be viewed as a steroid receptor reservoir releasing or retaining the receptor [35]. Compounds acting on transcriptional circuits should also influence nucleus-cytoplasm shuttling of involved molecules [25]. In our experiments, from the three drugs used, Tam modified ER and PgR nuclear-cytoplasm distribution with marked retention in the nuclear fraction (Figs. 2 and 3). It is a reflection of the active role of Tam on ER conformation that allows the Tam-ER complex to be delivered to the DNA to regulate the transcription message [36]: down-regulation of ER and up-regulation of PgR content. Both effects are an expression of the partial estrogenic agonist action of Tam [37]. The effect of Tam on ER nuclear retention is dominant and was not changed by the addition of MPA or 8Cl. These two latter compounds produced a small ER nuclear retention (12–13%) when administered separately, but when given together nuclear retention reached 39% of the total ER content. Neither of these two compounds acts directly on the ER pathway, but their combined effect on PgR nuclear retention (39%) probably influenced also the ER distribution by cross-talk or protein-protein interaction between both receptor molecules. From the drugs used in this study, 8Cl was the one that produced the least effects on the registered parameters when administered separately. This was to be expected because it acts through a transduction route (cAMP pathway) that was not measured on this occasion. However, 8Cl induced a significant nuclear retention (34%) of PgR as an indication that receptor nucleo-cytoplasmic shuttling is a dynamic parameter sensitive to protein-protein interactions, as stated above.

To sum up, pharmacological association of drugs acting upon two different transduction routes can alter certain interrelated circuits, but not others, as reflected by changes in the expression of some parameters without modifying the remainder. The present data are not sufficient for drawing conclusions on the mechanisms underlying the observed effects, and a further molecular approach is needed. Moreover, the observed effects correspond to one dose at a fixed time. Undoubtedly, other experimental protocols with the same drugs would produce different effects due to the ductile character of the target routes. Our purpose was to stress the contrasting response of related parameters when influenced by compounds acting on other transcriptional circuits. The use of drugs directed at the same time to more than one transcriptional pathway [38, 39] represents a novel approach for studying how cross-talk between involved components is reflected in their functional expression.

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