Eur J Cancer Clin Oncol, Vol. 21, No. 8, pp. 985-990, 1985.
Printed in Great Britain.

0277-5379/85$3.00+0.00
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In Vivo and *in Vitro* Antiestrogenic Action of 3-Hydroxytamoxifen, Tamoxifen and 4-Hydroxytamoxifen*

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Abstract—*This study demonstrates* in vivo *and* in vitro *properties of the non-steroidal antiestrogens tamoxifen (TAM), 4-OH-tamoxifen (4-OH-TAM) and 3-OH-tamoxifen (K 060 E). In immature rabbit uteri 4-OH-TAM and K 060 E bound to the respective estrogen receptors with a ten-fold higher affinity than TAM. Furthermore, K 060 E exhibited less agonistic (estrogenic) but higher antagonistic (antiestrogenic) activity in the immature rat uterus than TAM and 4-OH-TAM (change of uterine weight). The ratio of agonistic vs antagonistic effect of K 060 E was distinctly lower than in TAM and 4-OH-TAM. In addition, K 060 E reduced by approximately 45% the growth of the transplantable Fisher rat mammary tumor (R 3230 AC) as compared with TAM (33%). We assume that, due to the higher antitumor activity, K 060 E (3-OH-TAM) is a better antiestrogen than TAM.*

INTRODUCTION

ANTIESTROGENS such as tamoxifen (TAM) hold a considerable potential for the treatment of hormone-dependent human breast cancer. However, even in cases where the presence of estrogen receptors has been demonstrated, the TAM treatment has led to regression in only about 50% of the patients. Further reduction of tumor growth can be obtained in some of these patients by subsequent additional endocrine therapies (e.g. ovariectomy, adrenalectomy or aminoglutethimide) resulting in a further elimination of endogenous estrogens [1-3]. These facts indicate that the incomplete efficacy of TAM could be either an unsatisfying antiestrogenic activity or some intrinsic estrogenicity. The latter seems very probable since there is evidence that TAM is only a partial antagonist in rat and man [4]. Therefore it seems useful to seek antiestrogens with less estrogenic activity with regard to the endocrine therapy of estrogen receptor-positive human breast cancer. The mechanism of antiestrogenic action in countering estrogen-stimulated growth is not completely understood but from previous reports we know that TAM interacts with the estrogen receptor in the respective target cells [5].

The estrogenic and antiestrogenic activities of TAM have been compared with 3-hydroxy-tamoxifen (K 060 E), a compound which, in earlier *in vitro* experiments, proved to be a better antiestrogen than TAM [6, 7]. In addition, 4-OH-tamoxifen (4-OH-TAM) was included as a comparative control to K 060 E.

MATERIALS AND METHODS

Materials

Tamoxifen (TAM), 3-OH-tamoxifen (K 060 E = trans-1[4'-(2-dimethylaminoethoxy)phenyl]-1-1-(3'-hydroxyphenyl)-2-phenyl-1-buten) and 4-OH-tamoxifen (4-OH-TAM) were applied as a base or citrate, and were manufactured by Klinge Pharma GmbH, Munich (F.R.G.). 17β-Estradiol was bought from Sigma, Munich (F.R.G.), and all other reagents were used in p.a. quality of known companies.

Methods

Uterine weight test. TAM, K 060 E and 4-OH-TAM were given orally to immature female Sprague-Dawley (SD) rats (20 days old; 40 g body

Accepted 18 February 1985.
*This work was supported in part by the Swiss National Science Foundation, Grant No. 3.409.0.83.
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wt; Charles River Wiga, Sulzfeld, F.R.G.) for 3 consecutive days to test estrogenic activity. In addition to each antiestrogen dose, a standard dose of 1 mg/kg estradiol was administered orally to juvenile SD rats to determine the antiestrogenic effect of the compounds. The compounds were suspended in 0.25% agar for the administration. The animals were killed on day 4, the uteri removed, cleared of any intrauterine fluid and subsequently weighed in a dry condition. Estrogenic activity was estimated by the increase in uterine weight (uterotropic effect) initiated by the respective daily doses of TAM, K 060 E and 4-OH-TAM. The antiestrogenic effect of the compounds was tested by the reduction of the uterine weight (antiuterotropic effect) in the presence of 1 mg/kg estradiol.

Antitumor activity test. The transplantable mammary adenocarcinoma R 3230 AC [8, 9] was obtained by Dr Hartus (Max-Planck-Institute for Biochemistry, Martinsried, F.R.G.). Tumors were transplanted into the thighs of female Fisher 344 rats (body wt about 90 g; Charles River Wiga breeding, Sulzfeld F.R.G.) by i.m. injections (0.2 ml per injection) of about 1×10^6 tumor cells. The cells were obtained in suspension by digestion (0.1% hyaluronidase, 0.12% collagenase and 0.02% DNase in Richter's improved MEM cell culture medium) of tumor fragments. After a 3-hr digestion at 37°C the cells were filtered through sterile gauze, washed once with saline and taken up in saline for injection into the animals. The tumors appeared approximately 14 days after tumor transplantation and were subsequently measured with calipers in two perpendicular dimensions three times a week. Tumor area (mm^2/animal) was calculated as the product of the two perpendicular measurements. Drug treatment (six times a week) was started 24 hr after tumor cell transfer. All test compounds were suspended in 0.25% agar. Groups of ten rats received vehicle (0.25% agar) or K 060 E and TAM orally (stomach tube). During the test the body weight of all animals was recorded twice a week.

Estrogen receptor-binding assay. Estrogen receptors (ER) were measured in the cytosol of uterine tissue of female immature white New Zealand rabbits (3 months of age). The uteri were separated from surrounding fatty tissue, rinsed in ice-cold phosphate-buffered saline, minced and immediately transferred into liquid nitrogen. The frozen uterine tissue was put into a capped Teflon cylinder pre-cooled in liquid nitrogen that was vibrated (50 Hz) for at least 30 sec in a microdismembrator (Braun, Melsungen, F.R.G.) in the presence of a tungsten carbide ball. The resulting power was mixed with 4 units (1:4/w:v) of Tris buffer (0.01 M Tris, 0.001 EDTA, pH 7.5), homogenized with a Dounce homogenizer and centrifuged at 105,000 *g* for 1 hr. The supernatant (cytosol) was decanted and the protein concentration adjusted to 5 mg protein/ml. The protein concentration was measured according to Lowry *et al.* [8]. Aliquots of cytosol were pipetted into plastic tubes, 2.5×10^{-9} M [17β-^{3}H]estradiol, and a range of concentrations of unlabeled estradiol and antiestrogens were added. The relative binding affinity of the antiestrogens to the estrogen receptor was carried out with the dextran-charcoal method at 2°C as described by Devleeschouwer *et al.* [10].

All steps were carried out in triplicate. The relative binding affinity was defined as the ratio of the concentrations of radioinert 17β-estradiol to the competitor that is necessary to achieve a 50% inhibition of the specific [17β-^{3}H]estradiol binding. Bound radioactivity at the highest concentration of 17β-estradiol (2.5×10^{-7} M) was taken as unspecific binding and subtracted from all values.

RESULTS

Estrogen receptor-binding affinity

The ability of 17β-estradiol, TAM, 4-OH-TAM and K 060 E to inhibit the specific binding of [17β-^{3}H]-estradiol to the estrogen receptor (ER) of rabbit uterus is shown in Fig. 1. The competition curves clearly demonstrate that K 060 E and 4-OH-TAM have similar binding affinities to ER as compared with TAM. To obtain 50% inhibition of the specific 17β-estradiol binding the following competitor concentrations were required: 4×10^{-9} (17β-estradiol), 2.4×10^{-8} (K 060 E), 3.6×10^{-8} (4-OH-TAM) and 4.5×10^{-7} M (TAM), indicating again that the hydroxylated TAM derivatives have a ten-fold higher binding affinity to ER than the parent TAM.

Influence of antiestrogens on uterine weight

Estrogenic activity. The effect on uterine weight of three consecutive daily administrations (stomach tube) of various amounts of TAM, 4-OH-TAM and K 060 E to immature rats is illustrated in Fig. 2. TAM and 4-OH-TAM stimulated dose-independent uterine growth by approximately 64% using concentrations of 1, 6 and 12 mg/kg body wt. The same doses of K 060 E increased uterine weight only about 35%. In addition, Fig. 2 demonstrates that TAM and 4-OH-TAM induced their maximal estrogenic effect at about 1 mg/kg, in contrast to K 060 E at 6 mg/kg.

Antiestrogenic activity. The antiestrogenic activity of TAM, K 060 E and 4-OH-TAM was determined by administration of various amounts of these substances in the presence of 1 mg/kg

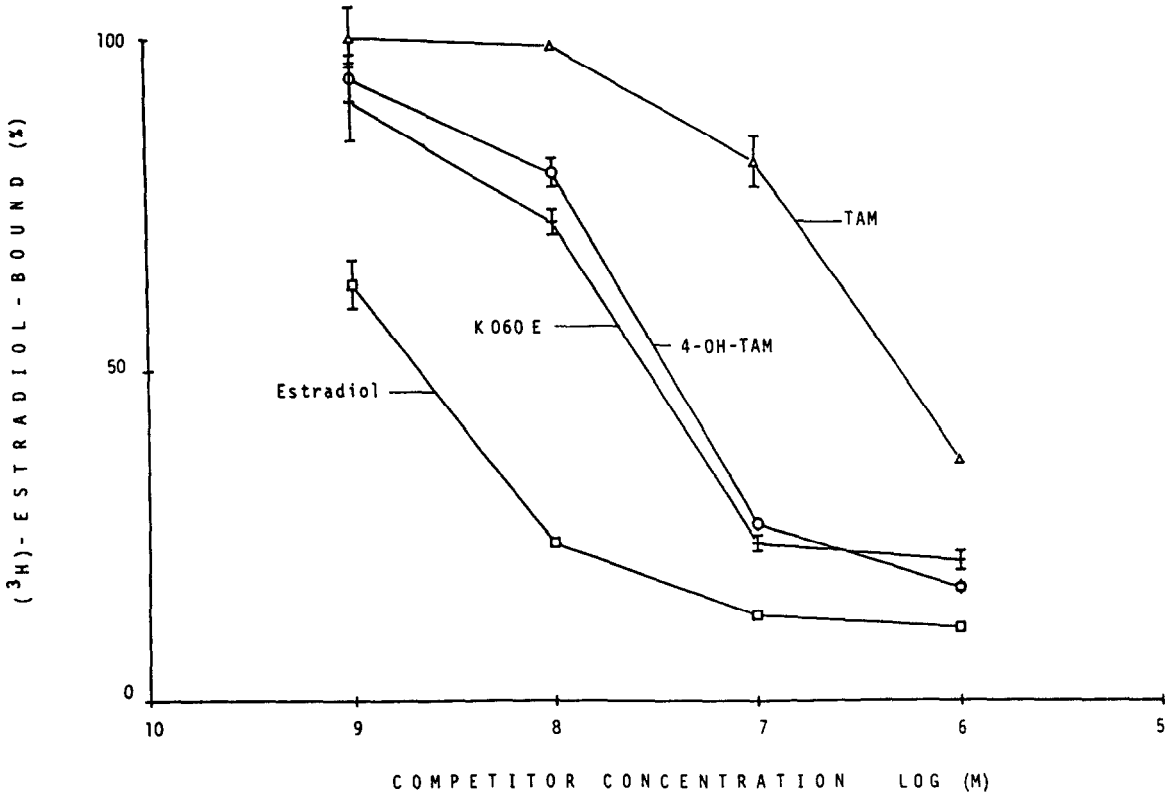

Fig. 1. Competitive binding assay showing the affinities of TAM (△), 4-OH-TAM (○), K 060 E (+) and 17β-estradiol (□) to cytosolic estrogen receptor of rabbit uterus. The cytosol was incubated for 18 hr at 2°C with 2.5 nM [17β-^{3}H]estradiol and increasing concentrations of 17β-estradiol, TAM, 4-OH-TAM and K 060 E. The protein concentration was 5 mg/ml. Means of triplicates ± S.D. are indicated in the plot.

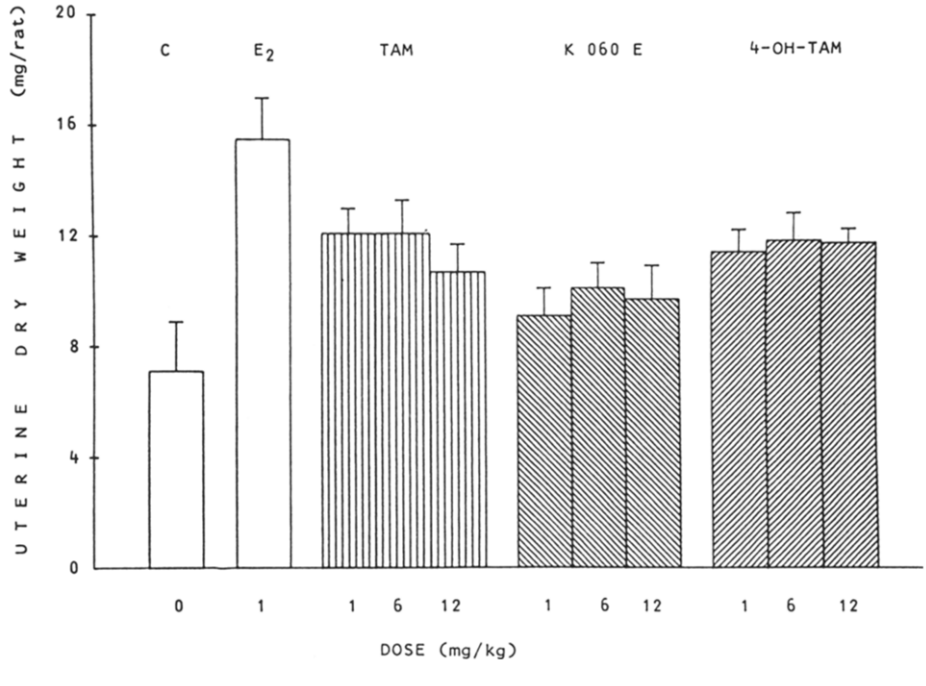

Fig. 2. Uterotropic activity of TAM, 4-OH-TAM, K 060 E and estradiol in immature rats. 1 mg/kg estradiol (E_2) increasing doses (1, 6 and 12 mg/kg) of test compounds (TAM, K 060 E and 4-OH-TAM) and vehicle (C) were administered orally to juvenile rats for 3 consecutive days as described in Methods. Each dose-response experiment was carried out with ten animals, and the results are expressed as means ± S.D.

estradiol to immature female rats. Both TAM and 4-OH-TAM reduced the estradiol-stimulated growth to a similar extent over the tested dose range 1–12 mg/kg (Fig. 3). These effects seemed to be dose-independent. In contrast, the decrease of the uterus by K 060 E was dose-dependent. Only 6 and 12 mg/kg of K 060 E were significantly antiestrogenic; these concentrations reduced uterine weight equivalently to TAM and 4-OH-TAM. Furthermore, Fig. 3 shows that TAM and 4-OH-TAM induced a maximal antiuterotropic effect at 1 mg/kg; and K 060 E at 12 mg/kg.

For the treatment of estrogen-dependent cancers it is of interest to know the antiestrogenic (growth inhibition) activity as well as the estrogenic (growth stimulation) activity of the antiestrogen applied. If the maximum of the uterotropic (estrogenic) and antiuterotropic (antiestrogenic) activities of TAM and 4-OH-TAM (1 mg/kg) and of K 060 E (12 mg/kg) are compared (Figs 2 and 3), the quotient of estrogenic to antiestrogenic activity of K 060 E is smaller and subsequently more favorable than those of TAM and 4-OH-TAM.

Influence of antiestrogen treatment on R 3230 *AC tumor growth.* The administration of K 060 E and TAM resulted in a dose-related inhibition of the growth of the ovarian-independent but estrogen-sensitive R 3230 AC rat mammary tumor. Figure 4 demonstrates the average tumor area per rat receiving 1.5 and 9.0 mg/kg K 060 E and TAM respectively. Tumor growth was reduced more effectively with K 060 E than with TAM using both doses. The lowest TAM concentration (1.5 mg/kg) seemed to be without effect. Use of 9.0 mg/kg TAM or 1.5 mg/kg K 060 E reduced the tumor growth to the same extent. In general, the weight decrease or increase was negligible under the influence of all compounds tested.

DISCUSSION

The newly developed triphenylethylene derivative K 060 E (3-OH-TAM) proved, in earlier experiments, to be a better ligand for the cytosolic ER than TAM in human mammary tumor cells [6]. The present results are consistent with these findings since K 060 E demonstrated equal relative binding affinities to the ER as 4-OH-TAM but ten-fold higher ones than TAM in the cytosol of rabbit uterine tissue. The binding affinities of 4-OH-TAM and TAM are not significantly different from the values published by Jordan *et al.* [11] and Wakeling *et al.* [12], whereas in the comparative studies of Ruenitz *et al.* [9] 4-OH-TAM had a higher affinity to the ER than estradiol and K 060 E. In conclusion, there is no doubt that hydroxylation of TAM either in the 3- or 4-position leads to a considerable increase in the affinity of the respective compound to the ER.

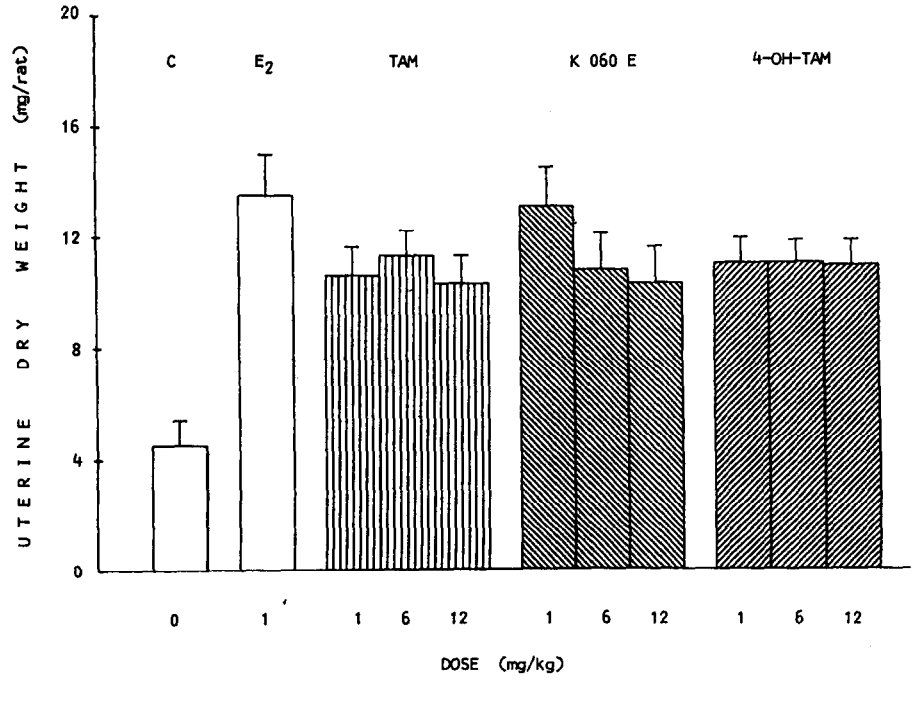

Fig. 3. Antiuterotropic activity of TAM, 4-OH-TAM and K 060 E in immature rats. 1 mg *estradiol/kg* (E_2), *increasing doses* (1, 6 *and* 12 mg/kg) *of test compounds* (*TAM* ▥, *K 060 E* ▨ *and* 4-OH-*TAM* ▨) *together with* 1 mg *estradiol/kg and vehicle* (*C*) *were administered orally to juvenile rats for* 3 *consecutive days as described in Methods. Each test represents the mean* ± *S.D. of ten animals.*

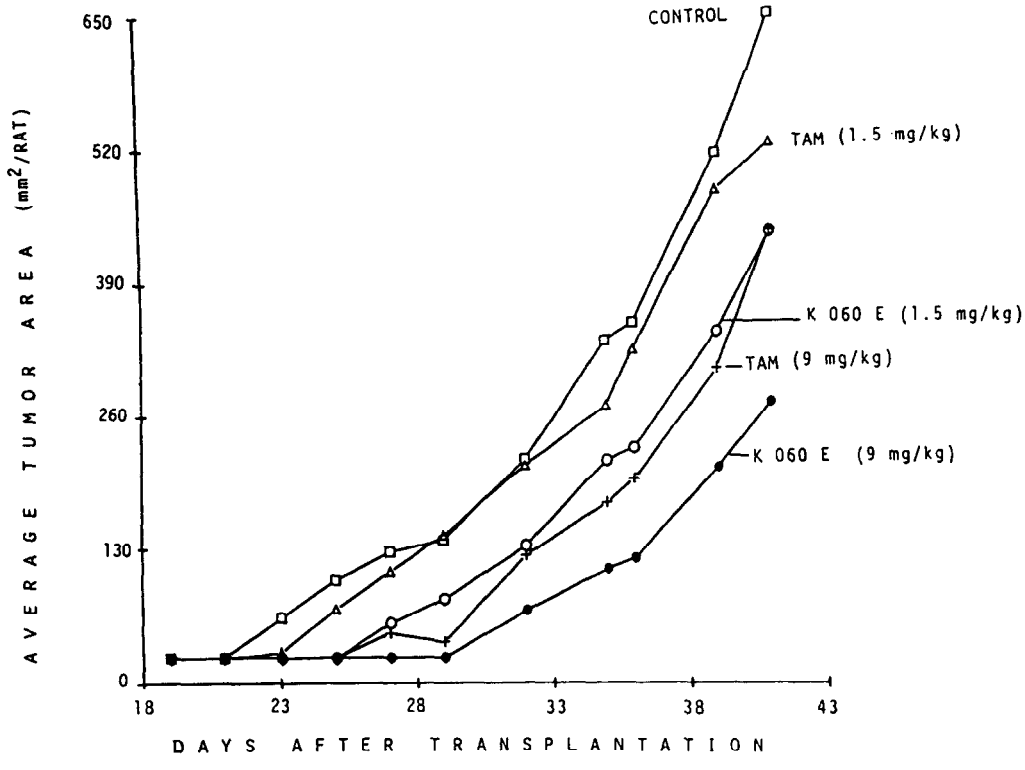

Fig. 4. Comparison of the effect of TAM and K 060 E administration on tumor growth of R 3230 AC mammary tumors. Tumors were implanted s.c. in female Fischer 344 rats using a single cell suspension. One day after the tumor transplantation the rats received the indicated daily doses (1.5 and 9 mg/kg respectively) of antiestrogens for 40 days. Control (□–□), TAM 1.5 mg/kg (△), 9 mg/kg (+) respectively, K 060 E 1.5 mg/kg (○), 9 mg/kg (●), respectively.

The antiestrogenic activity of TAM, 4-OH-TAM and K 060 E could be demonstrated as clearly inducing a reduction of the uterine weight (Fig. 3). K 060 E, however, exhibited a dose-dependent effect on uterine weight of juvenile rats, in contrast to TAM and 4-OH-TAM. The highest dose of K 060 E (12 mg/kg) showed similar antiuterotropic effects to 1 mg/kg (lowest dose) TAM and 4-OH-TAM. In these studies TAM and 4-OH-TAM induced similar anti-uterotropic (Fig. 3) as well as uterotropic (Fig. 2) effects, indicating that the lowest dose had already reached the maximum dose response of TAM and 4-OH-TAM in the rat uterus. This finding is in agreement with Jordan *et al.* [11], who described such a dose response for TAM and 4-OH-TAM, though below 1 mg/kg. This reasoning seems valid in spite of the fact that Jordan *et al.* used a different route (s.c.) of administration.

The estrogenic effect exerted by K 060 E in the rat uterus was significantly lower than that of TAM or 4-OH-TAM (Fig. 2), which is confirmed by Ruenitz *et al.* [9]. In addition, the lower agonistic/antagonistic ratio of K 060 E as compared with TAM and 4-OH-TAM gives evidence for improved therapeutic possibilities.

Since comparative studies of ER-binding affinities and antiuterotropic activities revealed no direct conclusion [12–15] concerning the antitumor activity of an antiestrogen, it was of special interest to use the ovarian-independent R 3230 AC mammary tumor as a model system to test the antitumor activity described by Tsai *et al.* [16]. In addition, the R 3230 AC mammary tumor model system provides a more accurate assay method than the various chemically induced tumors with their differences in growth rates and heterogenous hormone responsiveness.

According to our experimental procedure K 060 E reduced the tumor growth of the R 3230 AC tumor to a greater extent than TAM (Fig. 4). This may be due to the improved agonist/antagonist ratio of K 060 E.

Since K 060 E inhibited the growth of ER-positive human breast cancer cell lines MCF-7 and ZR-75 more efficiently [6, 7], and reduced the R 3230 AC tumor growth of the Fisher rat better than TAM, we conclude that K 060 E is a better antitumor agent than TAM.

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