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ORIGINAL ARTICLE

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Antitumor activity, distribution, and metabolism of 13-*cis*-retinoic acid as a single agent or in combination with tamoxifen in established human MCF-7 xenografts in mice

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Abstract *Purpose*: The efficacy of 13-*cis*-retinoic acid (13-CRA) given as a single agent or in combination with tamoxifen (TAM) was determined in athymic nude mice bearing advanced s.c. MCF-7 human breast cancers. *Methods*: 13-CRA alone was given by gavage at doses ranging from 26.4 to 200 mg/kg. TAM alone was given by gavage at doses of 7.5, 15, 30, or 60 mg/kg. For combination studies, each dose of TAM was followed 4 h later by 13-CRA at doses of 25, 50, 100, or 200 mg/kg. All treatments began on day 12 and were continued for 3 weeks. *Results*: The median time to two doublings recorded for the control and for 13-CRA and TAM given as single agents at the highest dose were 22.2, 29.2, and 54.7 days, respectively. In combination, 100 and 200 mg/kg 13-CRA with 7.5 mg/kg TAM resulted in a delay in tumor growth at least as high as that achieved with highest-dose TAM alone, but the effect was not synergistic. Pharmacokinetic analysis of 13-CRA was performed in plasma, liver, and tumor from mice bearing 0.5- to 2.0 g carcinomas following a single dose of 100 mg/kg 13-CRA. Results showed that 13-CRA was metabolized differently in various tissues, but concentrations of 13-CRA detected in tumor were in the range reported to be active in vitro. *all-trans*-Retinoic acid (ATRA) concentrations were about 5% of the 13-CRA concentrations detected in plasma, 68% of those found in liver, and 20% of those found in tumor. 4-oxo-CRA represented between 2% and 10% of 13-CRA concentrations detected in plasma and liver but was not detected in tumor. Furthermore there was no difference in peak plasma 13-CRA concentrations found in the same tissues at 30 min after a single dose or after the eighth dose of 100 mg/kg 13-CRA or 13-CRA and TAM. Mean 13-CRA concentrations detected in liver and tumor were 50–90% and 16–30% of plasma peak concentrations, respectively. No difference in 4-oxo-CRA concentration was observed between the treatment groups. *Conclusions*: These data suggest that 13-CRA is not effective against established human breast tumor xenografts despite the stability of the pharmacokinetics of 13-CRA and the generation of ATRA as a metabolite. The addition of 13-CRA to TAM did not improve the efficacy of TAM against these estrogen-receptor-positive xenografts.

Key words 13-*cis*-Retinoic acid · Tamoxifen · MCF-7 xenografts · Breast cancer

Abbreviations *13-CRA* 13-*cis*-retinoic acid · *TAM* Tamoxifen · *4-oxo-CRA* 4-oxo-13-*cis*-retinoic acid · *ATRA all-trans*-retinoic acid · *ER* estrogen receptor · NCI National Cancer Institute (USA) · *DTP* Developmental Therapeutics Program · *RAR* retinoic acid receptor · *HPLC* high-performance liquid chromatography · *AUC* area under the plasma concentration × time curve

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Introduction

Retinoic acid compounds have recently been investigated as anticancer agents for a variety of cancers. Compounds such as *all-trans*-retinoic acid (ATRA) and 13-*cis*-retinoic acid (isotretinoin, 13-CRA) have shown activity against a wide variety of human malignancies [2, 6, 9, 14, 26–29], including nonmelanoma skin cancer, bladder carcinoma, and acute promyelocytic leukemia. In addition, chemopreventive activity of retinoids has been demonstrated for premalignant skin lesions and oral leukoplakia and in the prevention of second primary carcinomas of the upper aerodigestive tract [13, 23, 26].

Numerous studies have suggested that the retinoic acid compounds may be of use in human breast cancer [10, 11, 17–19, 22, 24–26, 30]. In vitro, Fontana [10] demonstrated that ATRA at concentrations of 1 μM effectively inhibited the growth of estrogen-receptor (ER)-positive MCF-7 cells in culture and that the effect of combined treatment with ATRA and TAM was additive. Wetherell and Taylor [30] also showed that ATRA and 13-CRA were effective against the ER-positive human mammary cell line T47D and that cotreatment of the cells with either 13-CRA or ATRA plus TAM produced additive effects. Toma et al. [28] showed inhibitory effects of 13-CRA in MCF-7 cells in vitro, but no study has either documented in vivo activity of 13-CRA against ER-positive human breast xenografts or measured the concentrations of 13-CRA achieved in animals. However, one study showed that, although 13-CRA inhibited the growth of the ER-negative human breast carcinoma cell line MDA-MB-231 in vitro, it was ineffective when given in feed to mice bearing xenografts of the same tumor [11]. 13-CRA was also inactive as a single agent in a clinical trial in breast cancer [5]. For clinical applications, 13-CRA may represent a better candidate than ATRA because the pharmacokinetics of 13-CRA are stable over time, whereas those of ATRA are variable [1, 7, 12, 20, 26]. In the present study we examined the efficacy of 13-CRA both as a single agent and in combination with TAM in athymic nude mice bearing established MCF-7 human breast carcinoma xenografts. We also determined the pharmacokinetics of 13-CRA in plasma, liver, and tumor after the administration of 13-CRA to both fasted and nonfasted tumor-bearing athymic nude mice. Furthermore we examined peak concentrations of 13-CRA in mice after one or eight doses of the agent given alone or in combination with TAM so as to determine whether its metabolism or absorption would be altered by continued and/or combination treatment.

Materials and methods

Mice

Female athymic nude mice (NCr nu nu, 5–6 weeks of age specific pathogen-free) were obtained from the NCI Animal Production Branch (Frederick, Md.) and were allowed to acclimate to the University of Maryland animal facility for 1 week prior to initiation of the study. Mice were housed in microisolator caging and allowed autoclaved food and water ad libitum. Animal rooms were maintained at 25 ± 2 °C on a 12-h light/dark cycle and at least 12 air changes/h. All animals were handled in accordance with the Guide to the Care and Use of Laboratory Animals (National Institutes of Health, 1985) except that the s.c. tumors were allowed to grow to as large as 5 g in the control groups before the study was ended. Monthly analysis of sentinel mice housed in 1/5 dirty bedding from study mice confirmed that the study mice remained MAP (murine antibody profile)-negative.

Tumor

MCF-7 human breast-carcinoma tumor fragments (G00135) were obtained from the Division of Cancer Treatment Tumor Repository, National Cancer Institute (NCI) Frederick Cancer Research Facility (Frederick, Md.) and were implanted s.c. on the right flank. Using metafane anesthesia, slow-release 1.7-mg 17-β-estradiol pellets (Innovative Research of America, Sarasota, Fla.) were implanted s.c. above the left flank. Tumors used in these studies were harvested from passage mice using aseptic techniques. Fragments of tumor (approximately 25 mg) and estradiol pellets were implanted in study mice on day 0. Tumors were measured twice weekly, and tumor volumes were calculated from the formula $Length \times (width)^2/2$, where *length* is the largest dimension and *width*, the smallest dimension perpendicular to the length. Mice were observed until the tumors measured between 22 and 394 mm^3 as determined by digital caliper measurement. At that time, 12 to 18 days postimplantation, the mice were stratified into treatment groups of 8 or 10 mice/treatment group and 20 mice/control group such that the mean and median values recorded for the groups were not different from those noted for the other groups in terms of either body weight or tumor volume. For the pharmacokinetics studies, additional mice were implanted with MCF-7 fragments and estradiol pellets. These mice were dosed when the tumor volumes had reached between 300 and 500 mm^3 as determined by caliper measurement.

Drugs

13-CRA (Isotretinoin, NSC 329481) was obtained from the Developmental Therapeutics Program (DTP) of the NCI or was purchased from Toronto Research Chemical Inc. (Downsview, Ontario, Canada). ATRA (NSC 122758) was obtained from the DTP, NCI. TAM was purchased from Sigma Chemical Co. (St. Louis, Mo.) as the acetate salt. Mice received 13-CRA and/or TAM by gavage 5 days a week for 3 weeks beginning on day 12 or 18 post tumor implantation. An initial dose-ranging study was performed at doses of 26.4, 39.6, 59.4, 89, 133.2, and 200 mg/kg per dose of 13-CRA, prepared as fine suspensions in cremophor:ethanol:water (1:1:6, by vol.) such that the animals received 0.01 ml/g body weight. For TAM the initial dose-ranging study was performed at doses of 7.5, 15, 30, or 60 mg/kg in sterile water. For the combination studies, doses of 13-CRA were 25, 50, 100, and 200 mg/kg. TAM doses were 7.5, 15, 30, or 60 mg/kg. Doses were given as oral boluses using a 22-gauge 1.5-inch gavage needle and were based on each day's exact body weights. The positive control groups received the vehicle(s) only at 0.01 ml/g body weight. Single agents and combinations were given to cohorts of mice for 3 weeks, 5 days/week. Mice were weighed and observed daily during the dosing period, and tumor measurements were recorded twice weekly. After treatment had been completed, tumor measurements and body weights were recorded twice weekly until the end of the study. Any moribund animals were euthanized by inhalation of CO_2. Gross necropsies were conducted on all mice.

Pharmacokinetics studies

Mice bearing s.c. MCF-7 tumors ranging in volume between 500 and 2000 mm^3 were fasted overnight prior to dosing. 13-CRA at

100 mg/kg in cremophor:ethanol:water (1:1:6, by vol.) was given by oral gavage. Two mice from each group were euthanized with CO_2, and blood was collected by cardiac puncture using heparinized syringes at the following times after dosing: 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, and 960 min. For comparison, mice bearing s.c. MCF-7 xenografts were given 13-CRA without prior fasting to determine if there were differences in 13-CRA absorption between fasting and fed mice. These animals were killed at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 960, and 1440 min after dosing, and blood was collected by cardiac puncture. Animals receiving the vehicle only were killed at 5 min postdosing to determine if the vehicle altered the chromatography of the retinoic acid compounds. All procedures were performed in dim light because of the light sensitivity of the retinoic acid compounds. Livers and tumors from all the animals were quickly removed and weighed. The gall-bladder was removed from the liver samples. Both liver and tumor were snap-frozen in liquid nitrogen and stored in aluminum-foil-wrapped cryotubes at −70 °C until analysis. Blood was centrifuged at 1500 g for 10 min, and plasma was stored in foil-wrapped tubes at −70 °C until analysis. Additional mice, dosed with either 13-CRA at 100 mg/kg and/or TAM at 30 mg/kg for either one dose or eight doses, were killed at 0.5 h after the single dose or the eighth dose to determine if there were any changes in peak plasma concentration with repetitive dosing and if administration of TAM interfered with the metabolism of 13-CRA.

Sample preparation

Samples of plasma, liver, and tumor were thawed in a dark environment. Liver and tumor samples were weighed, homogenized in 2 vols of filtered, distilled water; and stored at −70 °C until analysis.

HPLC assay

A 35-µl aliquot of the internal standard (9.45 µ*M* Ro 11-5036, kindly provided by Hoffmann LaRoche, Inc., Nutley, N.J.) was added to 100-µl aliquots of plasma or tissue homogenate. Retinoids and internal standard were extracted from plasma or tissue homogenates with 0.7 vol. acetonitrile: 1-butanol (50:50, v:v) in the presence of 0.3 vol. K_2HPO_4 (pH 11.1). The organic phase was analyzed by reversed-phase gradient HPLC using a modification of the method of Bugge et al. [4]. The HPLC system employed Beckman solvent-delivery model 110B pumps (Beckman Instruments, Fullerton, Calif.) programmed with a 10-min linear gradient from 30% to 100% B at a flow rate of 1.5 ml/min. Mobile phase A was acetonitrile: 0.02 *M* ammonium acetate:acetic acid (pH 4.6; 50:50:0.5, by vol.), and mobile phase B was acetonitrile:0.2 *M* ammonium acetate:acetic acid (pH 8.4; 95:5:0.04, by vol.). The column employed was a Zorbax ODS special analytical column (MacMOD Analytical, Chadds Ford, Pa.) of 25 cm × 4.6 mm inside diameter with 5-µm spherical particles. The sample injection volume was 100 µl. Detection of retinoids was accomplished with a UV detector set at 360 nm. The detector output was processed with a SP4290 integrator (Spectra-Physics Inc., San Jose, Calif.). Concentrations of retinoids were determined by the ratio of the peak height of the compound to the peak height of the internal standard, with reference to a concomitantly performed standard curve. Under these conditions, good baseline separation of 4-oxo-13CRA, internal standard, 13CRA, and ATRA was achieved, with retention times being approximately 6, 11, 13.3, an 14.5 min, respectively. There was no endogenous interfering peak, and the inter- and intraday assay CV% were < 10% for 13-CRA and ATRA. The lower limit of quantitation was 30 ng/ml (0.10 µ*M*) for both 13-CRA and ATRA. The assay was linear at concentrations between 0.10 and 10 µ*M*. Samples expected to contain concentrations higher than 10 µ*M* were diluted prior to analysis.

Pharmacokinetic analysis

Noncompartmental analysis was performed on the plasma concentrations of 13-CRA and metabolites using the program LaGran [21], which uses the LaGrange function [31]. Compartmental analysis was performed with ADAPT II [8] using a one-compartment open linear model with first-order absorption.

Optimal T/C percent

The percentage of treated/control value (%T/C) was calculated by division of median treated tumor weight by the median control tumor weight on each observation day and multiplication by 100. For display the optimal value or minimal value obtained after the first course of treatment is shown and the day on which the optimal %T/C occurred is shown in parentheses.

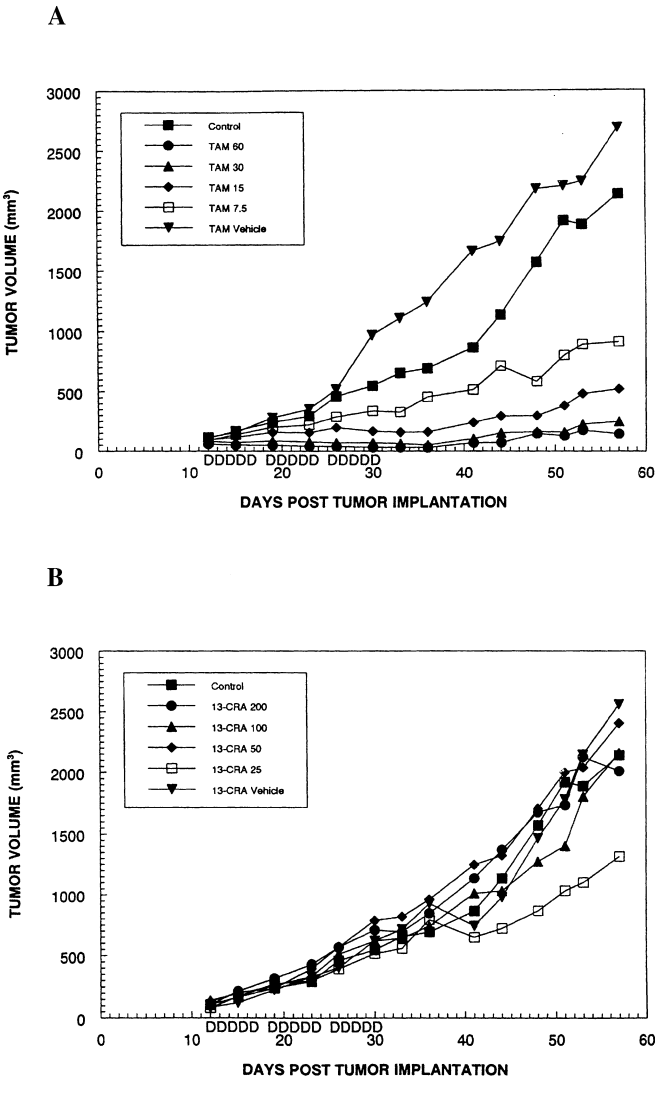

Fig. 1 **A** Effect of TAM as a single agent on MCF-7 human xenografts in athymic nude mice. Mice were implanted with tumor and treated with doses of TAM ranging from 7.5 to 60 mg/kg daily for 5 of 7 days/week for a period of 3 weeks, after tumors had reached a volume of 22–394 mm^3. The vehicle control was water. **B** Effect of 13-CRA as a single agent on MCF-7 human xenografts in athymic nude mice. 13-CRA was given at doses ranging from 25 to 200 mg/kg daily for 5 days/week for 3 weeks, after tumors had reached a volume of 150–300 mm^3. The vehicle control was cremophor:ethanol:water (1:1:6, by vol.). Results are expressed as median tumor volumes

Growth delay

Growth delay was expressed as the percentage by which the treated-group median tumor volume was delayed in achieving a specified tumor size as compared with the controls. We used the formula $(T - C)/C \times 100$, where T and C are the median times (in days) to a predetermined target tumor volume (in cubic millimeters) for the treated and control groups, respectively.

Net log cell kill

This is an estimate of the number of $\log_{10}$ units of cells killed by the test agent and is calculated using the following formula:

$$\frac{[(T - C) - \textit{Duration treatment}] \times 0.301}{\textit{Doubling time}},$$

where the doubling time is calculated as the time required for the tumor volume to increase from 500 to 1000 mm^3 and T and C are the median numbers of days needed to reach the specified tumor size defined above.

Statistical evaluation

Median tumor volumes and tumor growth delays were calculated. The data were analyzed by one-way analysis of variance with pairwise comparisons using Dunnett's test and by the nonparametric method, Kruskal-Wallis test. Paired comparisons were done using the Mann-Whitney test. Significance was set at $P \leq 0.05$. Calculations were done using the statistical software package MINITAB (Minitab, Inc., State College, Pa.).

Results

Single-agent efficacy

Treatment with TAM for 15 days was very effective in inhibiting the growth of MCF-7 xenografts (Fig. 1A, and Table 1, Addendum 1). The tumor growth was suppressed within 3 days of treatment at all dose levels tested and regrowth was very slow after treatment had been stopped. The growth delay was greater than 91% for the three highest doses of TAM and 64% for the lowest dose of 7.5 mg/kg per day. The time to two doublings was greater than 49 days for the highest doses of TAM versus 25.6 days for the controls. The vehicle (water) did not result in any growth delay.

When 13-CRA was given as a single agent the effects were much less dramatic (Fig. 1B, Table 2, Addendum 2). The median time to two doublings was increased to 37 days as compared with 23.7 days for the controls. The vehicle controls also exhibited a growth delay, presumably due to the mild anticancer activity of cremophor.

Combination-agent efficacy

When TAM and 13-CRA were given concurrently for 15 days the tumor growth delays were of a magnitude similar to those observed with TAM alone, except when 13-CRA was combined with the lowest dose of TAM. At a daily dose of 7.5 mg/kg TAM the addition of 13-CRA resulted in an increased time to three doublings and in increased growth delays. When 7.5 mg/kg TAM was combined with 200 and 100 mg/kg 13-CRA the median time to three doublings increased from 23.9 days to 56 and 50 days, respectively. The growth delays increased from 8% to 152% and 125%, respectively. At the higher doses of TAM the addition of 13-CRA did not result in efficacy greater than that of TAM alone (Fig. 2, Table 3, Addendum 3–6).

Pharmacokinetic results

The 13-CRA plasma concentration-time profiles obtained from both fasted and nonfasted animals are

Table 1 Response of advanced-stage s.c. MCF-7 breast tumor xenografts to tamoxifen[a]

TAM dose[b]	Optimal %T/C (day)[c]	Median days to 2 doublings[d]	Growth delay %T–C/C	NLCK[e]
Control	–	25.6 (4.5–>49)	–	–
60	7(41)	>49 (9.8–>49)	>91%	0.3
30	−3(41)	>49 (10.9–>49)	>91%	0.3
15	3(41)	>49 (5.3–>49)	>91%	0.3
7.5	18(41)	41.9 (16.1–>49)	64%	−0.1
Vehicle	–	19.2 (9.4–>49)	–	–

[a] MCF-7 human breast tumor xenografts and 1.7-mg 17-β-estradiol pellets were implanted into athymic nude mice as described in Materials and methods. Mice were randomized for study at 10 mice per treatment group and 20 mice in the control group. Treatment with tamoxifen p.o. as based on exact body weight was begun on day 18, qdX5, and repeated for 3 weeks. Vehicle-treated mice received 0.01 ml/g body weight water p.o. on the same treatment schedule. Tumor length and width were measured twice weekly using digital calipers and tumor volumes were calculated from the formula $length \times width^2/2$, where *length* is the largest dimension and *width* is the smallest dimension perpendicular to the length

[b] Dose of tamoxifen is given in mg/kg per day of treatment

[c] Optimal %T/C is the minimal %T/C obtained for that treatment group, and the day on which it occurred is indicated in parentheses

[d] The median number of days required for 2 doublings is given, and the range of days needed for 2 doublings is indicated in parentheses

[e] NLCK is the net log cell kill as based on the values recorded for the doubling times calculated from tumor-doubling times

Addendum 1 Data for Fig. 1A: median values and their 95% confidence intervals. The number of animals surviving at each time point is given in parentheses under the 95% confidence interval

Treatment	Days post tumor implantation													
	12	15	19	23	26	30	33	36	41	44	48	51	53	57
Control	107.5 85.5–175 (20)	168 117–232 (20)	242 185–357 (20)	294 240–413 (20)	460 347–637 (19)	548 421–766 (19)	655 452–1014 (15)	693 413–1105 (15)	867 433–1525 (15)	1138 560–1779 (11)	1570 687–2330 (11)	1918 645–2980 (11)	1885 882–3014 (11)	2559 889–3171 (11)
TAM 60	58.5 38.5–120.5 (8)	49.5 35–140 (8)	49 35–180 (8)	40.5 28–148 (8)	38.5 17–130 (8)	32 13–100 (8)	32 14–116 (8)	30 13–92 (8)	69 50–129 (8)	71 50–143 (8)	146 10–202 (8)	128 91–217 (8)	174.5 138.5–237.5 (8)	144 105–284 (8)
TAM 30	81.5 40–101 (8)	71 38–179 (8)	85 54–258 (8)	76 46–298 (8)	69 45–372 (8)	69 39–374 (8)	63 26–361 (8)	50 22–444 (8)	103 34–199 (8)	153 65–600 (8)	161 48–851 (8)	160 52–1014 (8)	226 97–1285 (8)	249 106–1636 (8)
TAM 15	91.5 33–200 (8)	116 39–220 (8)	156.5 36–303 (8)	155 50–242 (8)	197 54–304 (8)	166.5 96–250 (8)	158 83–393 (8)	159.5 93–295 (8)	240 94–359 (8)	294 144–378 (8)	297 189–452 (7)	383 155–558 (7)	484 264–613 (7)	521.5 270–886 (7)
TAM 7.5	91 56.5–123 (8)	137.5 61–238 (8)	200.5 92–321 (8)	220.5 101–416 (8)	289 126–431 (8)	337.5 103–667 (8)	339 83–809 (7)	455 86–787 (7)	516 90–1095 (7)	714 121–1238 (7)	585 132–1657 (7)	804 136–1845 (7)	895 139–2419 (7)	917 197–2699 (7)
TAM vehicle	116 62–190 (8)	155 73–266 (8)	280.5 72–587 (8)	353.5 50–666 (7)	518 58–867 (6)	970 230–1070 (6)	1109 560–1150 (6)	1240 640–1891 (5)	1660 690–1960 (5)	1742 750–2363 (5)	2184 920–2541 (5)	2209 921–2521 (5)	2247 1116–2975 (5)	2691 1277–3416 (5)

Table 2 Response of advanced-stage s.c. MCF-7 breast tumor xenografts to 13-CRA[a]

13-CRA dose[b]	Optimal %T/C(day)[c]	Median days to 2 doublings[d]	Growth delay %T–C/C	NLCK[e]
Control	–	23.7 (12.2–>38.0)	–	–
200	−9(21)	37.0 (21.8–>38.0)	56%	−0.2
133.2	59(21)	31.0 (24.6–>38.0)	31%	−0.5
89	27(21)	36.9 (13.0–>38.0)	55%	−0.2
59.4	27(21)	22.6 (17.2–37.0)	−5%	−0.9
39.6	80(21)	27.4 (18.5–35.0)	16%	−0.7
26.4	31(21)	36.2 (21.8–>38.0)	53%	−0.3
Vehicle	–	31.8 (19.6–>38.0)	34%	–

[a] MCF-7 human breast tumor xenografts and 1.7-mg 17-β-estradiol pellets were implanted into athymic nude mice as described in Materials and methods. Mice were randomized for study at 10 mice per treatment group and 20 mice in the control group. Treatment with 13-CRA p.o. as based on exact body weight was begun on day 12, qdX5, and repeated for 3 weeks. Vehicle-treated mice received 0.01 ml/g body weight cremophor:ethanol:water (1:1:6, by vol.) p.o. on the same treatment schedule. Tumor length and width were measured twice weekly, and tumor volumes were calculated as described in Table 1

[b] Dose of 13-CRA is given in mg/kg per day of treatment

[c] Optimal %T/C is the minimal %T/C obtained for that treatment group, and the day on which it occurred is indicated in parentheses. %T/C is the change in treated tumor over the change in control tumor using the median tumor volumes

[d] The median number of days required for 2 doublings is given, and the range of days needed for 2 doublings is indicated in parentheses

[e] NLCK is the net log cell kill as based on the values recorded for the doubling times calculated from tumor-doubling times

shown in Fig. 3. It is apparent that the absorption of 13-CRA is much more variable in fed mice (Fig. 3B) than in fasted animals (Fig. 3A). Furthermore, 13-CRA peak concentrations are lower in the fed animals than in the fasted mice, suggesting that food may interfere with the absorption of 13-CRA.

The decline of 13-CRA concentrations in plasma over time fit a one-compartment open linear model with a half-life of 73 min and first-order absorption. Peak concentrations were obtained at 30–45 min in plasma when mice had been fasted prior to dosing. The area under the plasma concentration-time curve (AUC) averaged 975 μg ml^{-1} min, with the apparent clearance being 103 ml min^{-1} kg^{-1}. A major metabolite, likely the glucuronide of 4-oxo-CRA, was identified at a retention time of approximately 3.3 min (METAB X in Table 4). AUCs were also calculated for 4-oxo-CRA, ATRA, and METAB X and were, respectively, 117, 87, and 3262 μg ml^{-1} min. The peak 13-CRA concentrations detected in tumors were 20% of the peak plasma concentrations, whereas those found in liver were equivalent to the plasma values (Table 4). Peak ATRA concentrations represented 5% of 13-CRA concentrations detected in plasma, 68% of those found in liver, and 20% of those found in tumor (Table 4). 4-oxo-CRA represented 2–10% of 13-CRA concentrations detected in plasma and liver but was not observed in tumor.

Mean peak plasma 13-CRA concentrations in the nonfasted mice occurred at 30 min and were 4.19 ± 1.66 μg/ml. Mean peak plasma 13-CRA concentrations were not different on day 1 or day 8 and were not affected by coadministration of TAM. The peak concentrations obtained on days 1 and 8 of treatment in mice treated with 13-CRA only were, respectively, 4.73 ± 1.92 and 3.10 ± 3.09, whereas those obtained in mice treated with both 13-CRA and TAM were 3.99 ± 1.17 and 4.53 ± 0.02 μg/ml, respectively. Mean concentrations of 13-CRA detected in tumor and liver were equivalent to those found in the initial pharmacokinetics study. No difference in 4-oxo-CRA was observed between mice that had been given 13-CRA alone and those that had been treated with 13-CRA and TAM.

Discussion

Several studies have suggested that retinoids may enhance the anticarcinogenic effects of antiestrogen treatment. In 1983, McCormick et al. [19] noted that oral retinyl acetate combined with ovariectomy significantly reduced the number of second primary breast tumors in rats. Fenretinide and TAM have been shown to be more effective than either agent alone against methyl nitrosourea-induced mammary tumors [18]. Various retinoids have been shown to be growth-inhibitory to mammary tumors in vitro [10, 17–19, 22, 24–26, 29]. Generally, retinoids are inhibitory only to ER-positive tumors [10, 26], although some agents classed with retinoids are effective against either ER-positive or ER-negative cells lines [11, 25]. Sheikh et al. [24] demonstrated that transfection of ER into ER-negative breast-cancer cell lines resulted in increased expression of retinoic acid receptor (RAR) alpha and in sensitivity to growth inhibition by ATRA. Rubin et al. [22] showed that both ATRA, a ligand for RARs, and 9-*cis*-retinoic acid, a ligand for both RARs and RXRs, down-regulate ER mRNA and protein in MCF-7 cells. These agents also down-regulate estrogen

Addendum 2 Data for Fig. 1B: median values and their 95% confidence intervals. The number of animals surviving at each time point is given in parentheses under the 95% confidence interval

Treatment	Days post tumor implantation													
	12	15	19	23	26	30	33	36	41	44	48	51	53	57
Control	107.5 85.5–175 (20)	168 117–232 (20)	242 185–357 (20)	294 240–413 (20)	460 347–637 (19)	548 421–766 (19)	655 452–1014 (15)	693 413–1105 (15)	867 433–1525 (15)	1138 560–1779 (11)	1570 687–2330 (11)	1918 645–2980 (11)	1885 882–3014 (11)	2559 889–3171 (11)
13-CRA 200	105.5 57–223 (8)	216 118–321 (8)	317.5 226–468 (8)	430 330–608 (8)	568 388–675 (8)	710 586–898 (8)	695 543–945 (7)	849 725–1175 (7)	1138 795–1586 (7)	1372 824–1753 (7)	1675 1201–2083 (7)	1733 984–2316 (6)	2125 1224–2730 (5)	2009 1203–3479 (5)
13-CRA 100	141.5 92–244 (8)	197 111–451 (8)	261 149–603 (8)	333.5 206–682 (8)	513 256–788 (8)	619.5 293–934 (8)	643 400–1011 (8)	741 388–1101 (8)	1014.5 517–1446 (8)	1035.5 563–1265 (8)	1276 703–1820 (8)	1404.5 708–2707 (8)	1801 896–2994 (8)	2009 1054–3436 (8)
13-CRA 50	130.5 58–282 (8)	206 47–489 (8)	240.5 57–488 (8)	395.5 90–660 (8)	568 131–900 (8)	788 256–967 (8)	821 372–1229 (7)	965 395–1341 (7)	1249 322–2179 (6)	1325 347–2237 (6)	1703 544–2678 (6)	1998 564–3014 (6)	2037 789–3485 (6)	2405 831–4043 (6)
13-CRA 25	81.5 68.5–141 (8)	173 94–275 (8)	275.5 120–464 (8)	298 169–596 (8)	394 165–965 (8)	517.5 236–1091 (8)	561 254–1114 (8)	794 342–1399 (6)	651 341–1516 (4)	724 391–1865 (4)	870.5 518–2386 (4)	1038 602–2410 (4)	1107 700–2516 (4)	1319 715–2976 (4)
13-CRA vehicle	85.5 62.5–104.5 (8)	123 66–178 (8)	228 117–310 (8)	330 145–512 (8)	405 193–520 (8)	621 234–667 (7)	715.5 83–809 (6)	931 373–1223 (6)	744 111–1572 (5)	984 194–2074 (5)	1467 440–2175 (5)	1778 542–2189 (5)	2145 672–2270 (5)	2559 889–3171 (5)

Table 3 Response of advanced-stage s.c. MCF-7 breast tumor xenografts to combination treatment with tamoxifen and 13-CRA[a]

TAM[b]	13-CRA[b]	Optimal %T/C (day)[c]	Median days to 3 doublings[d]	Growth delay %T–C/C	NLCK[e]
Control	Control	–	22.2 (8.1–>56.0)	–	–
–	200	105(33)	29.2 (9.3–>56.0)	32%	−0.6
–	100	76(19)	30.9 (27.9–38.8)	39%	−0.5
–	50	103(19)	30.0 (17.1–41.1)	35%	−0.5
–	25	85(33)	> 22.0 (10.9–>38.2)	−1%	−0.9
–	Vehicle	–	19.1 (15.0–>33.6)	−14%	–
60	–	−49(36)	54.7 (47.0–>56.0)	146%	0.8
30	–	−36(36)	50.6 (21.7–>56.0)	128%	0.5
15	–	10(36)	53.5 (27.9–>56.0)	141%	0.7
7.5	–	60(23)	23.9 (13.2–>56.0)	8%	−0.8
Vehicle	–	–	29.0 (15.0–>33.6)	31%	–
60	200	−17(33)	> 55.9 (46.8–>56.0)	152%	0.8
60	100	−22(30)	> 56.0 (42.0–>56.0)	152%	0.8
60	50	−13(30)	> 56.0 (> 56.0)	152%	0.8
60	25	0(36)	54.1 (45.8–>56.0)	144%	0.7
30	200	−16(19)	48.9 (35.9–>56.0)	120%	0.5
30	100	1(30)	> 56.0 (38.0–>56.0)	152%	0.8
30	50	−43(23)	> 56.0 (54.9–>56.0)	152%	0.8
30	25	−35(33)	> 55.4 (35.5–>56.0)	150%	0.8
15	200	7(33)	53.5 (42.4–>56.0)	141%	0.7
15	100	−28(33)	> 56.0 (46.8–>56.0)	152%	0.8
15	50	12(33)	52.3 (33.6–>56.0)	136%	0.6
15	25	−4(30)	48.5 (34.1–>56.0)	118%	0.4
7.5	200	24(19)	> 56.0 (31.9–>56.0)	152%	0.8
7.5	100	22(30)	50.0 (21.7–>56.0)	125%	0.5
7.5	50	43(36)	40.9 (28.0–>56.0)	84%	0.0
7.5	25	48(33)	39.5 (19.3–53.9)	78%	0.0

[a] MCF-7 human breast tumor xenografts and 1.7-mg 17-β-estradiol pellets were implanted into athymic nude mice as described in Materials and methods. Mice were randomized for study at 8 mice in each treatment group and 20 mice in the control group. Treatment with 13-CRA and TAM p.o. was was begun on day 12, qdX5, and repeated for 3 weeks. Vehicle-treated mice for TAM received 0.01 ml/g body weight water, whereas vehicle-treated mice for 13-CRA received 0.01 ml/g cremophor:-ethanol:water (1:1:6, by vol.) p.o. on the same treatment schedule. Tumor length and width were measured twice weekly, and tumor volumes were calculated as described in Table 1

[b] Doses of TAM and 13-CRA are given in mg/kg/day of treatment

[c] Optimal %T/C is the minimal %T/C obtained for that treatment group, and the day on which it occurred is indicated in parentheses

[d] The median number of days required for 3 doublings is given, and the range of days needed for 3 doublings is indicated in parentheses

[e] NLCK is the net log cell kill as based on the values recorded for the doubling times calculated from tumor-doubling times

response genes. Down-regulation of the ER and/or its response genes may result in an increased TAM effect. If the ER were down-regulated, there would be fewer ER receptors; therefore, at the same concentration, TAM could more effectively compete with endogenous estrogens for binding. Likewise, if response genes were down-regulated, the signal from the ER would be attenuated, possibly resulting in down-regulated tumor growth. Therefore, it is plausible that retinoids, which are thought to act through specific receptors, may well enhance the antiestrogen effect of TAM against breast cancer.

ATRA and fenretinide have been the major retinoids investigated in combination with TAM. In other tissues, most notably in oral premalignancy, 13-CRA has shown cancer-inhibitory effects [13]. 13-CRA may represent a more attractive clinical compound as compared with ATRA because its pharmacokinetics are stable over time [12], whereas those of ATRA are variable [1, 7, 20]. However, 13-CRA does not bind to the RARs with as much affinity as does ATRA [3]. Fenretinide, a semi-synthetic retinoid analogue, appears to exert its effects by mechanisms other than those involving RARs [25]. Although each of these agents is considered to be a retinoid, it is necessary that we discover which of these agents, if any, may be best suited for combination with TAM for possible therapeutic use in breast cancer.

Our data described experiments in xenografts of human ER-positive breast carcinomas in immune-suppressed mice. Peak tumor concentrations of 13-CRA and ATRA were in the range of effective concentrations reported for both agents in in vitro studies [10, 11, 30]. 13-CRA alone was ineffective against established MCF-7 human breast cancer xenografts in these studies. The data demonstrated a benefit in the combination of 13-CRA with TAM only for the lowest dose of TAM

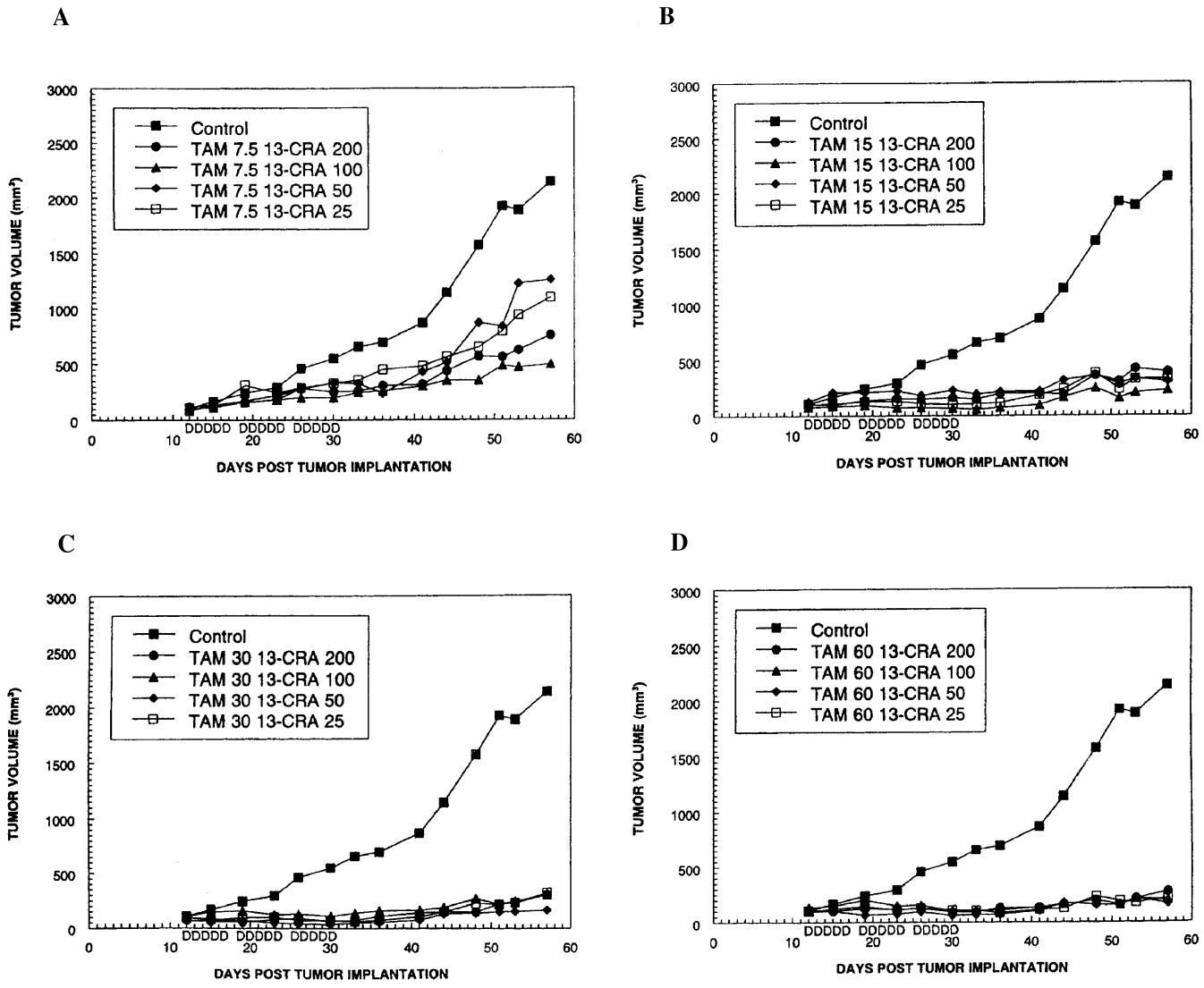

Fig. 2A–D Effect of the combination of daily doses of 25–200 mg/kg 13-CRA and **A** 7.5, **B** 15, **C** 30, or **D** 60 mg/kg TAM on established MCF-7 human breast cancer xenografts in athymic nude mice. Results are expressed as median tumor volumes

combined with the two highest doses of 13-CRA. Because TAM alone is well tolerated and is effective in ER-positive breast cancer, it is not likely that 13-CRA and TAM would be useful against established breast cancers clinically.

Although a reasonable delivery of 13-CRA to the tumor was demonstrated in these studies, there remain many unanswered questions. Protein binding may play a role in the observation of little activity in in vivo as compared with in vitro studies. 13-CRA probably circulates with 90% of the drug bound to albumin in vivo versus about 40% protein binding in culture. Concentrations of TAM were not measured. It is possible that these were altered by the addition of 13-CRA. However, the similarity of the median time to three doublings observed in tumors treated either with TAM alone or with TAM and 13-CRA would argue against this possibility. At low concentrations, TAM is an estrogen agonist [15]. The increased activity of the 13-CRA and TAM combination as compared with TAM alone at a low dose may be related to estrogen agonist effects rather than to the antagonistic affects of TAM at this dose. Two other possibilities may influence drug interactions in clinical trials but would not be addressed by either in vitro or these in vivo experiments. These possibilities (a) relate to the necessity for estrogen supplementation to the mouse for the xenografts to grow and (b) to the immunosuppressed state of the animal. The relationship of ambient estrogen concentrations to retinoid or TAM activity has not been defined. Likewise, retinoids do have effects on immune function ([26] and references therein) that may or may not influence tumor response. Finally, the active drug may be not 13-CRA but rather ATRA or 9-*cis*-retinoic acid or a combination of these. ATRA concentrations detected in tumor were only about 20% of 13-CRA concentrations found in plasma, and 9-*cis*-retinoic acid was not detectable. Thus, ATRA concentrations would be at or near an effective "in vitro" concentration (300 ng/g, or 1 μ*M*) for only

Addendum 3 Data for Fig 2A: median values and their 95% confidence intervals. The number of animals surviving at each time point is given in parentheses under the 95% confidence interval

Treatment	Days post tumor implantation													
	12	15	19	23	26	30	33	36	41	44	48	51	53	57
Control	107.5 85.5–175 (20)	168 117–232 (20)	242 185–357 (20)	294 240–413 (20)	460 347–637 (19)	548 421–766 (19)	655 452–1014 (15)	693 413–1105 (15)	867 433–1525 (15)	1138 560–1779 (11)	1570 687–2330 (11)	1918 645–2980 (11)	1885 882–3014 (11)	2140 1028–3935 (11)
TAM 60 13-CRA 200	114 54–190 (8)	104.5 49–182 (8)	126 40–208 (8)	117 36–193 (8)	124.5 41–204 (8)	91 60–169 (8)	91.5 31–186 (8)	130 46–173 (8)	130 57–195 (8)	162.5 79–246 (8)	247 119–316 (8)	173 77–302 (8)	245.5 103–405 (8)	275 95–466 (8)
TAM 60 13-CRA 100	132.5 72–161.5 (8)	150.5 62–187 (8)	202 80–265 (8)	148.5 83–221 (8)	154.5 83–226 (8)	103.5 21–154 (8)	107.5 52–215 (8)	107.5 70–228 (8)	133.5 67–222 (8)	162.5 93–236 (8)	198.5 97–348 (8)	157 83–304 (8)	213 100–410 (8)	199 83–533 (8)
TAM 60 13-CRA 50	97 65–152.5 (8)	102.5 42–175 (8)	68 33–179 (8)	75 32–193 (8)	94.5 31–219 (8)	61.5 42–341 (8)	67.5 30–168 (8)	70.5 20–164 (8)	102 38–198 (8)	176 46–257 (8)	154.5 64–326 (8)	152 38–327 (8)	213 60–350 (8)	169 0–408 (7)
TAM 60 13-CRA 25	99.5 72–143.5 (8)	124 79–216 (8)	145 83–252 (8)	113 62–307 (8)	130.5 62–310 (8)	109.5 19–158 (8)	106 45–400 (8)	84.5 52–343 (8)	114.5 67–407 (8)	127 83–330 (8)	231.5 121–434 (8)	190.5 100–572 (8)	177 111–630 (8)	213 114–767 (8)
TAM 60 13-CRA vehicle	58.5 38.5–120.5 (8)	49.5 35–140 (8)	49 35–180 (8)	40.5 28–148 (8)	38.5 17–130 (8)	32 13–100 (8)	32 14–116 (8)	30 13–92 (8)	69 50–129 (8)	71 50–143 (8)	146 10–202 (8)	128 91–217 (8)	174.5 138.5–237.5 (8)	144 105–284 (8)

Addendum 4 Data for Fig 2B: median values and their 95% confidence intervals. The number of animals surviving at each time point is given in parentheses under the 95% confidence interval

Treatment	Days post tumor implantation													
	12	15	19	23	26	30	33	36	41	44	48	51	53	57
Control	107.5 85.5–175 (20)	168 117–232 (20)	242 185–357 (20)	294 240–413 (20)	460 347–637 (19)	548 421–766 (19)	655 452–1014 (15)	693 413–1105 (15)	867 433–1525 (15)	1138 560–1779 (11)	1570 687–2330 (11)	1918 645–2980 (11)	1885 882–3014 (11)	2559 889–3171 (11)
TAM 30 13-CRA 200	70.5 52.5–123 (8)	64 43.5–107 (8)	59 25.5–123.5 (8)	77 32–154 (8)	92 21–166 (7)	67 19–158 (7)	74 23–178 (7)	110 34–187 (7)	139 46–235 (7)	150 61–353 (7)	146 93–474 (6)	217.5 72–594 (6)	244 115–525 (6)	303 115–464 (6)
TAM 30 13-CRA 100	106.5 67–230 (8)	146.5 57–289 (8)	156 52–256 (8)	122 70–208 (8)	129 52–233 (8)	110 62–222 (8)	137.5 67–279 (8)	159 78–319 (8)	164 113–320 (8)	186 136–319 (8)	267 193–433 (8)	215.5 152–564 (6)	244 125–862 (5)	301 97–714 (5)
TAM 30 13-CRA 50	96.5 60–131 (8)	87 40–148 (8)	68.5 25–153 (8)	48 20–122 (8)	40 15–104 (8)	36.5 10.5–102.5 (8)	46 14.5–92.5 (8)	53 21–105.5 (8)	74.5 29–147 (8)	129 60–208 (7)	137 48–194 (7)	147 43–231 (7)	151 62–262 (7)	163 55–282 (7)
TAM 30 13-CRA 25	94.5 49–126 (8)	76.5 31–122 (8)	94 43–166 (8)	99.5 52–208 (8)	73 54–172 (8)	68.5 40–213 (8)	61 37–198 (8)	74.5 39–208 (8)	104.5 50–282 (8)	149 90–331 (8)	224 192–531 (8)	213 149–486 (8)	229 170–552 (8)	332 239–703 (7)
TAM 30 13-CRA vehicle	81.5 40–101 (8)	71 38–179 (8)	85 54–258 (8)	76 46–298 (8)	69 45–372 (8)	69 39–374 (8)	63 26–361 (8)	50 22–444 (8)	103 34–199 (8)	153 65–600 (8)	161 48–851 (8)	160 52–1014 (8)	226 97–1285 (8)	249 106–1636 (8)

Addendum 5 Data for Fig. 2C: Median values and their 95% confidence intervals. The number of animals surviving at each time point is given in parentheses under the 95% confidence interval

Treatment	Days post tumor implantation													
	12	15	19	23	26	30	33	36	41	44	48	51	53	57
Control	107.5 85.5–175 (20)	168 117–232 (20)	242 185–357 (20)	294 240–413 (20)	460 347–637 (19)	548 421–766 (19)	655 452–1014 (15)	693 413–1105 (15)	867 433–1525 (15)	1138 560–1779 (11)	1570 687–2330 (11)	1918 645–2980 (11)	1885 882–3014 (11)	2559 889–3171 (11)
TAM 15 13-CRA200	108.5 67.5–162 (8)	97.5 57–212 (8)	136.5 70–256 (8)	153.5 81–214 (8)	147.5 83–241 (8)	162 68–237 (8)	148 74–204 (8)	198 87–245 (7)	201 102–387 (7)	188 137–477 (7)	364 199–629 (7)	309 118–721 (7)	423 162–649 (7)	393 121–891 (7)
TAM 15 13-CRA 100	78 57–145 (8)	84.5 41–186 (8)	93.5 42–177 (8)	71.5 40–163 (8)	72 46–127.5 (8)	65 39–103.5 (8)	56 34–116 (8)	71.5 35.5–119 (8)	93.5 60–165 (8)	164 118–227 (8)	249 165–298 (8)	157 95–258 (8)	207 160–286 (8)	227 120–955 (7)
TAM 15 13-CRA 50	125.5 85–201 (8)	210.5 111–347 (8)	211 125–425 (8)	222 90–381 (7)	182 76–418 (7)	228 93–411 (7)	193 100–416 (7)	215 113–425 (7)	218 148–371 (7)	318 225–543 (7)	361 298–987 (7)	286 224–856 (7)	333 247–973 (7)	306 249–1166 (7)
TAM 15 13-CRA 25	104.5 66–219 (8)	106 73–249 (8)	125.5 71–258 (8)	131 82–175 (8)	108 70–175 (8)	100 69–215 (8)	107 62–233 (7)	114 79–223 (7)	187 115–330 (7)	239 175–308 (7)	385 213–590 (7)	238 171–473 (7)	328 222–581 (6)	332 152–650 (6)
TAM 15 13-CRA vehicle	91.5 33–200 (8)	116 39–220 (8)	156.5 38–303 (8)	155 50–242 (8)	197 54–304 (8)	166.5 98–250 (8)	158 83–393 (8)	159.5 93–295 (8)	240 94–359 (8)	294 144–378 (8)	297 189–452 (7)	383 155–558 (7)	484 264–613 (7)	521.5 270–886 (6)

Addendum 6 Date for Fig. 2D: Median values and their 95% confidence intervals. The number of animals surviving at each time point is given in parentheses under the 95% confidence interval

Treatment	Days post tumor implantation													
	12	15	19	23	26	30	33	36	41	44	48	51	53	57
Control	107.5 85.5–175 (20)	168 117–232 (20)	242 185–357 (20)	294 240–413 (20)	460 347–637 (19)	548 421–766 (19)	655 452–1014 (15)	693 413–1105 (15)	867 433–1525 (15)	1138 560–1779 (11)	1570 687–2330 (11)	1918 645–2980 (11)	1885 882–3014 (11)	2559 889–3171 (11)
TAM 7.5 13-CRA 200	118 48–158 (8)	139.5 30–235 (8)	156 37–256 (8)	187 43–296 (8)	284 43–345 (8)	254 67–368 (7)	261 49–333 (7)	309 61–385 (7)	319 75–606 (7)	440 91–661 (7)	565 94–844 (7)	560 182–871 (7)	622 177–1087 (7)	755 195–1232 (7)
TAM 7.5 13-CRA 100	103.5 67.5–158.5 (8)	113.5 90–194 (8)	163 131–278 (8)	184 142–270 (8)	202 145–282 (7)	202 133–321 (8)	248.5 147–296 (8)	260 170–388 (8)	304 204–466 (8)	354 258–600 (8)	355 245–657 (8)	485 279–821 (7)	472 272–849 (7)	495 201–955 (6)
TAM 7.5 13-CRA 50	94 68–148.5 (8)	132 67–202 (8)	174.5 109–340 (8)	222 85–386 (8)	280 80–517 (7)	335 81–681 (7)	329 71–598 (7)	240 96–777 (7)	429 137–836 (6)	513 194–941 (6)	868 128–1323 (6)	836 159–1329 (6)	1221 169–1509 (6)	1253 166–1837 (6)
TAM 7.5 13-CRA 25	86 78.5–172 (8)	133 90–245 (8)	313.5 149–434 (8)	254.5 142–393 (8)	290 154–413 (8)	334 177–511 (8)	358 217–720 (8)	451.5 250–645 (8)	480.5 330–952 (8)	562 394–1147 (8)	648 438–1272 (8)	793 502–1639 (8)	938 603–2147 (8)	1094 755–2511 (8)
TAM 7.5 13-CRA vehicle	91 56.5–123 (8)	137.5 61–238 (8)	200.5 92–321 (8)	220.5 101–416 (8)	289 126–431 (8)	337.5 103–667 (8)	339 83–809 (7)	455 86–787 (7)	516 90–1095 (7)	714 121–1238 (7)	585 132–1657 (7)	804 136–1845 (7)	895 139–2419 (7)	917 197–2699 (7)

Table 4 Mean peak concentrations of 13-CRA and metabolites detected in the plasma, liver, and tumor of fasted athymic nude mice treated with CRA p.o.

Compound	Plasma (μg/ml)	Liver (μg/g)	Tumor (μg/g)	AUC(μg min ml^{-1})
13-CRA	9.84 (9.42–10.23)[b]	10.44 (5.76–15.15) [b]	1.2 (0.98–1.36) [b]	975
4-oxo-CRA	0.47 (0.34–0.57)	0.4 (0.38–0.43)	0 (0)	117
ATRA	0.44 (0.39–0.48)	3.3 (2.54–4.08)	0.27 (0.16–0.38)	87
METAB X[a]	9.48 (8.84–10.13)	40.96 (40.66–41.27)	0.75 (0.64–0.86)	3262

[a] Concentrations of METAB X are expressed in 13-CRA equivalents
[b] Ranges for the values are presented in parentheses

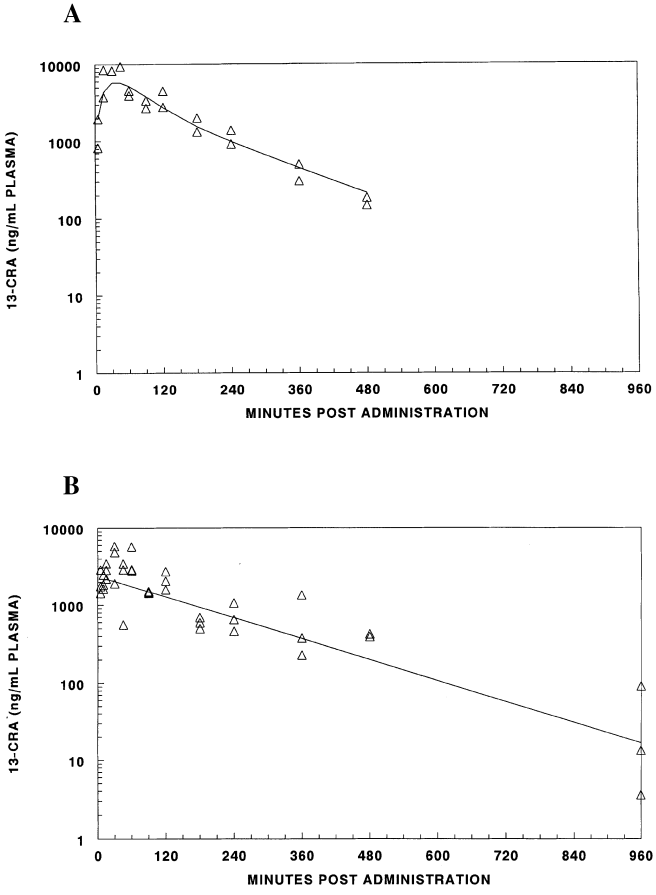

Fig. 3A,B Pharmacokinetics of 13-CRA given as a 100-mg/kg bolus to **A** fasted and **B** fed mice. Measurements of 13-CRA in plasma were obtained in 2 fasted mice at each time point and in 3 fed mice at each time point. Note the wider variability and lower peak in plasma concentration in fed animals

short periods. In general, though, delivery of drug to the tumor or its increased metabolism to inactive agents would not explain the lack of activity of this combination, given the activity of ATRA in acute promyelocytic leukemia [6, 14]. It is also possible that retinoids may be effective only in premalignant cells, not when cells have become fully malignant.

Because TAM and retinoic acid compounds are extensively metabolized in animals by the cytochrome P-450 mixed-function oxidase system, the activities of these agents might be altered by concurrent administration [12, 16]. Furthermore, the absorption of the agents might be altered by the presence of the other agent or of food in the stomach. Our data demonstrate that 13-CRA was absorbed more variably and to a lesser extent in fed mice than in fasted animals. Coadministration of TAM and continued administration of 13-CRA had no effect on 13-CRA pharmacokinetics or metabolism.

In summary, preclinical studies suggest that the combination of retinoids and antiestrogen theraphy are promising as a possible therapy for ER-positive breast cancer. However, further studies are necessary to define better regimens that are likely to be successful in the clinic.

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