

Novel function of the thyroid hormone analog tetraiodothyroacetic acid: a cancer chemosensitizing and anti-cancer agent

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Received: 15 January 2008 / Accepted: 17 March 2008 / Published online: 4 April 2008
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Abstract Previous studies from our laboratory have demonstrated that thyroid hormones play a key role in cancer progression. In addition, a deaminated form, tetraiodothyroacetic acid (tetrac), that antagonizes the proliferative action of these hormones was found to possess anti-cancer functions through its ability to inhibit cellular proliferation and angiogenesis. The present study was undertaken to investigate whether tetrac could also suppress the development of drug resistance, known as a causative factor of disease relapse. Tetrac was shown to enhance cellular response in vitro to doxorubicin, etoposide, cisplatin, and trichostatin A in resistant tumor cell lines derived from neuroblastoma, osteosarcoma, and breast cancer. The mechanism of action of tetrac did not involve expression of classical drug resistance genes. However, radiolabeled doxorubicin uptake in cells was enhanced by tetrac, suggesting that one or more export mechanisms for chemotherapeutic agents are inhibited. Tetrac was also found to enhance cellular susceptibility to senescence and apoptosis, suggesting that the agent may target multiple drug resistance mechanisms. Tetrac has previously been shown to inhibit tumor cell proliferation in

vitro. In vivo studies reported here revealed that tetrac in a pulsed-dose regimen was effective in suppressing the growth of a doxorubicin-resistant human breast tumor in the nude mouse. In this paradigm, doxorubicin-sensitivity was not restored, indicating that (1) the in vitro restoration of drug sensitivity by tetrac may not correlate with in vivo resistance phenomena and (2) tetrac is an effective chemotherapeutic agent in doxorubicin-resistant cells.

Keywords Drug resistance · P-glycoprotein · Tetrac · Thyroid hormone

Introduction

Thyroid hormones exert many physiological functions essential for normal development and proper functioning of critical organs (reviewed in [1–3]). A number of studies have demonstrated that genetic defects associated with dysregulated expression of thyroid hormone synthesis or their receptors can cause neurological, metabolic, and heart diseases [2–4]. Evidence has been provided that thyroid hormone (L-thyroxine, T₄; 3,5,3'-triiodo-L-thyronine, T₃) may also play a permissive role in breast [5], glial [6], and thyroid [7] tumor cell proliferation via a cell surface hormone receptor [8, 9]. In addition, the nuclear thyroid receptor (TR) has been implicated in tumor cell behavior by in vivo studies in which knocking-in a mutated form of the thyroid hormone nuclear receptor (TR β PV) accelerated the onset of aggressive thyroid cancers in mice [10]. Recent clinical evidence suggests that hypothyroidism affects glioblastoma multiforme [11] and breast tumor [12] growth in a clinically desirable manner.

The cell surface receptor for thyroid hormone that is relevant to tumor cell proliferation is on integrin $\alpha v \beta 3$ and

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the hormone signal is transduced by the ERK/MAPK pathway [8, 9]. Using the chick chorioallantoic membrane (CAM) model and human dermal microvascular endothelial cell assays, we have also implicated the $\alpha v\beta 3$ receptor-MAPK mechanism in thyroid hormone's pro-angiogenic effect [8, 13–15]. Studies from our laboratory have shown that tetrac, a deaminated non-agonist analog of T_4 , prevents binding of agonist T_4 and T_3 to $\alpha v\beta 3$ at the plasma membrane (8) and inhibits the hormonal effects on angiogenesis [8, 14, 15].

The present study was undertaken to explore another component of tumor cell behavior, namely, resistance to cancer chemotherapeutic agents. Several mechanisms of resistance exist [16–20], including expression of multi-drug resistance (MDR) pump genes whose gene products [21], inserted in the plasma membrane, export cancer chemotherapeutic agents to the extracellular space. We have shown that thyroid hormone, acting via its integrin receptor, has actions on plasma membrane ion transport systems, such as the Na^+/H^+ antiporter [22]. Therefore, we explored in the present studies the possibility that tetrac has membrane actions that are relevant to cellular handling of chemotherapeutic agents.

Materials and methods

Cells and reagents

Human neuroblastoma SKN-SH, osteosarcoma SaOS2, and breast carcinoma MCF-7 cells were purchased from ATCC (Rockville, MA). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from BioWhittaker (Walkersville, MD). The following drugs and reagents were obtained from the companies cited: doxorubicin, etoposide, cisplatin, and tetraiodothyroacetic acid (tetrac) (Sigma-Aldrich, St. Louis, MO), [^{14}C]doxorubicin (Amersham, Arlington Heights, IL), antibody to drug transporter P-glycoprotein (P-gp) (Signet Laboratories, Dedham, MA), SOD and GST- π antibodies (Santa Cruz Biotechnology Laboratories, Santa Cruz, CA), antibody to β -Actin from Sigma-Aldrich (St. Louis, MO), and secondary antibodies conjugated to horseradish peroxidase (BioRad, Hercules, CA).

Enhanced chemiluminescence reagents (ECL) were purchased from Amersham (Arlington Heights, IL) and immobilon-P transfer membranes for western blots were obtained from Millipore (Bedford, MA). Resistant cells were generated by continuous incubation of parental cell lines with stepwise increases in drug concentrations, ranging from 10^{-9} M up to 10^{-5} M, over a period of three-to-six months. At the end of selection, the cells were tested for resistance to drugs by using the MTT viability assay, as

described previously [20]. Briefly, the cells were seeded at 10^4 cells/well in 96-well plates and incubated with the drug for 96 h.

Ten microliter of MTT solution (5 mg/ml) were added to each well and incubated for 4 h at 37°C. The cells were then solubilized by the addition of 100 μ L of 10% SDS/0.01 M HCl and incubated for 15 h at 37°C. The optical density of each well was determined in an ELISA plate reader, using an activation wavelength of 570 nm and reference wavelength of 650 nm. The percentage of viable cells was determined by comparison with untreated control cells

Western blotting

Cell monolayers were grown in 25 cm² growth until 90% confluence, then washed with PBS, and the cells lysed in 50 mM HEPES pH 7.4 containing 150 mM NaCl, 100 mM NaF, 1 mM MgCl₂, 1.5 mM EGTA, 10% glycerol, 1% Triton \times 100, 1 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Equal quantities of protein were separated by electrophoresis on a 12% SDS-PAGE gel and transferred to Immobilon-P membranes. Proteins of interest were identified by reaction with specific primary and secondary antibodies linked to horseradish peroxidase and detected by chemiluminescence. The details of the procedure in our laboratory have been published [23, 24].

Measurement of doxorubicin accumulation

Doxorubicin-resistant cells were seeded in 12-well plates and incubated for 24 h, after which [^{14}C]doxorubicin (5 nCi) was added in the absence or in the presence of tetrac (30 μ g/ml) and incubated for 24 h. The cells were then washed four times with ice-cold PBS and lysed. Radioactivity in the lysate, which corresponds to cellular drug accumulation, was measured by scintillation spectrometry.

Senescence-associated- β -galactosidase (SA- β -Gal) staining

Cells were seeded into 24-well plates in DMEM culture medium and after 24 h, doxorubicin, tetrac, or both were added and the cells incubated for 5 days.

SA- β -Gal staining was performed as previously described [21]. In brief, cells were fixed for 5 min in 3% formaldehyde, washed and incubated at 37°C with X-gal (1 mg/ml), dissolved in a solution containing 40 mM citric acid pH 6.5, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM MgCl₂. After 24 h incubation, photographs were taken under a phase microscope.

Detection of apoptosis

Cells were seeded on 100-mm culture dishes and treated with doxorubicin, tetrac or both for 24 h, after which the cells were subjected to trypsin treatment at 37°C for 3 min, fixed with 4% formalin in PBS, and washed with PBS. Then they were incubated with 0.1 µg/ml Hoechst 33248 (bisbenzimidazole, Sigma-Aldrich) and spotted on slides for microscopy. Positive cells were counted and their fractions between non-treated and treated populations were compared [25].

Animal studies

Strain CD1 nude mice were obtained from Charles River Laboratories (Wilmington, MA) at approximately 5–6 weeks of age and weighing approximately 30 g. Animals received s.c. implantation of doxorubicin-resistant MCF7/R cells (10^6 cells in 100 µl). When tumors were approximately 50 mm³ in size, the animals were pair matched and divided into four groups of five mice as follows: a) vehicle treated controls, b) mice treated with tetrac (30 mg/kg), c) mice treated doxorubicin (2 mg/kg), d) mice treated with the combination of both drugs. A total of three injections (separated by 3 days) were performed. Mice were weighed, checked for clinical signs of drug toxicity and lethality. Tumor measurements were made with a caliper three times weekly for up to 3 weeks and converted to tumor volume by using the formula $W \times L^2/2$. Tumor growth curves were generated.

Statistical analysis

Statistical analysis was performed by one-way ANOVA using Statview software (Adept Scientific, Acton, MA), comparing the mean \pm SD of branch points from each experimental group with its respective control group. Statistical significance was defined as $P < 0.05$.

Results

Effect of tetrac on the proliferation of drug-sensitive and drug-resistant cells

We have tested the effect of tetrac on the proliferation of drug sensitive and resistant cell lines derived from neuroblastoma (SKN-SH), osteosarcoma (SaOS2), and breast cancer (MCF7). The resistant lines were selected by step-wise increase of doxorubicin concentrations over time. As shown in Fig. 1 (panels a, c, and e), cellular response to doxorubicin was significantly reduced in the resistant cells. These cells were also found to be resistant to other related

and unrelated agents such as etoposide, vinblastine, and the histone deacetylase inhibitor Trichostatin A (data not shown). However, the effect of tetrac on cell proliferation was similar in both sensitive and resistant lines (panels b, d, and f) suggesting that this hormone antagonist is capable of bypassing drug resistance. Interestingly, in the case of SaOS2 cells, the drug-resistant cells appeared to be more sensitive to tetrac than their parental drug-sensitive cells. This suggests that alterations in thyroid hormone signaling pathways that render cells more sensitive to tetrac might have occurred during the drug selection process.

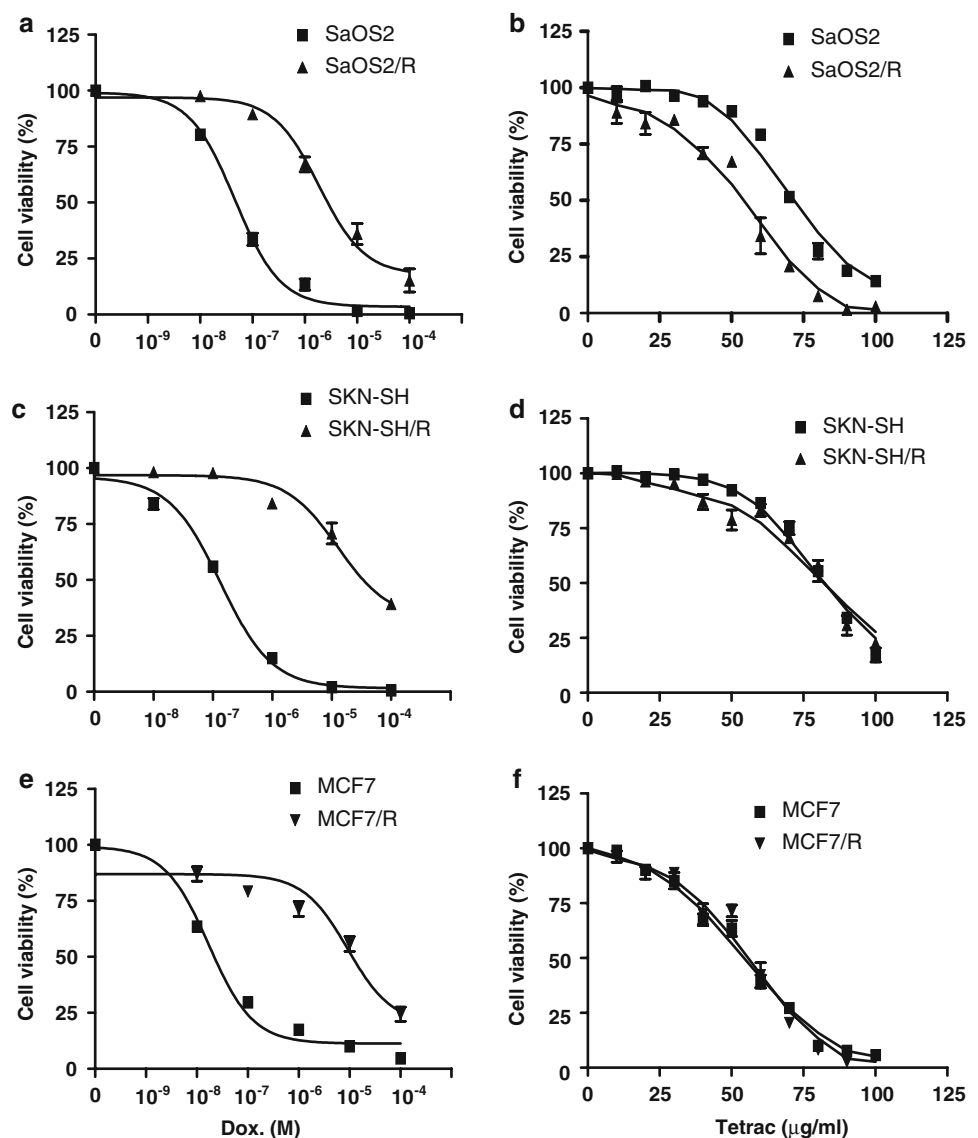
Enhancement of cancer cell response to classical anti-cancer agents by tetrac

The effect of tetrac on cellular response to known anti-cancer agents including doxorubicin, etoposide, cisplatin, and TSA were investigated in their drug-sensitive and drug-resistant counterparts. For this, the cells were first pretreated with tetrac (30 µg/ml), then subjected to treatment with classical anti-cancer agents and incubated for 4 days. As shown in Fig. 2, tetrac consistently enhanced cellular response to these drugs in the three resistant cell lines. Similar effects were also observed in drug-sensitive cell lines (data not shown). Since resistance to doxorubicin and etoposide is known to be associated with over-expression of the drug transporter P-gp, the possibility exists that tetrac may inhibit the function of this transporter; however, since resistance to cisplatin has been shown to be independent of this transporter, it is suggested that P-gp-independent mechanisms may also mediate the action of tetrac. Another possibility is that tetrac affects MDR pump activity by changing intracellular pH (see Discussion).

Putative mechanisms by which tetrac enhances cancer cell sensitivity to drugs

As mentioned above, over expression of the drug transporter P-gp is a hallmark for resistance to topoisomerase inhibitors such as doxorubicin and etoposide. We have found that this transporter was indeed over-expressed in doxorubicin-resistant MCF7/R compared to the parental drug-sensitive cells (Fig. 3a). In comparison, the expression of other drug resistance molecules such as SOD and GST- π did not change significantly between the two cell lines (Fig 3a), suggesting that alteration of drug transport could be the principal mechanism of drug resistance in these cells. Analysis of the effect of tetrac effect on expression of P-gp, SOD, and GST- π was carried out by western blots in MCF/R cells either subjected or not to treatment with this antagonist and/or doxorubicin. The data (Fig. 3b) indicate that none of the treatments affected the expression of these drug resistance genes. However, measure of drug transport in the presence of tetrac using radiolabeled

Fig. 1 Effect of tetrac on the proliferation of drug-sensitive versus drug-resistant cancer cells. Drug-sensitive and -resistant SKN-SH, SaOS2, and MCF7 cells were subjected to treatment with increasing concentrations of tetrac over a period of 4 days. Cell viability was then measured by MTT assay. The data represent the average of four determinations \pm SE



doxorubicin indicated that the accumulation of this drug was significantly enhanced in the treated cells (Fig. 3c), suggesting that the function of P-gp might be inhibited by this hormone antagonist. This observation sheds light on a previously unknown function of tetrac and provides compelling evidence that this analog has the potential to be considered in the treatment of drug-resistant tumors.

Taking into account the finding made in Fig. 2 that tetrac enhances cellular response to cisplatin, a non-P-gp substrate, additional drug resistance mechanisms must be considered. For example, recent studies from our laboratory and others have shown that regardless of the nature of cellular defense against stress, cancer cell susceptibility to undergo proliferation arrest, i.e., senescence, or cell death, i.e., apoptosis, represent key determinants in the onset and progression of drug-resistant tumors. To verify whether these pathways could be also affected by tetrac, we measured the expression

of molecular markers of growth arrest (cell cycle inhibitor p21/WAF1) and apoptosis (active caspase-3) in cells subjected to treatment with this compound, compared to non-treated cells. As shown in Fig. 4a, tetrac alone had no effect on expression of p21/WAF1, whereas its combination with a relatively low doxorubicin concentration had a strong effect on this cell cycle inhibitor. This finding was further substantiated by measurement of the senescence-associated beta galactosidase (SA- β -Gal) (Fig. 4b) indicating that cell exposure to this drug combination forces them into a senescence state. Also, under these same conditions, a strong increase in caspase 3 activation (Fig. 4a) and chromatin condensation (Fig. 4b) were observed, indicating that a fraction of treated cells were committed to apoptotic death. Together, these findings provided evidence that in addition to its effect on drug transport, tetrac may also synergize with cytotoxic drugs to induce cellular senescence and apoptotic cell death.

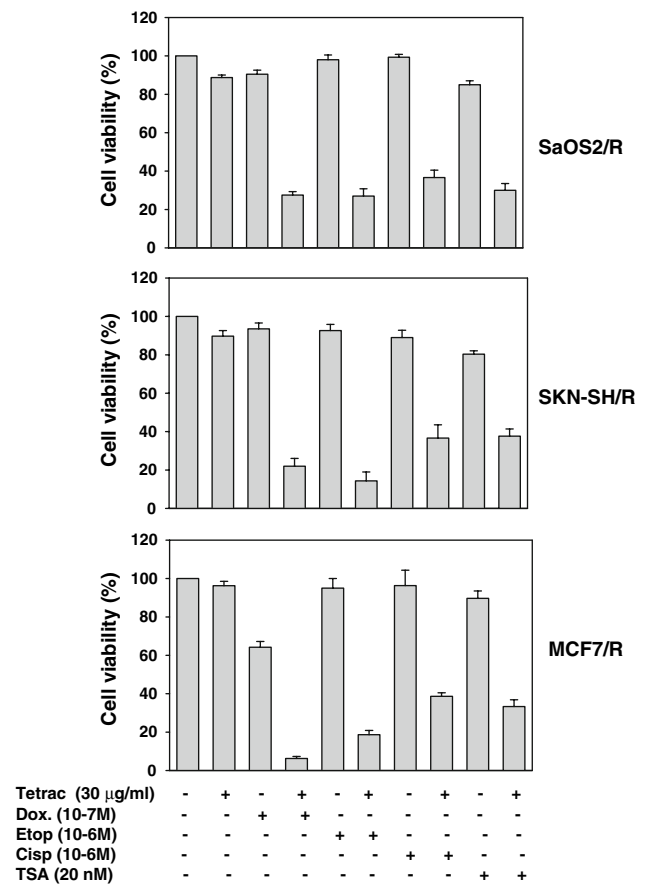


Fig. 2 Reversal of drug resistance by tetrac. Doxorubicin-resistant SKN-SH/R, MCF7/R, and SaOS2/R cells were subjected to treatment with tetrac either alone or in combination with each of doxorubicin (Dox), etoposide (Etop), cisplatin (Cisp), or trichostatin A (TSA) at the indicated concentrations. After 4 days cell viability was determined by the MTT assay and the data represented as average of three determinations ±SE

Effect of tetrac on the proliferation of drug-resistant tumors in vivo

To define further the *in vivo* relevance of tetrac in suppression of drug resistance, we tested its effect, either alone or in combination with doxorubicin, in nude mice bearing xenografts of drug-resistant cancer cells. Mice were injected with the doxorubicin-resistant breast cancer cell line MCF7/R and when the tumors became palpable, mice received three drug injections of doxorubicin alone, tetrac alone, or the combination of both. The maximal tolerated dose (MTD) for doxorubicin was 2.5 mg/kg; however, in the case of tetrac, no toxicity was detected for up to 60 mg/kg (data not shown). Concerning the efficacy of these treatments, the data indicated that doxorubicin alone (2 mg/kg) had no noticeable effect on tumor growth. In contrast, tetrac at 30 mg/ml alone reduced tumor growth by about 70% (Fig. 5). This effect was not further exacerbated by the combination of both drugs suggesting a lack of

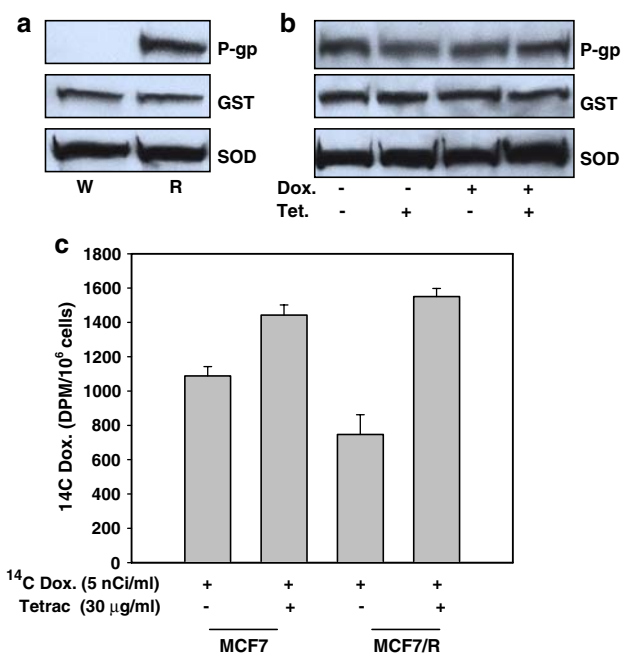


Fig. 3 Effect of tetrac on expression of classical drug resistance genes and on drug transport. Panel a. (full-length blots are presented in Supplemental Fig. 1a) Western blots showing the expression of P-gp, GST and SOD in wild type (W) drug sensitive and -resistant (R) MCF7 cells. Panel b. (full-length blots are presented in Supplemental Fig. 1b) Effect of tetrac and doxorubicin (Dox) on expression of these genes in MCF7/R cells. The cells were treated for 24 h with each drug alone or the combination of both, after which expression of drug resistance molecules was determined by Western blot using specific antibodies. Panel c. Effect of tetrac on intracellular accumulation of radiolabeled doxorubicin (¹⁴C Dox.) in drug sensitive MCF7 and -resistant MCF7/R cells. The cells were incubated for 24 h with doxorubicin in the absence or the presence of tetrac, after which they were washed and then solubilized. Radioactivity associated with the cell lysate was counted and compared between the two cell lines either treated or not with tetrac. The data represent an average of three determinations ±SE

synergistic effect at the concentrations used. Interestingly, the drug concentration of tetrac was well tolerated and no significant toxicity was noticed in the treated animals during the experiments. These findings suggest that tetrac is able to suppress the proliferation of drug-resistant tumors *in vivo* and thus, may hold promise for the treatment of drug-resistant tumors. Further investigations are warranted to determine the optimal dosage at which this compound may be used, either alone or in combination with other anti-cancer agents, for maximum anti-tumor efficacy.

Discussion

One of the remarkable features of cancer cells is their ability to adapt and thus to become resistant to virtually any type of stress. From the clinical standpoint, this is regarded as the principal cause of treatment failure and

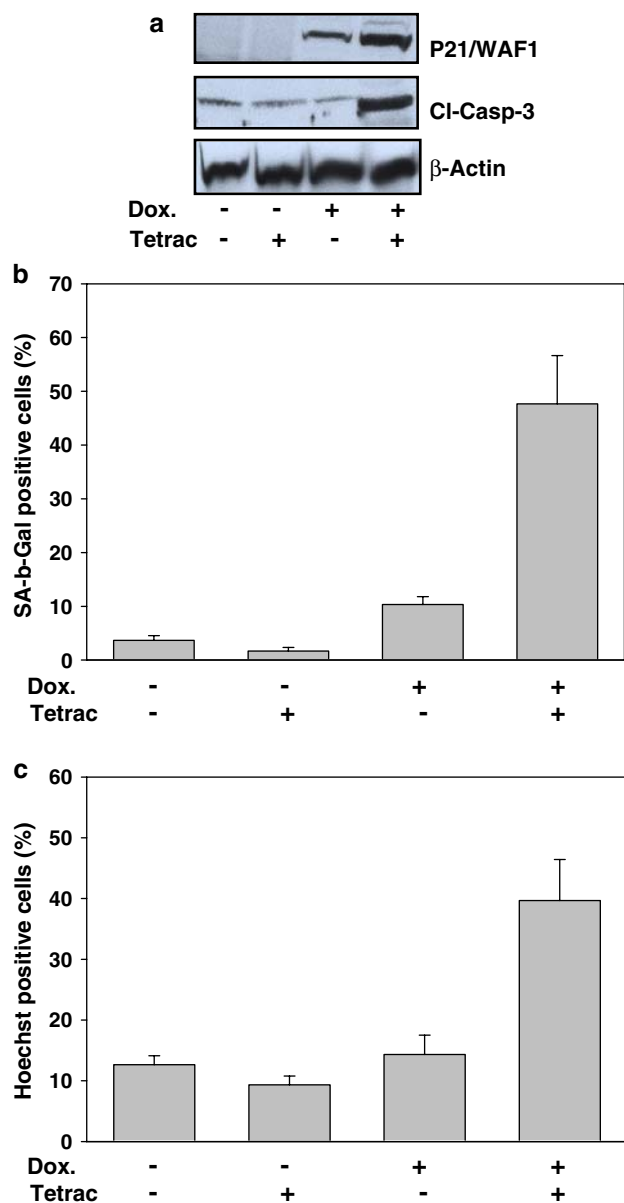


Fig. 4 Tetrac forces drug-resistant cancer cells to undergo senescence and apoptosis. Panel **a**. (full-length blots presented in Supplemental Fig. 2) SKN-SH/R cells were subjected to treatment with doxorubicin (Dox) alone, tetrac alone, or the combination of both for 24 h. Expression of p21/WAF1, cleaved caspase-3 (CI-Casp-3), and beta actin were measured by Western blot using specific antibodies. Panel **b**. The cells were seeded in 24-well plates and treated as mentioned above and expression of the senescence-associated beta galactosidase (SA- β -Gal.) was assayed as described in the methods section. Panel **c**. The cells were seeded on cover slips and treated with the drugs as above for 24 h, after which they were fixed and stained with Hoechst and the percentage of positive cells graphed. The data represent average of three determinations \pm SE

disease relapse; therefore, there is great interest in developing approaches to prevent and/or to reverse the development of drug resistance. In recent years, a number of drug candidates (most of which are inhibitors of ion

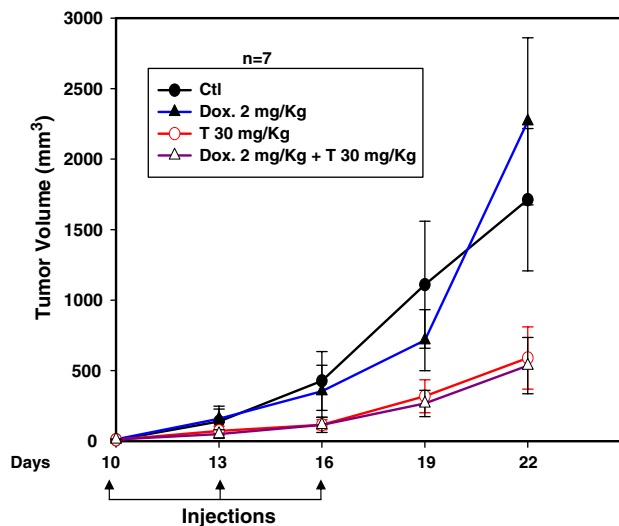


Fig. 5 Effect of tetrac on growth of doxorubicin-resistant tumors in nude mice. Mice were injected with doxorubicin-resistant MCF7/R cells (10^6) and after 10 days, they were assigned into groups of seven mice each and challenged with doxorubicin (2 mg/kg) or tetrac (30 mg/kg) either alone or in combination. Tumor volume was measured every 3 days for up to three weeks

channels) have been identified and, although most were found to be very effective in reversing drug resistance in vitro, they were unable to do so in vivo, often due to their high toxicity. In the search of novel, less toxic, drug resistance regulators, we have identified the thyroid hormone antagonist tetrac as a promising agent. Unlike other previously discovered drug-resistance-reversing agents, tetrac has no detectable toxicity and it exerts a dual action on drug transport and signaling pathways that control cellular susceptibility to drug-induced proliferation arrest and apoptotic death. This, in addition to its previously described effect on tumor angiogenesis [13, 15], makes tetrac a promising anti-cancer drug candidate.

The initial finding that tetrac exerted equivalent anti-proliferative activity in vitro against drug-sensitive and drug-resistant cells (Fig. 1) suggested that this antagonist can overcome drug resistance. The cellular responses to doxorubicin, etoposide, cisplatin, and TSA were significantly enhanced when these drugs were combined with tetrac (Fig. 2). However, since the mechanisms involved in resistance to these agents are not necessarily similar, it is suggested that tetrac may act by regulating more than one drug resistance pathway. To address this possibility, first we have studied the effect of tetrac on expression of P-glycoprotein, SOD, and GST- π and found that expression of none of these genes was significantly altered (Fig 3). Others have shown that agonist thyroid hormone can increase expression of P-gp [26, 27]. Tetrac, at the concentrations used, exerts its thyroid hormone antagonist activity primarily at the integrin receptor. Our results

indicate that P-gp gene expression is not modulated from the cell surface integrin receptor.

However, analysis of drug transport revealed that intracellular accumulation of radiolabeled doxorubicin increased significantly in the presence of tetrac as compared to non-treated cells (Fig. 3c) and suggested that tetrac may act as an inhibitor of P-gp activity. In light of the previous observation that thyroid hormones are able to bind to $\alpha v\beta 3$ integrin [8] and to P-gp [28], the finding that tetrac inhibits their binding to integrins raises the possibility that tetrac may also interfere with their binding to P-gp. Using the same logic, tetrac may also compete with drugs for the binding to P-gp and thus, disrupt the efficacy of this transporter. Whatever the mechanism, our finding that drug export was inhibited by tetrac is of a fundamental importance as it sheds light on the critical role of hormone homeostasis in the regulation of cancer response to chemotherapy.

Although this finding may represent a significant advancement in understanding the mechanism by which tetrac reverses drug resistance, it does not explain why this antagonist enhances cellular response to cisplatin, a non-p-gp substrate (Fig. 2). Since accumulating evidence indicates that cellular ability to undergo senescence or apoptotic death play key roles in chemotherapy outcome [29–32], we have tested the effect of tetrac on the corresponding pathways. As shown in Fig. 4, cellular ability to undergo doxorubicin-induced proliferation arrest (enhanced expression of p21/WAF1) [33, 34], SA- β -Gal [35], and cell death (caspase-3 activation and chromatin condensation) were dramatically enhanced upon exposure to tetrac. This evidence suggests that forcing cancer cells into senescence or apoptosis may represent additional mechanisms by which tetrac reverses drug resistance. With regard to this, preliminary studies have shown that increased expression of pro-apoptotic genes such as *bak* and *bax* were associated with increased response to chemotherapeutic agents in cancer of hematopoietic origins. However, in most solid tumors, a clear relationship between apoptosis and cellular response to chemotherapy was not established. In contrast, cellular ability to undergo senescence was found recently to be associated with treatment outcome of these tumors [29–32]. We found that tetrac regulates both processes as well as the mechanisms that regulate drug transport; therefore this antagonist may have a broad use for the treatment of aggressive cancers of both hematopoietic and solid origins. An example for this is provided by the *in vivo* data showing that tetrac is effective in suppressing the growth of drug-resistant tumors in nude mice (Fig. 5) without any noticeable toxic effect. Since the concentration of tetrac used in this experiment (30 mg/kg \times 3) demonstrated a relatively strong anti-tumor response of tetrac alone, it was not possible in this

case to determine whether a synergy exists between this compound and doxorubicin. Further optimization studies are ongoing to define the relative dosages of tetrac and doxorubicin required to elicit a synergistic effect in suppressing cancer resistance to chemotherapy *in vivo*.

Overall, these findings provided evidence that tetrac has anti-tumor activity *in vitro* and *in vivo*. By itself, this hormone analog is capable of suppressing proliferation of cancer cell lines known for resistance to conventional therapies. This, in addition to the fact that tetrac is also capable of suppressing angiogenesis and is non-toxic, make this hormone antagonist a compelling tool for the treatment of aggressive cancers.

Acknowledgments This work was supported in part by the Charitable Leadership Foundation and the Medical Technology Acceleration Program (to SA. Mousa and PJ Davis), the Pharmaceutical Research Institute at Albany College of Pharmacy (to SA. Mousa), and the Children's Memorial Research Center (to A. Rebbaa).

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