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New approaches to the understanding of tamoxifen action and resistance

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Abstract

Tamoxifen (TAM) provides an effective agent for treatment of hormone-dependent breast cancer but resistance uniformly ensues upon continued use. Additional studies are required to define more precisely the mechanisms involved in development of resistance. We conducted systematic experimental and clinical studies based on the hypothesis that tumors exposed to TAM long-term may develop resistance by becoming hypersensitive to its estrogenic effects. These investigations uncovered new features of the TAM resistance (TR) phenomenon and identified possible means for its prevention and/or elimination. Initially we confirmed that TR may be divided into two subtypes, primary and acquired resistance, and that these differ by certain important characteristics including the level of the possible involvement of adaptive and genetic components. Then we distinguished at least three consequent stages of this phenomenon: stage I when TAM behaves as an antiestrogen, stage II with development of increased sensitivity to the agonistic (pro-estrogenic) properties of TAM and stage III with an adaptive increase in sensitivity to estradiol (E2). During this evolutionary process, as shown in vitro, MAP kinase (MAPK) and aromatase activities increase. The time frame of the increase in MAPK activity as a rule outpaces the increase in aromatase activity during the course of the development of TR. This may occur as a response to estrogen deprivation or interruption of the process of estrogen signaling and can be one of the promoting factors of increased aromatase activation. On the other hand, the chronology of these events indicates that changes in the MAPK cascade can be more important for the early steps of the development and maintenance of the TR state. Changes in local estrogen production/sensitivity to E2 are perhaps essential for the later steps of this phenomenon.

We have explored the use of a growth factor-blocking agent to abrogate the adaptive changes in sensitivity. Farnesylthiosalicylic acid (FTS), an inhibitor of GTP-Ras binding to its membrane acceptor site, reduces the increase in the number of MCF-7 cells induced by long-term TAM treatment. It also decreases MAPK activity in TAM-treated MCF-7 cells and in established TR cell lines. Alone or in combination with letrozole (presumably, through the influence on MAPK pathway) FTS exerts moderate inhibitory effects on aromatase activity in estrogen-deprived or estrogen-exposed MCF-7 cells. Taken together, our observations suggest that FTS is a 'candidate drug' for the treatment of TR. Both the adaptive and genetic types of resistance may be amenable to this approach. Our studies underline the possible importance of starting the treatment/prevention of TR early on. From our clinical studies using immunohistochemistry, there is a rather strong rationale to include as a predisposing factor in the development of TR the increase in MAPK and aromatase activities in human primary breast tumors.

In summary, data obtained during the course of this project may be considered as evidence supporting the principle that processes resulting in responses to TAM as an agonist and the development of estrogen hypersensitivity of breast cancer cells could potentially be mechanistically linked.

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Berstein et al.: MAP kinase and aromatase in development of tamoxifen resistance

Introduction
Tamoxifen (TAM) represents a time-proven, effective, targeted therapy for breast cancer. One keen observer commented that 'Tamoxifen is one of the most effective and least toxic medical treatments discovered so far for any form of cancer' (Gray 1993). Nevertheless, after long-term treatment with this agent (Osborne et al. 1987, Gottardis & Jordan 1988, Ali & Coombes 2002) TAM paradoxically stimulates breast tumor growth in model systems and in patients. The mechanism to explain the growth-promoting effect of TAM is unknown but could involve one of the many adaptive processes which constitute TAM resistance (TR). The latter phenomenon is poorly understood at the present time, although considerable effort has attempted to elucidate its nature (MacGregor & Jordan 1998, Clarke et al. 2001). Suggested mechanisms include changes in estrogen receptor structure and function, post-receptor reactions and modifications, pharmacokinetics of antiestrogen, plasma membrane effects, and host endocrine status (Johnston 1997, Katzenellenbogen et al. 1997, Clarke et al. 2001). The majority of these ideas – although rather well-accepted initially – have not been convincingly supported by evolving evidence. Thus, additional studies are required to define more precisely the mechanisms involved in TR.

A more recent hypothesis regarding TR is that adaptive processes involving cross-talk between receptor signaling and growth factor pathways may be responsible. This process may parallel adaptive changes induced by exposure of breast cancer cells to a low-estrogen environment. Recently conducted experimental studies convincingly demonstrated that in breast cancer cells subjected to long-term estrogen deprivation (LTED), hypersensitivity to estradiol (E2) develops. Activation of MAP kinase (MAPK) renders cells more sensitive to the proliferative effects of estrogen (Masamura et al. 1995, Shim et al. 2000, Santen et al. 2001). In addition, 'adaptive' increases in aromatase activity occur in LTED cells through as yet unknown mechanisms (Yue et al. 1999, Santen et al. 2001).

The studies reported in this manuscript are based on the hypothesis that tumors exposed to TAM long-term may become hypersensitive to the estrogenic effects of TAM as well as to E2. Accordingly, processes resulting in responses to TAM as an agonist and the development of estrogen hypersensitivity of breast cancer cells could potentially be mechanistically linked (Santen 1996). We postulated that these linked mechanisms may result from activation of the MAPK cascade and could be influenced by modification of aromatase activity. With these concepts as a background, we performed systematic experimental and clinical studies to further understand the TR phenomenon and to identify possible means for its prevention and/or elimination. The results of these studies have led to the hypothesis that priming with aromatase inhibitors combined with Ras-MAPK blockade might prevent TAM failure and re-growth of breast cancer. These concepts suggest a practical new treatment strategy that could direct future clinical trials.

Materials and methods
The studies reported herein can be divided into four separate categories.

I. Effects of TAM in vitro
Wild-type MCF-7 cells were grown in IMEM with 5% fetal calf serum or in IMEM without phenol red with 5% dextrancoated charcoal (DCC)-stripped serum for 24 h, 1 week and 1, 3, 4, 5 and 6 months in the absence or in the presence of 10^{-7} M TAM. We quantitated cell number without or with E2 at concentrations of 10^{-8} and 10^{-11} M (Masamura et al. 1995). We also performed Western blots of MAPK (Jeng et al. 2000) and measured aromatase with the titrated water assay (Yue et al. 1999) at the time intervals indicated above.

II. In vitro studies with TR cell lines
Established TR cell lines (TAM-R-1, TAM-R-7, TAM-R-8), developed by Dr A Lykkesfeldt (Lykkesfeldt & Briand 1986, Madsen et al. 1997), were used together with their respective parental MCF-7 line. TAM-R lines were grown without TAM or after treatment with 10^{-9} M TAM during the previous 4–11 weeks. Methodological approaches similar to those mentioned in part I were utilized. In addition, in parts I and II, we utilized farnesylthiosalicylic acid (FTS, 50 or 10 \mu M), the compound that interferes with cell membrane binding of activated Ras (GTP-Ras) and with further signaling through the Raf, MEK and MAPK pathway (Kloog & Cox 2000). This compound was used separately or in combination with the aromatase inhibitor letrozole (10^{-7} M) to study effects on cell number, MAPK and aromatase activity. In the latter case, aromatase-transfected MCF-7 cells, or S Chen’s cells (Yue et al. 1999) were used as well.

III. Effects of TAM in vivo in tumor xenografts
Wild-type MCF-7 cells were implanted as xenografts in oophorectomized nude mice (Charles River Labs) aged 4–5 weeks at the start of experiment (Shim et al. 2000). Simultaneously with the transplantation of tumor cells, silastic capsules containing 2 mg E2 were inserted and left in for 4 weeks to allow tumors to be established (‘tumor establishment phase’). After 4 weeks of estrogen exposure, the pellets were removed and animals were divided into two groups for observation during phase I. The first group was given TAM via free access
In animals remaining alive after the 5 month time point, TAM and CHOL capsules were removed and each of these two groups was further divided into three subgroups (phase II). One subgroup received vehicle (CHOL), another E2 ‘clamped’ at a dose corresponding to plasma concentration at 1.25 pg/ml level (E2/CHOL ratio 1:319) and the third E2 equivalent to 20 pg/ml concentration (E2/CHOL ratio 1:19) (Shim et al. 2000). Tumor length and width were measured weekly in these animals during the next 7 weeks. By the end of the experiment, all animals were killed, and tumors and uteruses weighed. In aliquots of tumor material collected at the 1 and 5 month time points, aromatase (tritiated water assay) (Yue et al. 1999) and activated MAPK (immunocytochemistry with specific primary antibody) activities (Mandell et al. 1998, Shim et al. 2000) were determined. All animal experiments were conducted under Federal and Institutional guidelines and approved by the University of Virginia animal care and use committee.

IV. Immunocytochemical study of clinical material

Sections of human breast tumors (kindly provided by Drs M Dowsett and W R Miller, UK) were used. Material was received and evaluated in a paired mode (the same patient before and after treatment). Two groups of patients were represented, those exhibiting primary or de novo TR and those with secondary resistance. Paired samples from the primary resistance group included the initial mammary carcinoma biopsy and tumors excised after non-response to 3–4 months of neo-adjuvant TAM treatment (group of W Miller, n = 11 patients). The pairs reflecting secondary resistance included the initial tumor biopsy and tumor tissue obtained upon relapse after a long course of adjuvant TAM therapy (M Dowsett’s group, n = 38 patients). Patients were treated before clinical appearance of relapse on average for 33.6 ± 4.5 months. Aromatase (Santen et al. 1994) and activated MAPK (only in cases of primary TR; antibody from Cell Signaling Technology, Inc.) activities were evaluated in this material with immunocytochemical analysis (Santen et al. 1994, Mandell et al. 1998). Throughout all parts of the study appropriate methods of statistical evaluation of the data were utilized.

Results

In vitro studies with wild-type breast cancer cells

An increase in growth rate of wild-type MCF-7 cells pretreated with TAM (‘basal fraction’) was demonstrated starting from 1 month and continuing until the 5th month (Fig. 1). A modest additional increase in growth rate of these cells was observed in response to E2 when compared with cells not pretreated with TAM. This was observed primarily at the later time periods between 3.5 and 5 months (data not shown). In wild-type MCF-7 cells treated with TAM, aromatase activity gradually increased by 3–4 months, especially when grown under estrogen-deprived conditions. Between the 1st and 4th month time points, MAPK activity tended to be higher (not
statistically significant, data not shown) than in cells grown without TAM. The time frame of the increase in MAPK activity outpaced that for aromatase activity (Fig. 2).

**Studies with established TR cell lines**

In TR lines, MAPK activity was increased (in comparison with the parental line) in TAM-R-1 and TAM-R-8 cells largely if they were not pretreated with TAM (Fig. 3). Aromatase activity in TAM-R-7 and TAM-R-8 cells was found to be lower than in parental cells, and in TAM-R-1 was almost equal to the values in parental line (not shown).

**Effects of FTS**

The inhibiting effect of FTS on cell number under estrogen-deprived conditions (DCC serum medium) was demonstrated to be higher than in IMEM, especially in the presence of TAM (Fig. 4). MAPK activity was inhibited by FTS after 24 h treatment both in TAM pretreated wild-type MCF-7 cells (not shown) and in established TR cell lines (Fig. 5). The combination of FTS and the aromatase inhibitor letrozole was more effective than FTS and/or letrozole alone when taking MAPK (not shown) and aromatase (Fig. 6) activities as the endpoints.

**Xenograft studies in mice receiving TAM or CHOL**

During phase I of this experiment (i.e. after the tumor establishment phase), regression of the growth of tumor xenografts in animals receiving TAM occurred to a lesser extent than in the CHOL group (Fig. 7). This finding corresponds to the observations of others (Osborne et al. 1987, Gottardis & Jordan 1988). As detailed in the Materials and methods section, the CHOL or TAM implants were removed at the end of phase 1 (i.e. after 5 months). Phase II of the protocol then assessed the degree of sensitivity to exogenous
Figure 4  Effect of FTS on cell number in MCF-7 cell lines. Cells that were pretreated or not with $10^{-7}$ M TAM (T) during 3 months were used. For type of experiment and information about IMEM and DCC serum see description for Fig. 1. FTS was used at a concentration of 50 $\mu$M during the last 5 days; E$_2$ was also used during 5 days in concentrations of $10^{-11}$ M (E2-11) and $10^{-8}$ M (E2-8). CTL, control.

Figure 5  Effect of FTS on MAPK activity in established TR cells. The cell lines TAM-R-1, TAM-R-7 and TAM-R-8 developed by Dr A Lykkesfeldt from their parent MCF-7 cell line (P) and designated on the figure as 1, 7 and 8 were pretreated (T) or not pretreated with TAM ($10^{-6}$ M) during 11–12 weeks. FTS (F) was used during the last 24 h of the experiment in a concentration of 10 $\mu$M. MAPK activity was evaluated by Western blot (see Figs 3 and 4). A/T, ratio of active MAPK to total MAPK activity.
Figure 6 Effect of FTS and the aromatase inhibitor letrozole on aromatase activity (fmol/mg protein per h) in wild-type MCF-7 cells (a, b; two different experiments) and in aromatase-transfected MCF-7 cells (c). Wild-type MCF-7 cells kept in estrogen-deprived medium (DCC serum) during 6.5–7 months as well as aromatase-transfected MCF-7 cells (Yue et al. 1999) were seeded into six-well plates. Treatment of cells with FTS (50 µM) or letrozole (10⁻⁷ M) (Letro) or both (F + L) was performed during 4–5 days. Aromatase activity was evaluated by tritiated water release assay (see Materials and methods) (c) error bars represent S.E.M.

E2. Minimal growth occurred in animals in either group receiving vehicle or very low doses of E2 (1.25 pg/ml). Most importantly, tumors exposed to TAM during phase I responded to the subsequent 20 pg/ml E2 treatment with increased growth rate whereas the phase I vehicle group did not. The relative E2-induced increase in tumor size and weight in animals previously exposed to TAM in phase I was substantially higher during phase II than the uterine weight increase in the same animals (Table 1). Thus the enhanced sensitivity to E2 appeared to be selective for the breast tumor tissue and not for uterus.

Aromatase activity in tumor xenografts exhibited a tendency to increase slightly after 1 month of treatment with TAM but this did not persist at the 5 month time point. No statistically significant inter-group (TAM vs CHOL) differences were demonstrated in MAPK activity of tumor xenografts, although in ‘TAM-5 month’ samples this activity was lower than in ‘TAM-1 month xenografts’, P < 0.05 (Table 2).

Studies on specimens from patients with primary or secondary TR

Pairs of samples reflecting primary resistance

These observations involved patients with primary TR as evidenced by lack of tumor size reduction in response to neoadjuvant TAM treatment. In this group, we observed a
Table 1  Tumor and uterine weight (means ± S.E.M.) in mice at the end of experiment. E2 was clamped on two different blood concentrations (1.25 and 20 pg/ml).

<table>
<thead>
<tr>
<th>Weight (mg)</th>
<th>Vehicle CHOL</th>
<th>TAM</th>
<th>E2 (1.25 pg/ml) CHOL</th>
<th>TAM</th>
<th>E2 (20 pg/ml) CHOL</th>
<th>TAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.6 ± 5.0</td>
<td>39.0 ± 11.6</td>
<td>23.4 ± 3.4</td>
<td>57.3 ± 21.1</td>
<td>39.5 ± 16.1</td>
<td>160.5 ± 81.2</td>
</tr>
<tr>
<td>Uterus</td>
<td>18.7 ± 2.6</td>
<td>12.6 ± 0.85</td>
<td>114.3 ± 9.2*</td>
<td>76.6 ± 14.1*</td>
<td>151.8 ± 18.2*</td>
<td>69.7 ± 5.5*</td>
</tr>
</tbody>
</table>

*Difference between placebo and TAM groups is significant (P < 0.05). n = 5–7 per group.

Figure 7  Tumor growth dynamics in placebo (CHOL)- and TAM-treated groups. Data are presented as tumor volumes (mm³, vertical axis) starting from the moment of deletion of initial E2 supplementation and until the end of the 5 month period, when CHOL and TAM were deleted and replaced with either vehicle or E2 (1.25 pg/ml or 20 pg/ml) capsules. IMPL, implantation of tumor cells.

Table 2  MAPK staining and aromatase activity (means ± S.E.M.) in MCF-7 xenograft tissue of mice treated during 1 or 5 months with TAM or CHOL capsules.

<table>
<thead>
<tr>
<th>Group</th>
<th>MAPK Stained Score (fmol/mg protein per h)</th>
<th>Aromatase Epithelial Stroma Vessel Fat Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHOL, 1 month</td>
<td>32.5 ± 10.8</td>
<td>65.0 ± 13.1</td>
</tr>
<tr>
<td>TAM, 1 month</td>
<td>13.8 ± 1.6</td>
<td>41.7 ± 9.7</td>
</tr>
</tbody>
</table>

Difference between TAM1 and 5 month groups is significant (P < 0.05).

Pairs of samples reflecting secondary resistance

These observations involved 38 women treated long-term with TAM as adjuvant therapy who eventually relapsed. No differences in aromatase staining between primary tumors and tumor relapses were demonstrated according to the average data (not shown). However, subgroups with decrease (47.4% of all cases), increase (23.7%) and no change (28.9%) in aromatase values by the time of relapse could be distinguished. We then examined the pre- and post-treatment estrogen receptor immunochemistry and correlated this parameter with aromatase staining in the group with ‘increase or no change in aromatase’ (group A) and in the group with ‘aromatase decrease’ (group B). Interestingly, in group A estrogen receptor-positive frequency was 57.8 ± 11.3% prior to treatment, while in group B it was 70.5 ± 9.2% after treatment, demonstrating the inverse dynamics of these parameters.

Table 3  Results of MAPK and aromatase staining (means ± S.E.M.) of tumours in the groups of breast cancer patients receiving neoadjuvant therapy with tamoxifen.

<table>
<thead>
<tr>
<th>Group</th>
<th>MAPK Stained Score</th>
<th>Aromatase Stained Epithelial Stroma Vessel Fat Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (before</td>
<td>25.2 ± 2.8</td>
<td>25.1 ± 2.5</td>
</tr>
<tr>
<td>treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (after</td>
<td>13.5 ± 1.8</td>
<td>19.1 ± 1.6</td>
</tr>
<tr>
<td>treatment</td>
<td>(P = 0.002)*</td>
<td></td>
</tr>
</tbody>
</table>

*Difference is given for corresponding A and B values. n = breast cancer patients, each with before and after treatment slide.
to treatment and $47.4 \pm 11.4\%$, $P > 0.2$, during relapse. In group B, the corresponding frequencies were $88.8 \pm 7.4\%$ and $50.0 \pm 11.7\%, P < 0.05$. Thus, a decrease in estrogen receptor-positive frequency was observed only in the group with 'aromatase decrease'.

**Discussion**

TAM provides an effective therapy for women with hormone-dependent cancer in the neo-adjuvant, adjuvant and advanced disease settings (Fisher et al. 1996). However, with long duration of TAM use, patients ultimately relapse and may experience tumor stimulation due to its estrogen agonistic properties (Horwitz 1995, Santen 1996). In this situation, further hormonal treatment often induces additional tumor regressions (Santen & Harvey 1999). It has been suggested that tumors adapt to the initial hormonal manipulation by developing enhanced sensitivity to $E_2$. Under these conditions, secondary hormonal treatment leads to tumor regression by further lowering of $E_2$ or by more effective blockade of its action (Masamura et al. 1995, Santen et al. 2001).

While $E_2$ deprivation induces hypersensitivity in women following oophorectomy and in model systems, no prior studies examined whether blockade of its estrogen action with TAM could induce hypersensitivity to $E_2$. Accordingly, this study systematically examined the MAPK cascade, aromatase activity, and responsiveness to estrogen in a variety of experimental systems. In addition, the possible association of MAPK and aromatase up-regulation with the re-growth phenomenon led us to use the Ras antagonist FTS as a means to prevent development of TR. FTS dislodges activated Ras (GTP-Ras) from its cell membrane acceptor protein, galectin 1, and interferes with its further down-stream signaling through the Raf, MEK and MAPK pathway (Marom et al. 1995, Kloog & Cox 2000). In studies conducted by us, FTS was used separately or in combination with the aromatase inhibitor, letrozole.

In our studies, we could demonstrate differences in local estrogen biosynthesis and MAPK signaling both in cells exposed to TAM for several months and in established TR clones of MCF-7 cells. The former are thought to have previously undergone epigenetic adaptive changes and the latter may have genetic changes which induce TR. Specifically, we found that estrogen-deprived wild-type cells treated with TAM are more predisposed to the eventual increase in MAPK and aromatase activity (compared with respective controls) than cells of TAM-resistant lines. We could not demonstrate any increase in aromatase activity in the established TR cells. These observations highlight the fact that different mechanisms for TR are likely and may involve adaptive changes as well as ‘antiestrogen resistance genes’. Perhaps breast tumors in carriers of so called ‘antiestrogen resistance genes’ (Dorssers et al. 2001) have a lesser ability to modulate MAPK and aromatase as adaptive processes than those with ‘adaptive TR’.

Our in vitro studies demonstrated the ability of TAM to change the properties of MCF-7 cells. The stimulating effect of pretreatment with TAM on cell number under basal conditions at the 1–5 month time points was evident (Fig. 1). This results from TAM’s effect on cell proliferation and not on apoptosis (our unpublished observations using an ELISA assay for apoptosis). In the presence of $E_2$, the absolute number of cells pretreated with TAM was relatively higher only at the 5 month time point and only in cells exposed to IMEM (estrogen non-deprived medium) in comparison with samples without TAM pretreatment. Thus, in this in vitro model, it appears that the stage of increased sensitivity to the agonistic effect of TAM precedes the stage with higher sensitivity to $E_2$ and is more intensive than the latter.

The transient increase of MAPK activity observed in estrogen-deprived wild-type MCF-7 cells (TAM-treated or not) may be considered as an important impetus for subsequent adaptive biochemical changes. The temporary character of this increase may be explained by the time-dependent nature of this phenomenon and somewhat reflects the results observed in the clinical material (see below). The observed in vitro dynamics of MAPK activity may be the result of summation of the effects of estrogen deprivation, estrogen signaling interruption by TAM and the direct and rather controversial action of the TAM itself on MAPK (Duh et al. 1997, Berstein et al. 2002). The observation that an increase in MAPK activity precedes the increase in aromatase activity (Fig. 2) mechanistically is rather important and may be – according to our opinion and to some existing observations (Shozu et al. 2001) – one of the causes of the latter.

The effects of FTS demonstrated in MCF-7 cells (Figs 4–6) suggest that treatment with this compound should be started before signs of TR appear. As shown, this agent may be used in ‘genetic’ as well as in ‘adaptive’ types of TR. The data suggest that the action of the FTS presumably includes inhibition of aromatase activity (perhaps due to interference with the MAPK-mediated pathway; see also above).

As was mentioned in the Results section, partial agonistic effects of TAM were seen in the breast cancer xenograft model from the very start of the experiment (Fig. 7). This result has been previously reported by others (Osborne et al. 1987, Gottardis & Jordan 1988) and is manifested by a lesser degree of tumor regression in the TAM as opposed to the vehicle castration group. Tumors still regress under these conditions and consequently cannot be considered with confidence to have primary resistance. It is likely that the antiestrogenic properties of TAM are partially counterbalanced by weak agonistic effects during this phase. With continued exposure, TAM developed full agonistic properties and tumors re-grew. According to our data after short-term
(1 month) TAM treatment, MAPK activity was higher in tumor xenografts than after long-term (5 month) treatment (Table 2), suggesting the importance of early changes in this activity for the subsequent development of hypersensitivity to E\textsubscript{2} and resistance to TAM.

One of the primary aims of this study was to examine the effects of TAM on the level of sensitivity to E\textsubscript{2}. We clearly showed that this takes place in tumor xenografts exposed to TAM for 5 months. Rather low doses of E\textsubscript{2} (equivalent to 20 pg/ml in plasma) stimulate the growth of these TAM-treated tumors but not those previously exposed to vehicle. This increase in sensitivity appears to be specific for breast tumors since the uterus appeared more sensitive to E\textsubscript{2} in the vehicle-treated animals. Notably, hypersensitivity in response to estrogen was more evident in MCF-7 xenografts treated with TAM in vivo (Table 1 and performed with Dr Mark Conaway, analysis of individual slopes of tumor growth; LM Bernstein, J-P Wang, H Zeng, W Yue, M Conway & RJ Santen, data not shown) than in cells exposed to TAM in vitro. Perhaps long-term exposure to TAM is required in vitro to acquire the hypersensitive phenotype. We are continuing the in vitro exposure to TAM in order to assess this possibility.

We were not able to detect gradual increases in activation of MAPK or aromatase in human breast cancers unsuccessfully treated with TAM nor in tissue of tumor xenografts in nude mice by the end of TAM treatment. It should be mentioned that TAM has not been considered so far among factors influencing aromatase activity (Dowsett et al. 1993, Miller & Mullen 1993). It remains unknown whether and how the process of local estrogen production in breast cancer cells is involved in the first steps and further maintenance of TR. However, this remains an area for further investigation as suggested by several trends reflected by our data. For example, we observed a time-dependent increase of aromatase activity in association with TAM administration in wild-type MCF-7 cells or in 1 month TAM-treated xenografts. In addition, there was an association between shifts in aromatase and in tumor pathology (neo-adjuvant course) and between changes in aromatase and tumor estrogen receptor content (adjuvant course).

Several recent publications have described an ‘interrelationship’ between TAM exposure and MAPK activity (Donovan et al. 2001, Gee et al. 2001, Adeyinka et al. 2002, Atanaskova et al. 2002, Rabenoelina et al. 2002). As demonstrated in the present investigation, MAPK activity is higher in estrogen-deprived MCF-7 cells (which reproduces results of Jeng et al. 2000) as well as in the same cells treated with TAM (at least temporarily) and in established TAM-resistant cell lines (Fig. 3). Additionally this activity is higher in tumor xenografts in mice treated with TAM during 1 month (vs 5 months) and in primary human breast cancers before unsuccessful neo-adjuvant TAM treatment than after such courses (Tables 2 and 3). Based upon a summary of our own and published data, we suggest together with Rabenoelina et al. (2002) that MAPK activation might be involved in early steps during progressive reshaping of the cells toward a TR phenotype. Such reshaping may be the result of cellular adaptation that induces (after a stage of initial sensitivity to antiestrogenic effect of TAM) increased reaction to the agonistic action of the drug and finally to a growth-stimulating effect of estrogens.

In conclusion, our studies confirmed the complex nature of TR. The major findings of this investigation can be presented here in the following way. Two known types of TR exist: primary and acquired. These differ by certain important characteristics, which probably include the level of the involvement of adaptive and genetic components. Based upon our findings, at least three consequent stages of TR can be distinguished. These stages can be designated as stage I (when TAM behaves as an antiestrogen), stage II with increased sensitivity to agonistic (prooestrogenic) TAM effects and stage III with an adaptive increase in sensitivity to E\textsubscript{2}.

The time frame of the increase in MAPK activity in the course of the development of TR as a rule outpaces the increase in aromatase activity and can be one of the promoting factors for the latter. On the other hand, the chronology of these events indicates that changes in the MAPK cascade can be more important for the early steps of the development and maintenance of the TR state. Perhaps changes in local estrogen production/sensitivity to E\textsubscript{2} are essential for relatively later steps of this phenomenon.

FTS reduces the increase in number of MCF-7 cells induced by long-term TAM treatment. It also decreases MAPK activity in TAM-treated MCF-7 cells and in established TR cell lines. Separately or in combination with letrozole (presumably, through the influence on MAPK pathway) FTS moderately inhibits aromatase activity of estrogen-deprived or estrogen-enriched MCF-7 cells. Altogether, our observations suggest that FTS is a ‘candidate drug’ for the treatment of TR of both adaptive and genetic types.

Results from these studies suggest the possible importance of starting the treatment/prevention of TR early on. In addition, our data point to the need to find additional criteria predisposing to development of the resistant state. There is a rather strong rationale to include as a predisposing factor the increase in MAPK and aromatase activities in primary breast tumors. Both are directly or indirectly associated with sensitivity of tumor cells to E\textsubscript{2} and with genomic/non-genomic estrogenic effects (Santen et al. 2001, Berstein et al. 2002, Zhang et al. 2002).

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