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Metabolic and Antiproliferative Consequences of Activated Polyamine Catabolism in LNCaP Prostate Carcinoma Cells*

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Depletion of intracellular polyamine pools invariably inhibits cell growth. Although this is usually accomplished by inhibiting polyamine biosynthesis, we reasoned that this might be more effectively achieved by activation of polyamine catabolism at the level of spermidine/spermine N^1-acetyltransferase (SSAT); a strategy first validated in MCF-7 breast carcinoma cells. We now examine the possibility that, due to unique aspects of polyamine homeostasis in the prostate gland, tumor cells derived from it may be particularly sensitive to activated polyamine catabolism. Thus, SSAT was conditionally overexpressed in LNCaP prostate carcinoma cells via a tetracycline-regulatable (Tet-off) system. Tetracycline removal resulted in a rapid ~10-fold increase in SSAT mRNA and an increase of ~20-fold in enzyme activity. SSAT products N^1-acetylspermidine, N^1-acetylspermine, and N^1,N^{12}-diacetylspermine accumulated intracellularly and extracellularly. SSAT induction also led to a growth inhibition that was not accompanied by polyamine pool depletion as it was in MCF-7 cells. Rather, intracellular spermidine and spermine pools were maintained at or above control levels by a robust compensatory increase in ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities. This, in turn, gave rise to a high rate of metabolic flux through both the biosynthetic and catabolic arms of polyamine metabolism. Treatment with the biosynthesis inhibitor α-difluoromethylornithine during tetracycline removal interrupted flux and prevented growth inhibition. Thus, flux-induced growth inhibition appears to derive from overaccumulation of metabolic products and/or from depletion of metabolic precursors. Metabolic effects that were not excluded as possible contributing factors include high levels of putrescine and acetylated polyamines, a 50% reduction in *S*-adenosylmethionine, and a 45% decline in the SSAT cofactor acetyl-CoA. Overall, the study demonstrates that activation of polyamine catabolism in LNCaP cells elicits a compensatory increase in polyamine biosynthesis and downstream metabolic events that culminate in growth inhibition.

Cell growth is dependent on a sustained supply of polyamines, which is typically met by the integrated contributions of biosynthesis, catabolism, uptake, and export, each of which is sensitively regulated by effector molecules that, in turn, are controlled by intracellular polyamine pools (1). Thus, ornithine decarboxylase (ODC)[1] and *S*-adenosylmethionine decarboxylase (SAMDC) control biosynthesis, a polyamine transport system modulates uptake, and spermidine/spermine N^1-acetyltransferase (SSAT) regulates polyamine catabolism and export out of the cell. Neoplastic cell growth is associated with elevated polyamine biosynthetic activity, even when the surrounding normal tissue itself is rapidly proliferating, such as the intestinal mucosa (2–4). Thus, the rationale for targeting polyamines in antitumor strategies relates to their critical role in supporting neoplastic cell growth and to the overexpression of biosynthetic enzymes in tumor *versus* normal tissues (3, 5, 6).

The biology and metabolism of polyamines in the prostate is distinctly different from that of other tissues. In addition to synthesizing these molecules for epithelial cell replacement, the gland produces massive quantities of spermine (Spm) for export into reproductive fluids (7–10). The only major tissue that synthesizes polyamines for export, the prostate, and presumably tumors derived from it, may be dependent on novel and therapeutically exploitable homeostatic mechanisms. For example, we have observed that, in contrast to other cell lines, two of three prostate carcinoma lines failed to regulate polyamine transport in response to polyamine analogues or inhibitors (11). Very recently, Rhodes *et al.* (12) performed a meta-analysis of four independent microarray datasets comparing gene expression profiles of benign *versus* malignant patient prostate samples (12). Their study showed that polyamine biosynthesis was the most consistently and significantly affected metabolic, signaling, or apoptotic pathway. More particularly, the study revealed a synchronous network of genes contributing to polyamine biosynthesis was up-regulated while genes detracting from polyamine biosynthesis were down-regulated. In support of these findings, clinical studies by Bettuzzi *et al.* (13) indicate a significant increase in transcripts of the polyamine biosynthetic enzymes, ODC and SAMDC, in human prostatic cancer relative to benign hyperplasia.

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[1] The abbreviations used are: ODC, ornithine decarboxylase; CoA, coenzyme A; AcSpd, N^1-acetylspermidine; AcSpm, N^1-acetylspermine; dcSAM, decarboxylated *S*-adenosylmethionine, DiAcSpm, N^1,N^{12}-diacetylspermine; DENSPM, N^1,N^{11}-diethylnorspermine; DFMO, α-difluoromethylornithine; HPCE, high performance capillary electrophoresis; HPLC, high performance liquid chromatography; MTA, 5′-methylthioadenosine; PAO, polyamine oxidase; Put, putrescine; SAM, *S*-adenosylmethionine; SAMDC, *S*-adenosylmethionine decarboxylase; Spd, spermidine; Spm, spermine; SSAT, spermidine/spermine N^1-acetyltransferase; Tet, tetracycline; TRAMP, transgenic adenocarcinoma of mouse prostate; tTA, tetracycline-repressible transactivator; dansyl, 5-dimethylaminona-phthalene-1-sulfonyl.

In recognition of the unique physiology of the prostate gland, Heston and collaborators (14, 15) were among the first to propose that targeting polyamine biosynthesis may be particularly effective against prostate cancer. Most of these efforts have made use of known inhibitors of ODC or SAMDC (16–19). Gupta *et al.* (20) showed that the ODC inhibitor, α-difluoromethylornithine (DFMO) effectively suppressed development of prostate cancer in the TRAMP mouse model. As an alternative approach to the use of enzyme inhibition, we propose that disruption of polyamine homeostasis at the level of polyamine catabolism may have unique therapeutic potential against prostate carcinoma. It has been demonstrated, for example, that polyamine analogues such as N^1,N^{11}-diethylnorspermine (DENSPM) down-regulate polyamine biosynthesis at the level of ODC and SAMDC and, at the same time, potently (*i.e.* >200-fold) up-regulate polyamine catabolism at the level of spermidine/spermine N^1-acetyltransferase (SSAT) (1, 21–27). Several lines of evidence support the idea that analogue induction of SSAT and hence, activation of polyamine catabolism, is a critical determinant of DENSPM drug action. For example, DENSPM growth inhibition among tumor cell lines correlates with the extent to which SSAT is induced (23–25), and analogues that differentially induce SSAT inhibit cell growth in a correlative manner (22, 26, 27). As more direct evidence for this relationship, McCloskey *et al.* (28) showed that DENSPM-resistant Chinese hamster ovary cells are unable to induce SSAT. Recently, Chen *et al.* (29, 30) reported that small interference RNA interference with DENSPM induction of SSAT prevented polyamine pool depletion while blocking analogue-induced apoptosis in human melanoma cells.

The studies cited above relate to SSAT induction in the context of analogue treatment, but they do not address what happens when SSAT is selectively induced in cells. In an earlier report (31), we showed that conditional overexpression of SSAT leads to polyamine pool depletion and growth inhibition in MCF-7 breast carcinoma cells. On the basis of rationale suggesting that prostate carcinoma may react differently to perturbations in polyamine homeostasis, we investigated the consequences of conditional SSAT overexpression in LNCaP prostate carcinoma cells.

EXPERIMENTAL PROCEDURES

Materials—The inhibitor of polyamine oxidase (PAO), N^1-methyl-N^2-(2,3-butadienyl)butane-1,4-diamine (MDL-72527) was generously provided by Aventis Pharmaceuticals Inc. (Bridgewater, NJ). The ODC inhibitor DFMO was obtained from Ilex, Inc. (San Antonio, TX). Tetracycline (Tet), aminoguanidine, polyamines, and the acetylated polyamines N^1-acetylspermidine (AcSpd) and N^1-acetylspermine (AcSpm) were purchased from Sigma-Aldrich, whereas N^1,N^{12}-diacetylspermine (DiAcSpm) was provided as a gift from Dr. Nikolaus Seiler (Laboratory of Nutritional Oncology, Institut de Recherche Contre les Cancers, Strasbourg, France). SAM was purchased from Sigma-Aldrich, and the SAM metabolites, decarboxylated *S*-adenosylmethionine (dcSAM) and 5-methylthioadenosine (MTA), were synthesized and kindly provided by Drs. Canio Marasco and Janice Sufrin (Roswell Park Cancer Institute). Radioactive compounds L-[1-^{14}C]ornithine, [acetyl-1-^{14}C] coenzyme A, [α-^{32}P]dCTP were purchased from PerkinElmer Life Sciences, and *S*-adenosyl-L-[*carboxyl*-^{14}C]methionine was obtained Amersham Biosciences. Acetyl-coenzyme A (acetyl-CoA) was purchased from Sigma-Aldrich and solubilized as described by Liu *et al.* (32). Geneticin (G418) and hygromycin B were obtained from Clontech Laboratories, Inc. (Palo Alto, CA) and Invitrogen, respectively.

Cell Culture—LNCaP prostate carcinoma cells engineered to constitutively express the tetracycline-repressible transactivator (tTA) (33), designated LNGK9 (34), were cultured in RPMI 1640 media supplemented with 2 mM glutamine (Invitrogen), 10% Tet-approved fetal bovine serum (Clontech Laboratories, Inc.), penicillin at 100 units/ml, streptomycin at 100 units/ml (Invitrogen), and 150 μg/ml hygromycin B at 37 °C in the presence of humidified 5% CO_2. Aminoguanidine (at 1 mM) was routinely included in the media as an inhibitor of copper-dependent bovine serum amino oxidases to prevent conversion of extracellular polyamines to toxic products. Cells were harvested by trypsinization and counted electronically (Coulter Model ZM, Coulter Electronics, Hialeah, FL).

Transfections—LNGK9 cells expressing the tTA were seeded at 2×10^6 cells per 100-mm culture dishes in the absence of hygromycin B and Tet. The following day, fresh media were replaced and cells were co-transfected with the tTA-responsive pTRE-SSAT plasmid (31) and a G418-resistance selection plasmid pcDNA3 (Invitrogen) at a ratio of 20:1 using FuGENE 6 (Roche Applied Science) according to manufacturer's protocol. Stably transfected clones were selected in medium containing 500 μg/ml antibiotic G418, 150 μg/ml hygromycin B, and 1 μg/ml Tet. Healthy G418-resistant clones were selected and tested for SSAT mRNA by Northern blot analysis in the presence or absence of 1 μg/ml Tet. With the Tet-off system, SSAT transcription is induced in the absence of Tet (−Tet) but not in its presence (+Tet). A Tet concentration of 1 μg/ml was found to fully and consistently suppress SSAT gene expression during routine cell culture passage. Clones that expressed low basal level of SSAT mRNA under +Tet conditions and high induced levels of SSAT mRNA under −Tet conditions were selected for further study. Clones were maintained continuously under 1 μg/ml +Tet until experiments were initiated.

Northern Blot Analysis—Northern blot analysis was carried out as described by Fogel-Petrovic *et al.* (35) with modifications. Briefly, total RNA was extracted with an RNeasy® Mini kit (Qiagen Inc., Valencia, CA), and its concentration was determined by UV spectrophotometry. RNA samples (5 μg/lane) were separated on 1.5% agarose/formaldehyde gels and transferred to a Duralon-UV membrane (Stratagene, La Jolla, CA). The membrane was cross-linked in a Stratalinker™ 1800, hybridized to [^{32}P]dCTP random-labeled cDNA probes (Stratagene) for detection of SSAT mRNA (36), and exposed for autoradiography. A glyceraldehyde-3-phosphate dehydrogenase signal was used as a loading control.

Polyamine Enzymes and Polyamine Pools—SSAT, ODC, and SAMDC activities were assayed as described previously (27, 37). Polyamine enzyme activities were expressed as picomoles of AcSpd generated per minute/mg of protein for SSAT and as nanomoles of CO_2/h/mg of protein for ODC and SAMDC. Intracellular polyamines, including acetylated derivatives of spermidine (Spd) and Spm were extracted from cell pellets with 0.6 N perchloric acid, dansylated, measured by reverse phase high-performance liquid chromatography (HPLC) as described by Kramer *et al.* (38), and expressed as picomoles/10^6 cells. Extracellular polyamines and acetylated polyamines were extracted from media as described by Kramer *et al.* (39), containing fetal bovine serum, Tet, and L-glutamine but not G418 or hygromycin B. A total of 50 μl of dansylated sample was injected for HPLC, and data were collected and analyzed as noted above. Extracellular polyamine pools were expressed as nanomoles/equivalent volume (ml)/10^6 cells.

S-Adenosylmethionine and Metabolite Pools—Intracellular SAM and its metabolites, dcSAM and MTA, were extracted from cell pellets with 0.6 N perchloric acid and measured by HPLC according to chromatographic conditions reported by Yarlett and Bacchi (40) with modifications as described by Kramer *et al.* (38). Briefly, samples (50 μl) were eluted from a C18 column (40 °C) at a flow rate of 0.8 ml/min with a linear gradient starting with solvent A (0.1 M NaH_2PO_4, 8 mM octane sulfonic acid, 0.05 mM EDTA, 2% acetonitrile) at 80% and solvent B (0.15 mM NaH_2PO_4, 8 mM octane sulfonic acid, 26% acetonitrile) at 20%. Over the course of 30 min, the gradient increased to 100% solvent B for 10 min. Effluent was monitored with a Waters 2487 dual wavelength UV detector, and data were processed using instrumentation described for polyamine pool analysis and expressed as picomoles/10^6 cells.

Measurement of Acetyl-CoA—High performance capillary electrophoresis (HPCE) separation and quantitation of acetyl-CoA in biological samples followed the method of Liu *et al.* (32). Cells were lysed and processed by using a solid-phase extraction. Extracts were then analyzed on a Beckman P/ACE MDQ capillary electrophoresis system equipped with a photodiode array detector and an uncoated fused silica CE column of 75-μm inner diameter and 60 cm in length with 50 cm from inlet to the detection window (Polymicro Technologies, Phoenix, AZ). Electrophoretic conditions were according to Liu *et al.* (32) with modifications. Briefly, the capillary was preconditioned with 1 M NaOH and Milli-Q water for 10 min each at 20 p.s.i. and then equilibrated with 100 mM NaH_2PO_4 running buffer containing 0.1% β-cyclodextrin (pH 6.0) for 10 min. After each run, the capillary was rinsed with 1 M NaOH, Milli-Q water, and running buffer for 2 min each. The injection was done hydrodynamically at a pressure of 0.5 p.s.i. for 10 s. Injection volume was calculated using CE Expert Lite software from Beckman. Separation voltage was 15 kV at a constant capillary temperature of 15 °C. To establish the standard calibration curves, solutions contain-

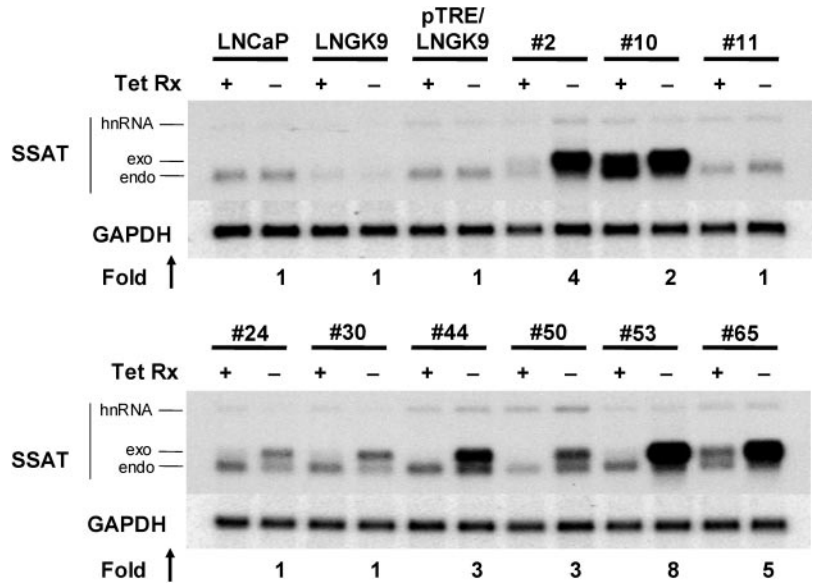

FIG. 1. **Conditional overexpression of SSAT mRNA in LNCaP clones transfected with Tet-regulatable human SSAT cDNA. LNGK9 cells, a subline of LNCaP, were transfected with the Tet-repressible human SSAT plasmid.** Stably transfected clones resistant to neomycin were selected and tested for their responsiveness to Tet by Northern blot analysis. Representative examples from over 100 selected clones were cultured for 48 h in the presence (+) or absence (−) of 1 μg/ml Tet. Note the endogenous (*endo*) and exogenous (*exo*, *i.e.* plasmid transcript) mature SSAT mRNA can be distinguished on the basis of size (*i.e.* ~1.3 *versus* ~1.5 kb, respectively). For quantitation, the exogenous and endogenous SSAT mRNA bands were scanned fluorometrically, normalized to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) signal, and expressed as -fold increase (*Fold* ↑) in −Tet relative to +Tet. Blots are representative of findings from three separate experiments. *hnRNA*, heteronuclear RNA.

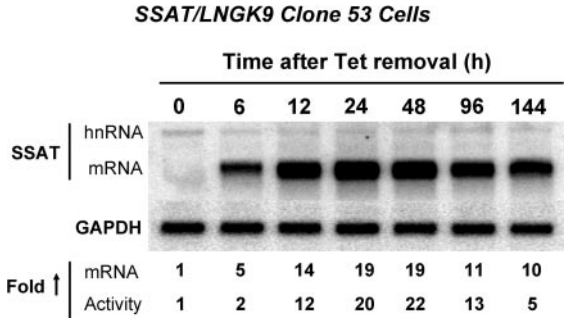

FIG. 2. **Time-dependent increases in SSAT mRNA and activity in SSAT/LNGK9 clone 53 following Tet removal.** Tet was removed for the indicated time, and cells were harvested for total RNA isolation and SSAT enzyme activity. An amount of 5 μg of total RNA was loaded onto each Northern blot lane. Note that following removal of 1 μg/ml Tet, both SSAT mRNA and activity increased rapidly before reaching a plateau between 24 and 48 h. For quantitation, SSAT mRNA bands were scanned fluorometrically, normalized to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) signal, and expressed as -fold increase (*Fold* ↑) relative to +Tet at 0 h (*lane 1*). As expressed by -fold increase, SSAT activity increased in parallel to SSAT mRNA. This blot is representative of findings from three separate experiments. *hnRNA*, heteronuclear RNA.

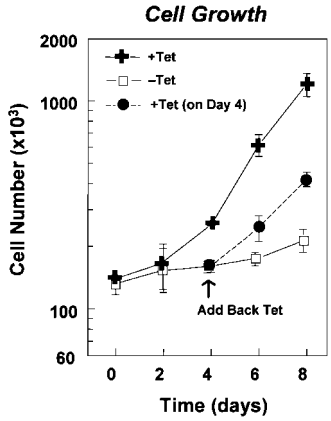

FIG. 3. **Effects of conditional SSAT overexpression on growth kinetics of LNCaP prostate carcinoma cells.** SSAT/LNGK9-clone 53 cells were cultured in the presence (+*Tet*, ⊕) or absence (−*Tet*, □) of 1 μg/ml Tet for the indicated time and collected for growth analysis. Removal of Tet (□) resulted in an inhibition of cell growth over the course of 8 days. Note that addition of 1 μg/ml Tet at 96 h (●) leads to a resumption of cell growth. Data represent means ± S.E., where *n* is 3.

ing the acetyl-CoA and the internal standard (isobutyryl-CoA, 41 nmol) were prepared at concentrations ranging from 1 to 200 nmol. Standards were processed as described above for cell lysates and resuspended in 10 μl of water. The detector response was ($r > 0.99$) for all acetyl-CoA species over the above concentration range. Coenzyme As were monitored with a photodiode array detector at the maximum absorbance wavelength (253.5 nm). Data were collected and processed by using Beckman P/ACE 32 Karat software version 4.0. Cellular acetyl-CoA levels were expressed as nanomoles/10^6 cells.

RESULTS

Derivation of Transfected Cells—Cells transfected with the human SSAT cDNA were selected in neomycin and grown as clones in the presence of 1 μg/ml Tet. Of the 100 SSAT/LNGK9 clones screened (data not shown), those most sensitive to Tet regulation were selected according to the differential expression between SSAT mRNA in +Tet (SSAT-off) *versus* mRNA in −Tet (SSAT-on) (Fig. 1). Based on these criteria, clone 53 was selected for further study, because it displayed low SSAT mRNA in +Tet and an 8-fold increase in total SSAT mRNA (exogenous and endogenous) under −Tet conditions for 48 h. Nearly identical responses were also obtained with several other clones. Transfected human SSAT cDNA (~1.5 kb) was distinguishable from the smaller endogenous transcript (~1.3 kb) by differences in polyadenylation, which became apparent during enzyme induction (35). Although the exogenous 1.5-kb transcript levels increased with Tet removal, the endogenous 1.3-kb transcript levels remained at basal levels, indicating that the presence or absence of the antibiotic did not affect endogenous gene expression. Tet-regulated expression of SSAT mRNA and activity was characterized from 0 to 144 h in clone 53 (Fig. 2). Following Tet removal, SSAT mRNA increased significantly by 6 h and plateaued by 24 h at levels ranging between 10- and 20-fold greater than the 0-h sample. Induction of mRNA was closely paralleled by increases in SSAT activity, which reached a maximum of ~20-fold by 24 h (see Figs. 2 and 4).

Effects of SSAT Overexpression on Cell Growth and Polyamine Metabolism—As shown in Fig. 3, SSAT overexpression caused significant inhibition of cell growth at ~2 days following Tet removal, which was sustained through the 6-day experiment. Growth inhibition appeared to be cytostatic rather than cytotoxic, because there was no obvious decline in cell number as would be expected with apoptosis and because addition of

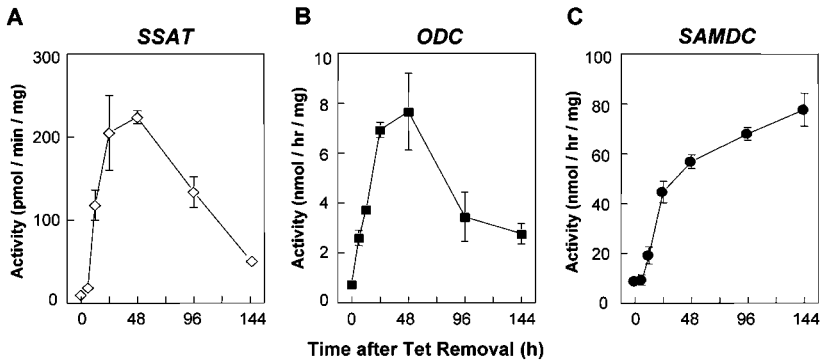

FIG. 4. **Time-dependent effects of conditional SSAT overexpression on polyamine biosynthetic enzyme activities.** Tet was removed from SSAT/LNGK9-clone 53 cells for the indicated time after which cells were harvested for polyamine enzyme activities for SSAT, ODC, and SAMDC. Following Tet removal, both SSAT activity (◇) increased sharply to a maximum of ~20-fold (*A*), and ODC activity (■) increased sharply to ~10-fold (*B*) at 48 h before undergoing a steady decline. By contrast, SAMDC activity (●) increased steadily over the course of 144 h to a maximum of ~18-fold that of basal levels (*C*). Enzyme activities of SSAT/LNGK9-clone 53 cells grown continuously in the presence of Tet remained relatively unchanged from 0 h (data not shown). Data represents mean values ± S.E., where *n* is 3.

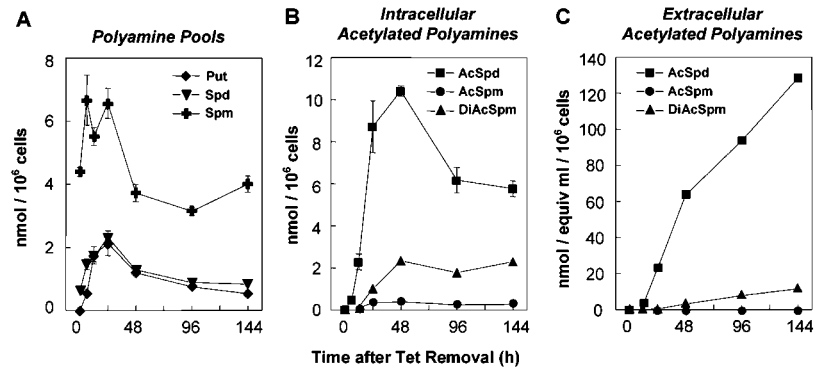

FIG. 5. **Time-dependent effects of conditional SSAT overexpression on intracellular and extracellular polyamines.** Similar to the experiment as described in Fig. 4, SSAT/LNGK9-clone 53 cells were grown in the absence of Tet for the indicated time and then harvested for polyamine pool analysis by HPLC. Intracellular polyamines pools increased transiently for 24 h following Tet removal and then decreased steadily. Note that, even at 144 h, Put, Spd, and Spm pools remained similar to or slightly above basal levels (0 h) (*A*). Intracellular AcSpd and AcSpm increased markedly following Tet removal (*B*). At the same time, huge amounts of AcSpd accumulated in the media (*C*) indicating that acetylated products are readily exported. Significant levels of DiAcSpm were detected intracellularly and extracellularly (*B* and *C*). (*AcSpd*, N^1-acetylspermidine; *AcSpm*, N^1-acetylspermine; *DiAcSpm*, N^1,N^{12}-diacetylspermine; *Put*, putrescine; *Spd*, spermidine; *Spm*, spermine). Data represent means ± S.E., where *n* is 3.

Tet at 96 h resulted in a rapid resumption of cell growth (Fig. 3). The time-dependent effects of Tet removal on enzyme activities and polyamine pools are shown in Figs. 4 and 5. SSAT increased steadily to 22-fold by 48 h before declining slowly from 48 to 144 h (Figs. 2 and 4). This steady decrease in SSAT activity and mRNA may be due to a homeostatic adjustment of gene expression and/or to a time-dependent selection of cells that express lower levels of SSAT. Consistent with the observed rise in enzyme activity, acetylated polyamines increased under −Tet conditions (Fig. 5). Intracellular AcSpd increased remarkably from undetectable levels (<10 pmol/10^6 cells) to 10,420 pmol/10^6 cells by 48 h. Other SSAT products, AcSpm and DiAcSpm, which are rarely seen in cells (31), accumulated to 390 pmol/10^6 cells and 2,340 pmol/10^6 cells, respectively, by 48 h and remained elevated during the course of the 144-h experiment. Putrescine (Put) pools also rose remarkably due presumably to back-conversion of Put from Spd via AcSpd (Fig. 5) and to forward synthesis due to increased ODC activity (described below). Despite the massive accumulation of acetylated polyamines, intracellular levels of Spd and Spm failed to decrease. In fact, the levels of Put, Spd, and Spm increased substantially during the first 24 h following SSAT induction, after which they declined slowly to levels that were above (*i.e.* Put and Spd) or close to (*i.e.* Spm) 0 h levels. Enzyme activity data in Fig. 4 (*B* and *C*) strongly suggest that these pools were sustained by compensatory increases in ODC and SAMDC activities, which rose ~10-fold and ~8-fold, respectively, during the first 48 h of SSAT overexpression (Fig. 4*A*).

Analysis of cell culture media revealed huge amounts of acetylated polyamines following SSAT overexpression (Fig. 5*C*). In particular, AcSpd and DiAcSpm, which were barely detectable in +Tet culture media, were as high as 128,210 pmol/equivalent ml/10^6 cells and 11,500 pmol/equivalent ml/10^6 cells, respectively, 144 h following Tet removal. These extracellular levels were actually 10-fold higher than intracellular levels on the basis of 10^6 cells. The finding is consistent with the tenet that acetylation by SSAT facilitates export of polyamines out of the cell (41–43). Taken together, the above findings indicate that SSAT induction leads to a strong metabolic flux through both the biosynthetic and catabolic arms of the polyamine pathway. As additional indication for this interpretation, we observed increased conversion of SAM to dcSAM via the SAMDC reaction and increased intracellular accumulation MTA, a well known by-product of dcSAM that is stoichiometrically released during the Spd and Spm synthase reactions (Fig. 6).

Effects of PAO and ODC Inhibition on SSAT-induced Growth Inhibition and Pool Dynamics—Data in Figs. 4 and 5 indicate that SSAT overexpression and inhibition of cell growth in LNCaP cells were *not* due to depletion of intracellular Spd and Spm pools. Thus, our next experiments were designed to investigate the basis for SSAT-induced growth inhibition in LNCaP

cells. Because polyamine oxidase (PAO) is functionally located downstream of SSAT, and because the enzyme liberates toxic by-products (hydrogen peroxide and reactive aldehydes) having clear cytotoxic potential (44), we first examined whether the PAO inhibitor MDL-72527 might prevent growth inhibition during SSAT-mediated deregulation of polyamine catabolism. As shown in Fig. 7*A*, the presence of 10 μM MDL-72527 during −Tet failed to abrogate growth inhibition. The substantial increase in acetylated polyamines during inhibitor treatment relative to −Tet alone confirms that PAO was effectively blocked (Table I). We next considered that growth inhibition might be due to metabolic flux resulting from up-regulation of ODC and polyamine biosynthesis. To test this hypothesis, the specific ODC inhibitor DFMO was added to the media during Tet removal to interrupt metabolic flux. As others have reported (45), wild-type LNCaP cells are inherently resistant to DFMO. SSAT-off (+Tet) cells treated with 1 mM DFMO grew similarly to cells not treated with DFMO (Fig. 7). The more unexpected finding was that DFMO treatment of SSAT-on (−Tet) cells effectively prevented growth inhibition. Thus, by inhibiting ODC, a relationship between metabolic flux and the antiproliferative effect was established. Importantly, ODC inhibition fully prevented accumulation of acetylated polyamines (Table I) and thereby provided direct evidence for interruption of polyamine flow from biosynthesis to catabolism. In experiments to be discussed below (see Fig. 10*D*), interference with flux by DFMO is further confirmed by the fact that there is no accumulation of MTA, the by-product of Spd and Spm synthesis.

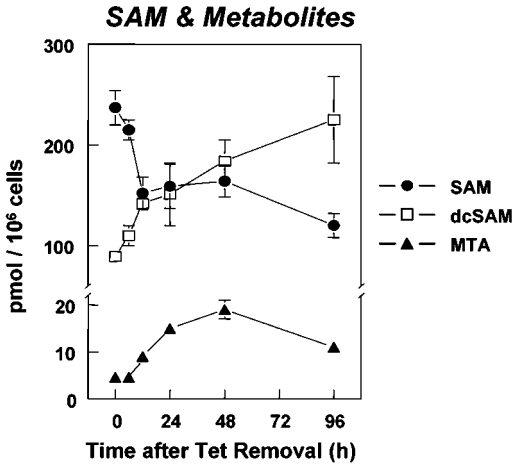

FIG. 6. **Effects of SSAT overexpression on SAM, dcSAM and MTA pools.** Tet was removed from SSAT/LNGK9-clone 53 cells for the indicated times after which cells were harvested and extracted for SAM pool analysis by HPLC. Note that upon Tet removal (−Tet), intracellular levels of the SAMDC substrate SAM (●) declined steadily to ~50% at 96 h while the SAMDC product dcSAM (□) increased steadily to a maximum of 250% at 96 h. At the same time, the biosynthetic by-product of dcSAM metabolism MTA (▲) increased to a maximum of ~400% at 48 h. The findings are consistent with accelerated polyamine metabolic flux due to increased polyamine biosynthesis and acetylation in −Tet cells. The limit of detection for MTA is <5 pmol/10^6 cells. Data represent means ± S.E., where *n* is 3.

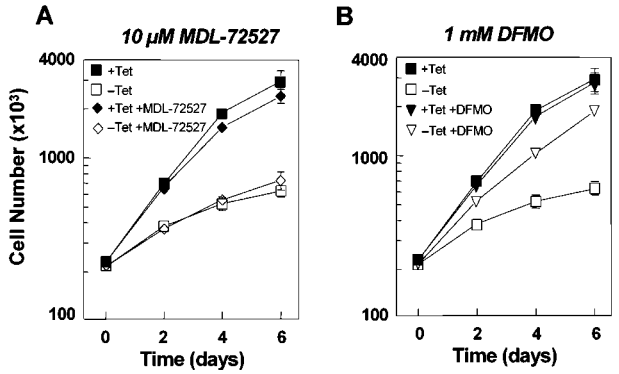

FIG. 7. **Effects of PAO or ODC inhibition on SSAT-induced cell growth inhibition.** SSAT/LNGK9-clone 53 cells were grown in the presence and absence of Tet and treated with either a PAO inhibitor (10 μM MDL-72527 (*A*)) or an ODC inhibitor (1 mM DFMO (*B*)) for the indicated time and then analyzed for prevention of cell growth inhibition. When included during Tet removal, the PAO inhibitor MDL-72527 failed to prevent growth inhibition by SSAT (*A*), whereas the ODC inhibitor DFMO was very effective in preventing SSAT-induced growth inhibition (*B*). Data represent means ± S.E., where *n* is 3.

The basis for sustained cell growth in the presence of DFMO is not clear by the polyamine analysis shown in Table I. At 48 h, Put and Spd pools are very low and Spm pools remain as high as those seen in the growth-inhibited −Tet cells. It is possible that, although most cells seem to rely on Spd for cell growth (18), LNCaP cells may grow under conditions of severe Put and Spd limitation by relying on Spm pools and a small amount of Spd back-converted from Spm via the SSAT/PAO pathway. Consistent with this idea, Spm pools were maintained at near to control levels for more than 96 h (data not shown).

Metabolic Flux and Depletion of Polyamine Precursor Stores—Enhanced metabolic flux may deplete metabolites and polyamine precursors and thereby limit their availability for cell growth. Such molecules include the biosynthetic precursors ornithine, methionine, SAM, and the SSAT cofactor acetyl-CoA. As shown in Fig. 8, the inclusion of 1 mM ornithine or methionine in the −Tet media failed to prevent SSAT-induced growth inhibition indicating that the amino acids were not limiting to cell growth.

The impact of SSAT overexpression on acetyl-CoA pools was investigated, because, in addition to serving as a cofactor to SSAT, the molecule is critically involved in fatty acid synthesis, histone acetylation, and other metabolic processes that could affect cell growth. As shown by electropherogram (Fig. 9*A*), acetyl-CoA was detectable by HPCE as a distinct and highly reproducible peak. Peak changes under −Tet *versus* +Tet conditions were quantitated relative to the internal standard

TABLE I
Polyamine pools under conditions of SSAT overexpression and enzyme inhibition in LNCaP cells

SSAT/LNGK9 clone 53	48-h treatment[a]	Polyamine pools (cells)[b]					
		Put	AcSpd	Spd	AcSpm	DiAcSpm	Spm
		pmol / 10^6 cells					
+Tet	Untreated	<10	<10	685 ± 10	<10	<10	4,180 ± 102
−Tet	Untreated	960 ± 25	16,910 ± 623	885 ± 70	525 ± 20	5,300 ± 320	3,050 ± 147
+Tet	1 mM DFMO	<10	<10	40 ± 2	<10	<10	3,850 ± 88
−Tet	1 mM DFMO	<10	320 ± 80	90 ± 15	40 ± 3	<10	3,455 ± 373
+Tet	10 μM MDL-72527	215 ± 15	<10	1,135 ± 68	<10	<10	4,390 ± 328
−Tet	10 μM MDL-72527	985 ± 160	21,795 ± 852	1,110 ± 57	1,030 ± 55	8,765 ± 345	2,625 ± 179

[a] Treatment began at Tet removal.
[b] Data are expressed as means ± S.E., where *n* is 3.

isobutyryl-CoA. Following analysis, intracellular acetyl-CoA pools were found to decrease by ~25% at 48 h and by ~45% at 96 h (Fig. 9*B*) suggesting a cause-and-effect linkage. The inability of acetyl-CoA to penetrate cells precluded more defining prevention studies such as those involving amino acids (Fig. 8). Attempts to prevent growth inhibition with exogenous 1 mM pyruvate as an acetyl-CoA precursor, proved unsuccessful (data not shown). It is puzzling, however, that these pools were not protected during DFMO prevention of growth inhibition (Fig. 10*A*). Because inhibition of ODC by DFMO results in a marked increase in SAMDC activity and dcSAM pools (Fig. 6), it is possible that acetylated polyamines may be back-converted to Put and Spd and then forward-converted to Spd and Spm due to excess dcSAM. These would then become available for re-acetylation by overexpressed SSAT. If sufficiently rapid, this cycling could account for the depleted acetyl-CoA pools during DFMO treatment.

As noted above, SAM pools were significantly reduced 12 h following Tet removal (Fig. 6). Repletion experiments were not feasible because, like acetyl-CoA, SAM does not penetrate cells effectively. Although SAM pools fell ~50% at 96 h (Figs. 6 and 10*B*), Spd and Spm pools did not similarly decline indicating that there was at least sufficient levels to sustain polyamine biosynthesis. A further disconnect between SAM levels and growth inhibition was noted in the finding that SAM pools remained reduced during treatment with DFMO (Fig. 10*B*), even though growth inhibition was prevented. Finally, we note that, as SAM pools declined, dcSAM pools increased in a correlative manner. Because the accumulation of dcSAM may cause growth inhibition (46), we considered that the significant rise in dcSAM seen following SSAT induction might be toxic (Fig. 6). This possibility, however, was also excluded by the finding that DFMO markedly increased dcSAM pools while preventing growth inhibition (Fig. 10*C*). Accumulation of the dcSAM metabolite, MTA, is also capable of exerting an antiproliferative effect (47, 48), but this seems unlikely in the present system, because there was no indication of Spd and Spm pool depletion, which is usually regarded as a major indication of MTA toxicity via feedback inhibition of the Spd and Spm synthases (49).

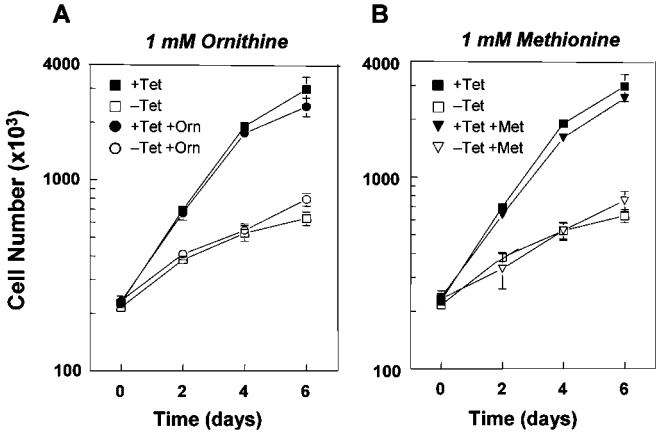

FIG. 8. **Effects of polyamine precursors on SSAT-induced cell growth inhibition.** SSAT/LNGK9-clone 53 cells grown in the presence and absence of Tet were simultaneous treated with 1 mM of the polyamine precursors, ornithine (*A*) or methionine (*B*), for the indicated time and analyzed for prevention of growth inhibition. Note that both amino acids failed to prevent SSAT-induced growth inhibition when included during Tet removal. Data represent means ± S.E., where *n* is 3.

DISCUSSION

We have previously shown that activation of polyamine catabolism by conditional overexpression of SSAT led to growth inhibition in MCF-7 breast cancer cells (31). We undertook the present study to examine whether LNCaP prostate tumor cells might respond differently to such perturbations in polyamine homeostasis. The data presented here are consistent with this possibility. Although both cell lines expressed a similar ~20-fold increase in SSAT activity, growth inhibition in MCF-7 breast carcinoma cells correlated closely with a depletion in intracellular polyamine pools (31). By contrast, growth inhibi-

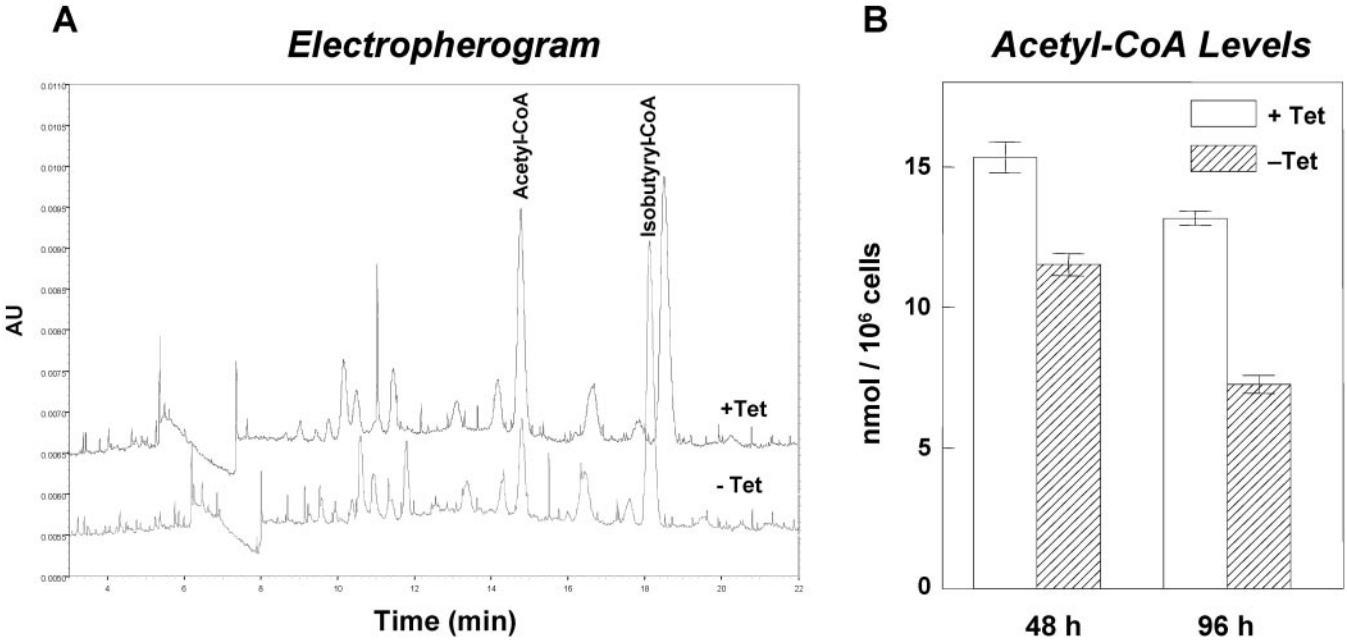

FIG. 9. **Effects of SSAT overexpression on acetyl-CoA levels.** SSAT/LNGK9-clone 53 cells were grown in the presence (+*Tet*, *open bar*) and absence of Tet (−*Tet*, *hatched bar*) for 48 and 96 h and analyzed by HPCE for acetyl-CoA. *A*, an electropherogram from HPCE showing levels of acetyl-CoA in clone 53 cells and an exogenous internal standard, isobutyryl-CoA in the presence (+*Tet*) and absence (−*Tet*) of Tet at 96 h. The internal standard isobutyryl-CoA was added to each sample to allow for calculation of loss during extraction. Note that the acetyl-CoA peak is much lower in −Tet cells compared with +Tet cells. Quantitation of the peaks (*B*) reveals a 25% decline in acetyl-CoA by 48 h and a 45% decline by 96 h in −Tet cells (*hatched bars*) when compared with their levels in +Tet cells (*open bars*). Data represent means ± S.E., where *n* is 3.

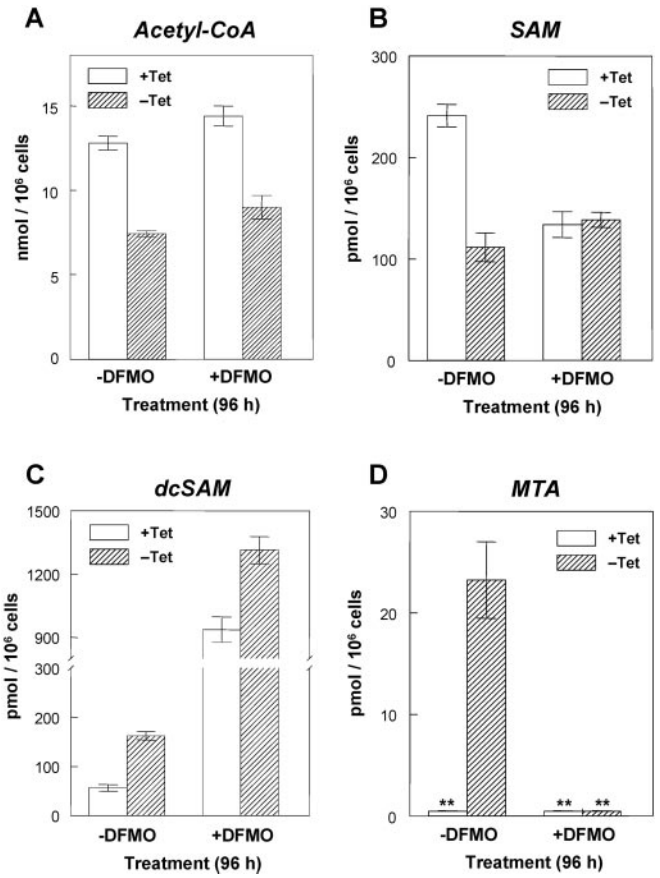

FIG. 10. **Effects of DFMO treatment on the levels of acetyl-CoA (*A*), SAM (*B*), dcSAM (*C*), and MTA (*D*) during SSAT overexpression.** SSAT/LNGK9-clone 53 cells were grown in the presence (+*Tet*, *open bar*) and absence of Tet (−*Tet*, *hatched bar*) with or without 1 mM DFMO for 96 h and analyzed by HPCE for acetyl-CoA and by HPLC for SAM, dcSAM, and MTA. Data represent means ± S.E., where *n* is 3.

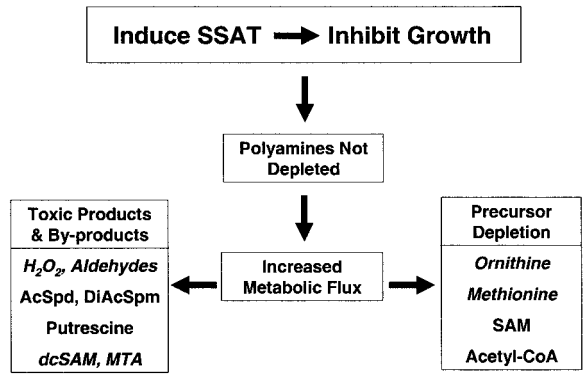

FIG. 11. **Diagrammatic representation of the possible causes of growth inhibition in SSAT overexpressing LNCaP cells.** Activation of polyamine catabolism by overexpressing SSAT causes growth inhibition in LNCaP cells, which is not accompanied by polyamine pool depletion. It is, however, associated with compensatory increase in polyamine biosynthetic activity that leads to heightened flux thru polyamine metabolism. When biosynthesis is interrupted by the ODC inhibitor, DFMO, growth inhibition is prevented (not shown), thus linking flux to the antiproliferative effect. The possible causes of growth inhibition emanating from heightened flux are presented as accumulation of product or by-product excess (*left panel*) or as depletion of critical precursors and cofactors (*right panel*). Of the product/by-product possibilities, overproduction of hydrogen peroxide (H_2O_2) and reactive aldehydes (such as acetamidopropanal), dcSAM, and MTA have been experimentally eliminated (*italic type*). Of the possible precursor possibilities, depletion of ornithine and methionine has been experimentally excluded (*italic type*). Thus, the possibilities that were not excluded and that may contribute to growth inhibition include accumulation of acetylated polyamine products, decreased levels of the polyamine aminopropyl donor, SAM, and/or a reduction in stores of the SSAT co-factor, acetyl-CoA.

tion in LNCaP prostate carcinoma cells took place in the absence of polyamine pool depletion. As will be discussed below, the difference appears to be due to the ability of LNCaP cells to metabolically compensate for activated catabolism or conversely, to the inability of MCF-7 cells to mount such a response.

The idea of activating polyamine catabolism derived from observations made with polyamine analogues (23–27). We note that conditional overexpression of SSAT produces a 10- to 20-fold increase in SSAT activity, whereas induction by polyamine analogues such as DENSPM reaches ~1000-fold in certain cell lines. A significant portion of DENSPM-induced enzyme protein, however, is inhibited by analogue binding and is unable to acetylate polyamines (50). Accumulation of acetylated products represents a better indication of SSAT functional overexpression in cells. Thus, the seemingly modest 20-fold increase in SSAT activity seen here in LNCaP cells resulted in exceedingly high levels of intracellular and extracellular acetylated polyamines, which undoubtedly had a profound impact on the metabolic equilibrium of polyamines. We also note that enzyme induction is also associated with polyamine species such as DiAcSpm that are rarely seen in cells unless SSAT is overexpressed (31) or analogue-induced (29).

It was expected that the massive acetylation of Spd and Spm and their export into the media would deplete intracellular pools as was previously seen in MCF-7 cells (31). In LNCaP cells, however, depletion of Spd and Spm was averted by a compensatory increase in polyamine biosynthesis: ODC activity rose by ~16-fold at 48 h following Tet removal and SAMDC, by ~8-fold. Thus, instead of decreasing, intracellular polyamine pools actually increased rapidly despite the diversion of huge amounts of Spd and Spm to intracellular and extracellular acetylated polyamines. Although a similar up-regulation of polyamine biosynthesis has been reported in SSAT stably transfected cell lines and most tissues of transgenic mice (51–53), it is likely to have evolve over time via a process of selection. By contrast, the increase in ODC and SAMDC activities in LNCaP cells began almost simultaneously with SSAT activation. The net effect of this response was heightened flux through the biosynthetic pathway as indicated by the decline in SAM pools and by the related rise in MTA, a by-product of the Spd and Spm synthase reactions. The relationship between this flux and cell growth was clearly established by the observation that DFMO, an inhibitor of ODC, effectively prevented SSAT-induced growth inhibition. The finding is particularly significant, because DFMO typically inhibits, rather than prevents, cell growth (16). Whether this linkage is common to prostate-derived tumor cells or whether it is MCF-7 cells that are unusual remains to be determined. If the former is confirmed, the finding is consistent with other known biochemical idiosyncrasies regarding polyamine metabolism in prostate carcinoma cell lines (11, 15).

SSAT-induced growth inhibition in the absence of polyamine pool depletion raises obvious questions regarding the basis for the antiproliferative effect. As diagrammed in Fig. 11, the high rate of metabolic flux through both the biosynthetic and catabolic arms of the pathway suggests that accumulation of pathway products or by-products may reach toxic levels (*left panel*) or that certain metabolites may become growth-limiting (*right panel*). The various possibilities that were experimentally eliminated as causes of flux-induced growth inhibition include: elaboration of PAO toxic by-products, accumulation of dcSAM, and depletion of the amino acids ornithine and methionine (Fig. 11, *italic type*). Possibilities that were not clearly elimi-

nated include high levels of acetylated polyamines and Put, depletion of SAM pools, and decreases in acetyl-CoA pools (Fig. 11).

Our HPCE analysis of acetyl-CoA pools revealed that these pools decreased by 45% during SSAT induction in LNCaP cells. To our knowledge, this is the first time that such a linkage has been demonstrated between polyamine metabolism and depletion of acetyl-CoA stores. We examined this possibility with the view that the massive amounts of acetylated polyamines being generated may render the SSAT cofactor acetyl-CoA limiting for critical cellular functions such as fatty acid synthesis, cholesterol synthesis, and histone regulation. Indeed, fatty acid synthase expression and lipidogenesis are known to be increased by androgens and highly relevant to the normal prostate biology and prostate cancer (54–56). In addition, Ettinger *et al.* (57) recently showed that androgen independence in LNCaP xenograft models is closely associated with dysregulation of enzymes that coordinately control lipogenesis and cholesterol synthesis. These various findings imply a high dependence of prostate cancer on acetyl-CoA stores.

Taken together, findings indicate that activation of polyamine catabolism at the level of SSAT leads to: (*a*) altered polyamine pool homeostasis, (*b*) a compensatory increase in polyamine biosynthesis, (*c*) heightened metabolic flux through both the biosynthetic and catabolic pathways, (*d*) synthesis of enormous quantities of acetylated polyamines, (*e*) significant depletion of critical metabolite pools such as the polyamine precursor *S*-adenosylmethionine (SAM) and the SSAT cofactor, acetyl-CoA, and (*f*) inhibition of cell growth. We emphasize that this analysis applies strictly to selective SSAT overexpression and not to enzyme induction by polyamine analogues such as DENSPM, which in addition to potently inducing SSAT also down-regulate polyamine biosynthesis and, thereby, preclude the heightened metabolic flux seen in LNCaP cells. Importantly, studies from our laboratory (58) have shown that cross-breeding SSAT transgenic mice that are genetically predisposed to develop prostate cancer (*i.e.* TRAMP mice (59) results in metabolic responses similar to those seen in LNCaP cells and leads to a marked suppression of prostate tumor outgrowth.

Although the present findings are based on an artificial system (*i.e.* conditional overexpression of SSAT), there are many pharmacological examples of SSAT induction by classes of drugs other than polyamine analogues to levels comparable to those obtained here (43). For example, we and others have shown that anticancer drugs unrelated to polyamines can also elicit very significant increases in SSAT gene expression. Maxwell *et al.* (60) found that SSAT was the most potently induced gene by the antimetabolite 5-fluorouracil from among >3000 represented in a gene profiling study of MCF-7 cells. Similarly, we have shown that SSAT mRNA is among the top 10 genes induced by the DNA-alkylating platinum compounds, oxaliplatin and cisplatin (61). Finally, studies from our laboratory (58) have shown that cross-breeding SSAT transgenic mice that are genetically predisposed to develop prostate cancer (*i.e.* TRAMP mice (59)) markedly suppressed genitourinary tumors. These findings support the possibility that selective small molecule inducers of SSAT may have therapeutic and/or preventive potential against prostate cancer.

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