A Small Molecule Inhibitor of Isoprenylcysteine Carboxymethyltransferase Induces Autophagic Cell Death in PC3 Prostate Cancer Cells*§

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A number of proteins involved in cell growth control, including members of the Ras family of GTPases, are modified at their C terminus by a three-step posttranslational process termed prenylation. The enzyme isoprenylcysteine carboxymethyltransferase (Icmt) catalyzes the last step in this process, and genetic and pharmacological suppression of Icmt activity significantly impacts on cell growth and oncogenesis. Screening of a diverse chemical library led to the identification of a specific small molecule inhibitor of Icmt, cysmethynil, that inhibited growth factor signaling and tumorigenesis in an in vitro cancer cell model (Winter-Vann, A. M., Baron, R. A., Wong, W., dela Cruz, J., York, J. D., Gooden, D. M., Bergo, M. O., Young, S. G., Toone, E. J., and Casey, P. J. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4336–4341). To further evaluate the mechanisms through which this Icmt inhibitor impacts on cancer cells, we developed both in vitro and in vivo models utilizing PC3 prostate cancer cells. Treatment of these cells with cysmethynil resulted in both an accumulation of cells in the G1 phase and cell death. Treatment of mice harboring PC3 cell-derived xenograft tumors with cysmethynil resulted in markedly reduced tumor size. Analysis of cell death pathways unexpectedly showed minimal impact of cysmethynil treatment on apoptosis; rather, drug treatment significantly enhanced autophagy and autophagic cell death. Cysmethynil-treated cells displayed reduced mammalian target of rapamycin (mTOR) signaling, providing a potential mechanism for the excessive autophagy as well as G1 cell cycle arrest observed. These results identify a novel mechanism for the antitumor activity of Icmt inhibition. Further, the dual effects of cell death and cell cycle arrest by cysmethynil treatment strengthen the rationale for targeting Icmt in cancer chemotherapy.

Posttranslational processing of so-called CaaX proteins has received much attention in the past two decades due to the important roles these proteins play in biological regulations and diseases (1, 2). This processing is initiated by isoprenoid modification of the cysteine residue of the C-terminal CAAX motif of the protein, subsequent proteolytic removal of the three C-terminal amino acids, i.e. the −AX residues, and the methylation of the newly exposed carboxyl group of the prenylated cysteine residue. The overall process, termed protein prenylation, has been shown to be important for the localization, stability, and ultimate functions of a broad array of CaaX proteins (3).

Most members of the Ras superfamily of GTPases are CaaX proteins, and Ras proteins themselves, which are farnesylated, have been extensively studied due to the high prevalence of dysregulated Ras signaling in human cancers (4). Inhibitors of protein farnesyltransferase (FTase)2 have been under development as anticancer agents for over a decade, but their efficacy, especially in solid tumors, has been disappointing (5, 6). The realization that some CaaX proteins, including forms of Ras in which mutations are prevalent in human tumors, are subject to alternative prenylation by protein geranylgeranyltransferase I when FTase is inhibited (7) spurred efforts to target the post-prenylation processing steps of proteolysis and methylation since each of these steps is catalyzed by a single enzyme that acts on both farnesylated and geranylgeranylated proteins (8, 9). In particular, targeting of CaaX protein methylation via inhibition of the enzyme responsible, isoprenylcysteine carboxylmethyltransferase (Icmt), through both genetic and pharmacological approaches, has been shown to dramatically impair oncogenesis in several tumor cell models (10, 11).

The mechanism(s) through which inhibition of Icmt impacts on cell proliferation and oncogenesis are still far from clear. Interference with cell cycle progression, however, is a cornerstone of many chemotherapeutic agents, and both FTase inhibition and geranylgeranyltransferase I inhibition have been

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1 The abbreviations used are: FTase, farnesyltransferase; Icmt, isoprenylcysteine carboxymethyltransferase; LC3, microtubule-associated protein 1 light chain 3; LC3-II, activated form of LC3; J3, cysmethynil analog 1-octylm-tolyl-1H-indole; atg5, autophagy related 5 homolog; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; GAPDH, glyceroldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco’s modified Eagle’s medium; 3-MA, 3-methyladenine; siRNA, small interfering RNA; DMSO, dimethyl sulfoxide.
demonstrated to arrest many types of tumor cells at G1 phase of
the cell cycle; FTase inhibitors also trigger a G2/M arrest in
certain cell types (5, 6, 12). Another important property of can-
cer chemotherapeutic agents is the ability to induce cell death.
The process of apoptosis in particular has been widely studied
in this regard, and many current anticancer agents, including
CaaX prenylation inhibitors, enhance apoptosis in cells (6, 13).
Quite recently, autophagic cell death has stepped into the spot-
light as a type of programmed cell death with the potential to be
enhanced by cancer therapeutics (14, 15). As with many biologi-
cal regulatory processes, autophagy seems to be a double-
edged sword in terms of an impact on cell functions. Most cell
types display a baseline level of autophagy to clear damaged
organelles and unwanted proteins, but dysregulation of the
autophagy process can be detrimental to cell survival. Conse-
quentially, manipulation of autophagy is now considered to pre-
sent therapeutic opportunities in several disease states, includ-
ing cancer (16).

We recently reported the identification of a specific small
molecule inhibitor of Icmt, cysmethynil, and demonstrated a
mechanism-based impact on tumorigenesis in an in vitro can-
cer cell model (11). We now report that treatment of PC3 pros-
tate cancer cells with cysmethynil impairs cell cycle progression
and, unexpectedly, activates an autophagic process in the cells
and promotes autophagy-dependent cell death. Further, treat-
ment of mice harboring xenograft tumors with cysmethynil
results in dramatic impairment of tumor growth. These results
identify a novel mechanism for the antitumor effects of Icmt
inhibition and strengthen the support for targeting Icmt in
cancer chemotherapy.

EXPERIMENTAL PROCEDURES

Materials—Cysmethynil and biotin S-farnesylcysteine were
synthesized by the Duke Small Molecule Synthesis Facility via
established methods (11, 17). Cysmethynil analog 1-octyl-m-
tolyl-1H-indole (J3) was synthesized via standard chemical pro-
cedures and characterized to confirm identity and purity (see
the supplemental text and scheme). Stock solutions were pre-
pared at 10 mM in DMSO and stored at −20 °C. Antibodies
recognizing cyclins D1 and B1, p27, poly(ADP-ribose) poly-
merase-2, caspase 3, eukaryotic initiation factor 4E-binding
protein 1 (4EBP1), and GAPDH were all from Cell Signaling.
The LC3 antibody was from Abgent.

Cell Culture and Proliferation Assays—The PC3 human
prostate cell line was obtained from American Type Culture
Collection (Rockville, MD). Cells were maintained at 37 °C with
5% CO2 in DMEM (Sigma) supplemented with 10% fetal bovine
serum (Hyclone), 50 units/ml penicillin (Invitrogen), and 50
µg/ml streptomycin (Invitrogen). For proliferation assays, cells
were seeded at 15–20% confluency in DMEM containing 5%
plasma bovine serum in 96-well plates for 24 h prior to treatment
with specific agents (e.g. cysmethynil) or vehicle at the concen-
trations and length of time indicated in the legends for Figs.
1–2. The relative number of the live cells was determined using
the CellTiter™ 96 AQueous One Solution cell proliferation
assay (Promega). Each condition was performed in quadrupli-
cate, and data presented that obtained from at least three sep-
arate experiments. For proliferation studies performed with
3-methyladenine (3-MA) in addition to cysmethynil, cells were
seeded as above and incubated with 0.5 mM 3-MA or vehicle at
37 °C overnight. Cells were then treated with the concentra-
tions of cysmethynil as indicated in the legend for Fig. 1 in the
continued presence of 0.5 mM of 3-MA for the indicated
durations.

Flow Cytometry Analysis—PC3 cells (5 × 10⁵) were seeded in
100-mm dishes in DMEM containing 5% fetal bovine serum
and incubated for 24 h, whereupon the cells were exposed to
cysmethynil, the J3 analog, or vehicle at the concentration and
for the time indicated in the appropriate figure legend. Cells
were harvested by centrifugation at 300 × g for 5 min, where-
upon the cells were then fixed in ice cold 70% ethanol overnight
before being resuspended in phosphate-buffered saline con-
taining 40 µg/ml propidium iodide and 0.1 mg/ml Ribonucle-
ase A (both from Sigma) for 1 h at 37 °C. Fluorescence was
measured by flow cytometry analysis using an Excalibur Instru-
ment (BD Biosciences).

Immunofluorescence of LC3—Cells subjected to the indicated
treatments were fixed with 4% paraformaldehyde and perme-
abilized with 0.3% Triton using a standard protocol (18). Incu-
bation with primary antibody to LC3 (MAP1LC3B, Abgent)
was performed at 4 °C overnight before incubation with Rho-
damine Red-X secondary antibody (Jackson ImmunoResearch
Laboratories). Analysis was performed using an Olympus fluo-
rescent microscope fitted with the appropriate excitation and
emission filters.

Acridine Orange Staining for Autophagy Detection—Acric-
dine orange (Sigma) staining was performed according to a
published protocol (19). Briefly, cells were washed twice with
phosphate-buffered saline and then stained with 1 µM/ml acri-
dine orange for 15 min at 37 °C. Analysis was performed via
fluorescence microscopy using 490-nm band-pass blue excita-
tion filters and a 515-nm long-pass barrier filter. In the study of
the effect of baflomycin co-treatment, cells were treated with
200 nM baflomycin A1 for 40 min prior to the addition of acri-
dine orange.

Atg5 Knockdown—Stealth® siRNA duplex oligoribonucleoti-
des targeting Atg5 (Invitrogen) were resuspended to make a 20
µM solution following the manufacturer’s instructions. Trans-
factions were carried out using the Lipofectamine 2000 proto-
col provided by Invitrogen. Conditions were optimized with
varying ratios of Lipofectamine and RNA, as well as different
time intervals after the transfections as determined by immu-
noblot analysis of atg5 protein levels. Cell proliferation on both
the atg5 siRNA-treated cells and the mock-transfected cells was
then assessed using the assay described above.

Cysmethynil Treatment of Xenograft-harboring Mice—PC3
cells were grown in DMEM and 10% fetal calf serum until near
confluence and then harvested with a standard method using
tryptsin. Cells (5 × 10⁶) were then mixed with Matrigel® (BD
Biosciences) to achieve 40% Matrigel in the final solution. The
cell preparation was then injected subcutaneously into the
flanks of 6–8-week-old SCID mice. For treatment, cysmethynil
was prepared in 4% DMSO, 1.4% Tween 80, and 1% sodium
carboxymethyl cellulose (Sigma) normal saline solution; the
vehicle control was with the same mixture lacking cysmethynil.
In a preliminary acute toxicity study, mice were injected intra-
Cysmethynil Exhibits Mechanism-based Antiproliferative Activity toward Prostate Cancer Cells—To facilitate a rapid evaluation of the mechanism-based consequences of cysmethynil treatment on cells, we sought a structural analog of the compound that lacked activity toward the enzyme. Molecular modeling of available structure-activity data on the chemical series from which cysmethynil was identified (22) suggested that the amide portion of the indole ring might be important in this activity. An analog lacking only the amide portion of the indole ring was synthesized, termed J3 (Fig. 1A), and evaluated for in vitro activity toward Icmt. This analysis, shown in Fig. 1B, revealed that the J3 analog was essentially devoid of Icmt inhibitory activity despite its chemical similarity to cysmethynil.

The impact of treatment, by cysmethynil and the inactive J3 analog, on the prostate cancer-derived cell line PC3 was evaluated using a cell viability assay. Cysmethynil treatment resulted in a dose- and time-dependent reduction in the number of viable PC3 cells, whereas the J3 analog at the highest corresponding dose was ineffective (Fig. 1C). These data, along with a previous study using a colon cancer cell line in which the antiproliferative activity of cysmethynil was markedly diminished by overexpression of Icmt in the cells (11), provide compelling evidence that the impact of cysmethynil treatment on cells is directly due to inhibition of Icmt. An additional finding from this study is that at moderate dosage, cysmethynil appears to exhibit primarily cytostatic activity, whereas at higher concentrations, both cytostatic and cytotoxic activity are observed (Fig. 1C).

Cysmethynil Is Efficacious in Controlling Tumor Growth in a Xenograft Mouse Model of Prostate Cancer—To investigate the ability of cysmethynil to inhibit tumor growth in vivo, we first conducted a dose escalation toxicity trial in Balb/C mice and found that an intraperitoneal dose of less than 0.3 mg/g was well tolerated. We then established a xenograft model of PC3 cells in SCID mice. Tumor cells were implanted subcutaneously in the flank. When tumors started to increase in size (usually 100–200 mm³), confirming the successful grafting of the tumor cells, mice were randomly assigned to control (vehicle) and cysmethynil treatment groups. Mice thus bearing established PC3 tumors were given intraperitoneal injections of vehicle only, 0.1, 0.2, or 0.3 mg/g of cysmethynil every 48 h for 28 days. The duration of the experiments were dictated by the tumor burden of the control mice. As shown in Fig. 2A, both doses of cysmethynil significantly suppressed the growth of PC3 tumors when compared to vehicle.
pared with the vehicle control group. Neither dose was accompanied by any appreciable toxicity as assessed by body weight determinations of the treated mice when compared with the control group (Fig. 2B). These data indicate that pharmacological inhibition of $I_{cmt}$ in vivo significantly impacts on tumor growth and further reinforces the notion of $I_{cmt}$ as an anticancer drug target.

Cysmethynil Treatment Induces Cell Cycle Arrest in PC3 cells—As noted in the Introduction, there is considerable evidence that inhibition of CaaX protein processing by either of the protein prenyltransferases can impact on cell cycle progression. This information, coupled with the finding that moderate doses of cysmethynil have apparently predominant cytostatic activity on PC3 cells (Fig. 1C), prompted us to examine more closely cell cycle parameters in cells treated with this $I_{cmt}$ inhibitor. Flow cytometry analysis of PC3 cells treated $48 \text{ h}$ with $20 \mu M$ cysmethynil showed a significantly increased population of cells in $G_1$ (Fig. 3A). Further examination of molecular markers associated with $G_1$ arrest showed remarkable changes, including increased p27 levels, reduced cyclin D1 levels, and showed almost complete loss of phospho-Rb (Fig. 3B). These data are all consistent with the $G_1$ arrest observed in the flow cytometry analysis.

Cysmethynil Treatment Induces Autophagic Cell Death—Although the ability of cysmethynil treatment to trigger a $G_1$ arrest in PC3 cells could account for the cytostatic capacity of this compound, the reduction in cell count following longer term and higher dose treatments in vitro suggested that an increase in cell death was also being triggered by $I_{cmt}$ inhibition. Our initial set of experiments to examine this potential outcome of cysmethynil treatment was focused on apoptotic pathways since as noted above, inhibition of CaaX protein processing had been linked to elevated apoptosis in many cell types (6). However, no consistent impact of cysmethynil treatment at the concentration of cysmethynil that decreased the number of viable cells was observed on apoptotic markers such as caspase 3 and poly(ADP-ribose) polymerase-2 cleavage in the treated PC3 cells (data not shown). Additionally, neither DNA fragmentation nor abnormal nuclear morphology was observed in PC3 cells following cysmethynil treatment at the concentration that resulted in cell death (not shown), arguing against a significant contribution of apoptosis to diminished cell viability.

The inability to link apoptosis to the cell death observed in the PC3 cells following cysmethynil treatment prompted us to consider whether the drug induced a non-apoptotic form of cell death. Specifically, we examined autophagy, a process that involves the degradation of cellular components through an autophagosome-lysosome pathway, as this process has recently become appreciated as important in cell growth and survival.
Since different cell types exhibit varying degrees of autophagy, we quantified the autophagosomes in the cysmethynil- and vehicle-treated cells by determining the fraction of cells exhibiting elevated level of LC3 aggregation; this quantitation showed that cysmethynil treatment significantly elevated cellular abundance of autophagosomes (35% versus 2%, Fig. 4B).

The process of autophagy starts with the autophagosome formation and then progresses to autophagolysosomes through the fusion of acidic lysosomes with autophagosomes (15). Therefore, acridine orange staining of the live cells was also employed to visualize acidic autophagolysosomes in control and cysmethynil-treated PC3 cells. As shown in Fig. 4C, cysmethynil treatment markedly elevated the amount of autophagolysosomes in the cells, providing further evidence that the autophagic process was being activated by drug treatment and that the autophagosome formation is not the result of the inhibition of lysosomal fusion.

Bafilomycin A1, an inhibitor of vacuolar H+ ATPase, prevents the transition of autophagosome to autophagolysosomes by disrupting the fusion of autophagosomes to lysosomes (24). Hence, bafilomycin A1 provides a useful way of studying the autophagy process. The treatment of the PC3 cells with bafilomycin A1 markedly reduced the quantity of acidic orange-positive vesicles (Fig. 4C), confirming that the autophagosomes produced by the treatment of cysmethynil undergo the same maturation process with the fusion with lysosomes. Indeed, in the bafilomycin A1-treated cells, LC3-II levels remain elevated despite a marked reduction of acidic vesicles (not shown), indicating that the earlier stages of autophagy prior to lysosomal fusion were not affected by this lysosome fusion inhibitor.

**Inhibition of Autophagy Protects PC3 Cells from Cell Death Elicited by Cysmethynil Treatment**—To assess whether the induction of autophagy observed in cysmethynil-treated PC3 cells actually contributed to the cell death elicited by treatment with the compound, we first assessed whether 3-MA, a known inhibitor of autophagy that acts through inhibition of type 3 PI3 kinase (25), could alleviate cysmethynil-induced cell death. PC3 cells were treated with vehicle alone, cysmethynil, 3-MA alone, or 3-MA plus cysmethynil, and viability of the cells was assessed 72 h later. As seen in Fig. 5A, 3-MA treatment alone had little impact on cell viability; the viability of cysmethynil-treated cells was markedly increased if 3-MA was present during the course of the treatment. Immunoblot analysis showed that the cells treated with both 3-MA and cysmethynil had much lower levels of the autophagy marker LC3-II when compared with the cells treated with cysmethynil alone, suggesting that the autophagic process stimulated by cysmethynil is subjected to the regulation by type 3 PI3 kinase (Fig. 5A).

We also employed a knockdown strategy to impair autophagy to further assess its impact on cysmethynil-induced cell death. RNA interference-mediated knockdown of atg5, a crucial component of the autophagy cascade (14), markedly reduced cell death elicited by cysmethynil treatment (Fig. 5B). This has provided additional evidence supporting a crucial role for autophagy-dependent cell death in the efficacy of cysmethynil. Knockdown of atg5 also resulted in the reduction of LC3-II production in cysmethynil-treated cells (Fig. 5B), confirm-
ing a direct impact of the knockdown on the autophagic process in the cells. Taken together, the survival benefits achieved with both 3-MA treatment and atg5 RNA knockdown in the cysmethynil-treated PC3 cells provide compelling evidence that cysmethynil not only induces autophagy but that the accompanying autophagy-dependent cell death contributes significantly to the efficacy of cysmethynil in inducing cancer cell death.

_Cysmethynil Treatment Impacts on mTOR Signaling in PC3 Cells_—The data presented above indicate that cysmethynil treatment induces both G1 cell cycle arrest and autophagy; a common link of these two processes is that they can be modulated by mTOR signaling. Two types of CaaX proteins are known to be important in regulating mTOR signaling, Ras GTPases and the Rheb GTPase. Ras activates PI 3-kinase, leading to the activation of Akt, which in turn activates Rheb by inhibiting tuberous sclerosis complex, a negative regulator of Rheb. Rheb positively regulates mTOR kinase, with a resultant positive impact on cell cycle progression and negative impact on autophagy (26). Inhibition of Ras methylation has been shown to impair Ras activity (11); Rheb could also be affected by Icmt inhibition, and this could further impact mTOR signaling. Indeed, cysmethynil treatment of PC3 cells resulted in a marked reduction of phosphorylation of Akt (Fig. 6A), as would be expected from inhibition of Ras. In addition, phosphorylation of the mTOR downstream effector ribosomal protein S6 was markedly reduced in cysmethynil-treated cells, and another effector, 4EBP1, showed the characteristic shift in phosphorylation pattern consistent with inhibition of the mTOR kinase (Fig. 6A). Furthermore, Rheb levels in PC3 cells were markedly reduced following 48 h of cysmethynil treatment, providing evidence that the general availability and activity of Rheb GTPase were reduced. Based on these data, we propose a model whereby inhibition of Icmt leads to a reduction of Ras and Rheb activity and consequent inhibition of Akt and mTOR signaling, contributing to the effects on cell cycle progression and autophagy (Fig. 6B).

**DISCUSSION**

Autophagy as a means of induction of cancer cell death has attracted increasing attention recently, especially in circumstances of apoptosis-resistant cancer cells. For example, the
Icmt Inhibition Causes Autophagic Cell Death

mTOR inhibitor rapamycin has been reported to induce cell proliferation in glioblastoma cell lines that are resistant to many therapies that induce apoptosis (27). In this regard as well, a recent study showed that temozolomide treatment alone of glioblastoma cell lines causes autophagic cell death (28). Although the realization that autophagic cell death may be an important component of the action of certain cancer drugs is relatively new, the connection between the PI3K/Akt/mTOR inhibition and autophagy induction has been well established (14).

The PI3K/Akt/mTOR signaling pathway activation enhances cell proliferation, survival, and growth and inhibits autophagy in many cells, and aberrant up-regulation of the PI3K/Akt/mTOR axis has been frequently linked to oncogenesis in many cancers (29). In many cases, up-regulation of the pathway has been linked to loss of the phosphatase and tensin homolog (PTEN) phosphatase. Although these aberrations do contribute to resistance to therapies many cancers, they might also render the cancers more sensitive to mTOR inhibition, as the sustained activation would render the cells more dependent on this pathway for proliferation (21, 30). On this note, the PC3 prostate cancer cell used in the current studies is PTEN-null for targeting Icmt as an anticancer strategy from a mechanistic standpoint but also demonstrates significant clinical efficacy of cysmethynil through its administration to mice bearing xenograft prostate cancer-derived tumors. Our data clearly indicate that an induction of autophagy by cysmethynil is a major contributor to the cell death that accompanies pharmacological inhibition of Icmt. Although the specific CaaX protein(s) underlying this phenomenon have not yet been unambiguously identified, the Ras and Rheb GTPases are potential players due to their abilities to control PI 3-kinase and mTOR signaling.

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