Chemoprevention of B-Cell Lymphomas by Inhibition of the Myc Target Spermidine Synthase

TachaZi Plym Forshell, Sara Rimpi, and Jonas A. Nilsson

Abstract

The oncogenic transcription factor c-Myc (Myc) is frequently overexpressed in human cancers. Myc is known to induce or repress a large set of genes involved in cell growth and proliferation, explaining the selection for mutations in cancer that deregulate Myc expression. Inhibition of ornithine decarboxylase, an enzyme of the polyamine biosynthetic pathway and a Myc target, has been shown to be chemopreventive. In the present study, we have dissected the role of another enzyme in the polyamine biosynthetic pathway, spermidine synthase (Srm), in Myc-induced cancer. We find that Srm is encoded by a Myc target gene containing perfect E-boxes and that it is induced by Myc in a direct manner. RNA interference against Srm shows that it is important for Myc-induced proliferation of mouse fibroblasts but to a lesser extent for transformation. Using the compound trans-4-methylcyclohexyamine, we show that Srm inhibition can delay the onset of B-cell lymphoma development in λ-Myc transgenic mice. We therefore suggest that inhibition of Srm is an additional chemopreventive strategy that warrants further consideration. Cancer Prev Res; 3(2): 140–7. ©2010 AACR.

Introduction

Signaling pathways such as the Wnt, Hh, transforming growth factor-β, and receptor tyrosine kinases, all converge on the MYC gene family which encodes oncogenic transcription factors (1, 2). Emerging evidence suggests that these pathways are deregulated in most forms of cancer, indirectly resulting in elevated levels of Myc proteins. MYC (encoding c-Myc), MYCN (encoding N-Myc), and MYCL (encoding L-Myc) also become overexpressed by direct means due to genomic translocations and amplifications in B-cell malignancies, neuroblastoma, breast cancer, and lung cancer (1). These facts, taken together, suggest that Myc overexpression is a hallmark of cancer.

Myc and its obligatory dimerization partner Max bind to CACGTG E-boxes present in a large subset of genes (3). Myc:Max dimers will then activate transcription by recruiting complexes containing histone acetyltransferases and the general transcription machinery (4). Max can also bind the Mad/Mnt family of transrepressors and the levels (and possibly affinities) of Myc proteins or Mad/Mnt proteins decide whether a Max-containing dimer will stimulate or inactivate a gene. If a cell is stimulated to grow, Myc is produced and Myc:Max dimers can relieve transrepression (5). Furthermore, some genes can also be repressed by Myc via interaction with proteins like Miz-1 (6).

Pre-genomics era transcriptional targets of Myc were difficult to identify and relied on biased assumptions based on Myc’s functions to stimulate cell growth and proliferation. Some of the first identified, including ornithine decarboxylase (Odc), lactate dehydrogenase A (LdhA), and carbamoyl phosphate synthetase/aspartate trans-carbamoylase/dihydroorotase (Cad) fit with a function of Myc in stimulating cell growth, whereas cyclin-dependent kinase 4 (Cdk4) and cyclin D2 (CcnD2) fit with a role of Myc in stimulating the cell cycle (7). Although these two groups of genes provided compelling evidence, they did not anticipate what was to come with the development of genomics tools. Serial analyses of gene expression, microarray analyses and genome-wide chromatin immunoprecipitation experiments have all indicated that the “Myc transcriptome” is very large and may comprise up to 15% of the genome (8). We are therefore faced with the possibility that Myc proteins are to be considered general transcription factors that stimulate the genes necessary for cell growth and proliferation. To lend further support of that view, Myc even transcriptionally induces the GCN5 histone acetyltransferase, which results in an “open” configuration of chromatin (4). Recently, Myc was also shown to cause a widespread repression of microRNAs, with the exception of a few, which instead had oncogenic capacities (9).

Despite the pleiotropic functions of Myc, inhibiting the products of a few downstream target genes may still be of use in chemoprevention or chemotherapy against cancer. A case for inhibiting Odc as a means to prevent the onset of tumors like colon cancer and lymphoma was established based on experimental evidence in mouse models
(10, 11) and clinical trials (12) using the drug α-difluoro-methylornithine (DFMO). Because the downstream products of Odc, polyamines, are involved in many aspects of cellular physiology, it is possible that targeting Odc also interferes with several of Myc’s functions and that Odc thus may be a more crucial target gene of Myc’s compared with most. It is therefore interesting to note that several genes of the polyamine biosynthetic pathway have been shown to be induced in B cells overexpressing Myc (11). In the present study, we are investigating the role of spermidine synthase (Srm), a Myc target gene encoding an enzyme which produces spermidine from the putrescine formed by Odc. Interestingly, we observe that Srm is more potently induced by Myc than Odc, and that inhibiting this enzyme also has a chemopreventive effect on lymphogenesis. Srm has been ignored because it is considered constitutively active; however, these data suggest that it could be a promising future drug target in chemoprevention.

Materials and Methods

Mice

μ-Myc, iMycEλ, and λ-Myc mice, all on a C57/BL6 background were obtained from The Jackson Laboratory, the National Cancer Institute mouse repository, and a kind gift from Dr. Georg Bornkamm (GSF, Munich, Germany), respectively. C57/BL6 mice (6 wk of age; Taconics, Denmark) were injected with 1 million lymphoma cells via the tail vein. λ-Myc mice and transplanted C57/BL6 mice were maintained in normal water (untreated) or treated with drinking water containing 10% fetal bovine serum, 2 mmol/L of L-glutamine, with drinking water containing were maintained in normal water (untreated) or treated the tail vein.

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Cell culture

NIH 3T3 fibroblasts and 293T human kidney cells were purchased from American Type Culture Collection and cultured in DMEM with 10% fetal bovine serum, 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate, and 1× antibiotic-antimycotic cocktail (penicillin/streptomycin/fungizone; Invitrogen). P493-6 cells expressing a tetracycline-regulated MYC oncogene (13) were a kind gift from Dr. Georg Bornkamm. B-cell lymphoma lines from Myc-transgenic mice were established by making single-cell suspensions from palpable lymphomas that originated in these mice. The lymphoma lines and P493-6 cells were seeded at a density of 2 × 105 cells/mL in RPMI 1640, 10% fetal bovine serum, 2 mmol/L of L-glutamine, 500 μmol/L of β-mercaptoethanol, and 1× antibiotic-antimycotic cocktail. Spermidine synthase inhibitor 4MCHA (TCI Europe) was added to cells to a final concentration of 100 or 250 μmol/L. Cells were dead. For RNAi, expression of spermidine synthase was analyzed using real-time quantitative reverse-transcriptase PCR (qRT-PCR). For Myc-ER experiments, puromycin-selected cells were grown in the presence or absence of cycloheximide and/or 4-hydroxytamoxifen (4-HT; both from Sigma) for 4 h, followed by monitoring of gene expression by qRT-PCR. For anchorage-independent growth of infected NIH 3T3 fibroblasts in soft agar, cells were suspended in 2.5 mL of 2× DMEM and 2.5 mL of 1.4% low melting point agarose (Sigma) and then plated in a 10 cm dish. They were incubated in a humidity chamber within a tissue culture incubator at 37°C with 5% CO2 for 2 wk. Transformed cell colonies were visualized by light microscopy and compared with uninfected NIH 3T3 cells.

RNA and protein preparation and analysis

RNA from magnetically sorted B cells, cultured cells, or tumors from λ-Myc mice were isolated using the RNA II spin kit (Macherey-Nagel). For qRT-PCR, cDNA synthesis was done using the iScript first-strand synthesis kit (Bio-Rad) and the PCR was run using the SYBRgreen PCR Mastermix (BioTech) on an iQ Real-time PCR machine (Bio-Rad). Primers used to detect Arf expression were sense, 5′-TTTCTTGGTGAGTGTTGATCCGATCC-3′ and anti-sense, 5′-CTCGACCGTATGTCGAGCGGAAAG-3′), whereas those used to detect Odc, Amd1, Srm, and ubiquitin (Ub)

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were as previously described (11). Relative mRNA levels were calculated using the ΔΔCT method, comparing expression of Odc, Amd1, Arf, and Srm with that of Ub.

Lysates were prepared from cultured cells, or tumors that originated in λ-Myc mice by pulverization in liquid nitrogen using a metal mortar followed by incubation in lysis buffer, vigorous vortexing, and sonication while it was kept on ice. Protein concentration was determined using the Bio-Rad protein determination kit. Fifty micrograms of protein per well was loaded on a Criterion 4% to 20% gradient Tris-HCl polyacrylamide gel and resolved by electrophoresis. Western blot was carried out according to standard methodologies and the antibodies used were from Sigma-Aldrich (β-actin, AC-15), Santa Cruz Biotechnology (p53, M-19; p19ARF, 5-C3-1; c-Myc, C-19; ER-α, MC-20), and from Dr. Gregor Laube at the Institute of Integrative Anatomy CCM, Berlin, Germany (Srm; ref. 15).

Polyamine analysis
The reversed-phase high-performance liquid chromatography (HPLC) method was used to quantify cellular polyamine content (16). Cell pellets were sonicated in 0.2 mol/L of perchloric acid and centrifuged at 20,000 × g (10 min, 4°C). The supernatant was analyzed using the Varian Vista 5500 liquid chromatography system, equipped with a Model 9090 Autosampler, a Model 2010 HPLC pump, a Model 2050 variable-wavelength UV detector, and a Fluorichrom fluorescence detector. The software Dynamax HPLC Method Manager and MacIntegrator (Rainin Instruments) were used along with a Macintosh SE/30 for method editing, HPLC control, as well as data collection and analysis (peak identification and quantification).

Results
Srm is a Myc target gene and RNAi-mediated knockdown reduces Myc-induced proliferation of fibroblasts
Odc is one of the most established Myc target genes, but a previous report (11) and the Myc target gene database suggest that other genes encoding polyamine biosynthetic

Fig. 1. Srm is a direct Myc target gene. A, qRT-PCR analysis of RNA from B cells of wild-type and λ-Myc mice and of tumors arising in the same mouse model. The mRNA levels of Odc and Srm were normalized to that of ubiquitin (Ub). B, genomic organization of the Srm gene showing schematically the position of five Myc-binding E-boxes. C, qRT-PCR analysis of RNA from NIH 3T3 cells infected with a Myc-ER–expressing retrovirus which had been treated with 4-HT to induce Myc nuclear translocation in the presence or absence of protein synthesis. Protein synthesis was blocked by the addition of cycloheximide (CHX). The mRNA levels of Srm and Amd1 were normalized to those of Ub. D, NIH 3T3 cells infected with a Myc-ER–expressing retrovirus were treated with 4-HT for 24 h. Protein levels were analyzed by Western blot using an Srm antibody, and an ER-α antibody for the detection of the Myc-ER fusion protein. E, P493-6 lymphoblastoid B cells were treated with tetracycline to repress the MYC transgene. After 48 h, the tetracycline was washed away to induce Myc expression and, after 24 h, Myc and Srm levels were determined by Western blot analysis.
enzymes may also be regulated by Myc, e.g., spermidine synthase (Srm) and S-adenosylmethionine decarboxylase (Amd1). In the present study, we have focused our attention on Srm (which forms spermidine from putrescine) and evaluated its regulation and importance in Myc overexpressing cells. To gain insight as to whether or not Srm might be involved in B-cell lymphomagenesis, we analyzed its mRNA levels by qRT-PCR in B cells from 6-week-old wild-type and λ-Myc mice, as well as in tumors that had arisen in the same mouse model. As previously reported for the Eμ-Myc mouse (11), Odc was induced by Myc in λ-Myc B cells before the onset of tumor development and was maintained at the same level or higher in most, but not all, tumors. Interestingly, Srm expression was elevated several-fold, and unlike Odc, there was not a single tumor expressing Srm to a lower or same degree as B cells from wild-type mice (Fig. 1A). This suggests that the Srm gene is as important for Myc-induced tumors as the Odc gene.

The fact that Srm is induced in B cells and tumors from λ-Myc mice does not reveal if the gene is a direct Myc target or if it is indirectly induced by, e.g., cell growth or a factor that is regulated by Myc. We therefore analyzed the mouse genome for the presence of E-boxes in the Srm regulatory region. As seen in Fig. 1B, Srm carries five canonical E-boxes (CACGTG), located just upstream and downstream of exon 1. To add additional formal evidence, we infected NIH 3T3 fibroblasts with a retrovirus expressing inducible Myc-ER. This construct expresses c-Myc fused to the ligand-binding domain of the estrogen receptor (ER), and the protein is therefore kept in the cytoplasm by heat shock proteins (17). Upon the addition of 4-HT, heat shock proteins could no longer bind the ER part, resulting in nuclear translocation and activation of Myc-induced gene transcription. Genes that are rapidly induced by 4-HT even in the presence of cycloheximide, a protein synthesis inhibitor, are dubbed true Myc targets. In this regard, Odc, Amd1, and Srm all qualify as such (Fig. 1C; refs. 18, 19). In addition, Myc activation also resulted in elevated protein levels of Srm in both fibroblasts expressing Myc-ER and in P493-6 lymphoblastoid cells expressing a tetracycline-regulated MYC oncogene (Fig. 1D and E).

Odc has been shown to be important for tumor development in mouse models of lymphoma, skin, prostate, breast, and colon cancer, and most recently, neuroblastoma (10, 11, 20–24). In the Eμ-Myc mouse, treatment with the suicide Odc enzyme inhibitor DFM0 reduced rates of proliferation of B cells and delayed lymphomagenesis significantly (11). We wanted to examine if Srm operates in a similar way by using RNAi as a genetic tool.
to assay the effect of the loss of Srm expression. We designed five different short hairpin RNAs against Srm and used retroviruses to deliver these into NIH 3T3 fibroblasts. Two short hairpin RNAs were very efficient in eliciting RNAi resulting in decreased Srm mRNA levels and protein as measured by qRT-PCR and Western blot analyses (Fig. 2A). Furthermore, measurements of polyamine levels by HPLC verified that loss of Srm expression indeed led to reduced enzyme activity. Expectedly, knockdown of Srm resulted in a substantial decrease in spermidine content of the cells and an accumulation of the precursor putrescine (Fig. 2B), which cannot be converted to spermidine in the absence of Srm.

With these RNAi tools in hand, we were able to determine the importance of Srm in Myc-overexpressing cells. We infected NIH 3T3 fibroblasts with control, Srm RNAi-1, or Srm RNAi-2–expressing retroviruses followed by retroviral infection with a Myc-expressing virus. Growth curves showed that Srm RNAi inhibited cell proliferation (Fig. 2C; Supplementary Fig. S1A). Because Myc can induce cellular transformation of murine fibroblasts with a defective Arf-p53 axis, e.g., NIH 3T3 cells which lack Arf, we were also able to study the effect of loss of Srm on Myc-induced transformation. Despite the potent effect of Srm loss on proliferation, the majority of Myc-overexpressing cells still became transformed cells in the absence of Srm but the colonies growing in soft agar were smaller (Fig. 2D; Supplementary Fig. S1B). Taken together, these data suggest that Srm contributes to Myc-induced proliferation and may therefore be an attractive target for inhibition in cancer cells that rely on Myc-induced proliferation.

**Pharmacologic inhibition of Srm is chemopreventive but not chemotherapeutic in vivo**

Human Burkitt lymphoma is characterized by a translocation of the MYC oncogene from its normal location on chromosome 8 to any of the three chromosomes (2, 14, or 22) carrying immunoglobulin light or heavy chains. The formal evidence that these translocations are oncogenic has come from the development of mouse models in which transgenic or knock-in mice have been created to mimic Burkitt lymphoma by placing Myc under the control of enhancers from IgM (Eμ) or IgL (λ) genes. In the present study, we used three different mouse models, the Eμ-Myc mouse, the iMycEμ mouse, and the λ-Myc mouse, which all develop Myc-induced B-cell lymphomas (Supplementary Fig. S2) of varying degrees of maturity and latency. Given the promising results with Srm RNAi in fibroblasts, we were interested in finding out if Srm inhibition would block Myc-driven B-cell lymphomas that had arisen in mouse models of Burkitt lymphoma. To that end, we first infected several B-cell lymphoma lines from Myc transgenic mice, exemplified here by Eμ#239 from the Eμ-Myc transgenic mouse, with control and Srm

![Fig. 3. Pharmacologic or genetic Srm inhibition reduces Myc-induced lymphoma cell growth in vitro and in vivo.](image-url)

**A**, a primary lymphoma line Eμ#239 was established from a tumor that had developed in an Eμ-Myc transgenic mouse. Eμ#239 was infected with either a control retrovirus or Srm RNAi-1, and growth rate was determined by total cell count over 48 h. **B**, to determine the effect of pharmacologic inhibition of Srm on Eμ#239 proliferation, cells were cultured with 100 or 250 μmol/L of 4MCHA. To avoid toxic effects due to amine oxidase–mediated degradation of 4MCHA, cells were grown in the presence of 1 mmol/L of aminoguanidine (a.g.). C, the in vivo chemopreventive effect of pharmacologic inhibition of Srm was determined using λ-Myc transgenic mice. λ-Myc mice were maintained on normal drinking water (untreated) or drinking water containing 0.025% 4MCHA. The mice were followed for signs of moribidity and were sacrificed if unhealthy. Median survival time was 96 d for untreated mice, and 136 d for 4MCHA-treated mice (P = 0.0001).
Role of Srm in Myc-Induced Cancers

RNAi-1—expressing retroviruses (Supplementary Fig. S3A) and assessed the effect on cell growth. Although there was a growth-suppressing effect in vitro (Fig. 3A), Srm RNAi-1 did not affect the ability of the tumor cells to engraft when transplanted into syngeneic recipients (Supplementary Fig. S3B), implying rescue by polyamine uptake. We also used a pharmacologic inhibitor of Srm, 4MCHA (25), to treat Eμ\textit{Myc} cells. As seen in Fig. 3B, 4MCHA concentrations previously used in the literature (26) slightly reduced the growth rates of these cells on the second day of treatment. We therefore decided to analyze the effect of 4MCHA in primary tumor cells transplanted into recipient mice. These cells have never been in culture, allowing us to exclude overestimations or underestimations of the data due to cell culture artifacts. As seen in Supplementary Fig. S3C, transplantation via tail vein injections of tumor cells from \textit{iMyc}\textsuperscript{Eμ} mice and \textit{λ-Myc} mice (data not shown) resulted in the reappearance of tumors in recipient C57/BL6 mice. However, administration of 0.025% 4MCHA in the drinking water did not delay tumor onset, again suggesting that uptake by tumor cells of polyamines in blood might compensate for Srm inhibition. We were not able to test higher concentrations because the mice would not tolerate higher doses, as previously described (25, 27, 28). We also tried the curative effect of 1% DFMO in the drinking water, alone or in combination with 4MCHA, with similar results (Supplementary Fig. S3D; data not shown). The lack of a curative effect of the polyamine synthesis inhibitors could not be attributed to the functional status of the p53 tumor suppressor because cells that had lost the upstream p53 regulator Arf or mutated p53 all developed into tumors when injected into mice (Supplementary Fig. S3E).

Previous studies have shown that Odc inhibition could block tumor development in mouse models and patients and thus show promise for chemoprevention (rather than chemotherapy) of human cancer (29). To test if Srm would be an alternative target in chemoprevention, we treated \textit{λ-Myc} mice with 4MCHA in their drinking water directly from weaning age onwards. As seen in Fig. 3C, 4MCHA effectively delayed tumor onset in \textit{λ-Myc} mice, from a median survival time of 95 to 136 days. These \textit{in vivo} data suggest that Srm is a promising target for chemoprevention against B-cell malignancies.

The most common proteins deregulated in B-cell lymphomagenesis in the \textit{Eμ-Myc} mouse are the tumor suppressors Arf and p53 (30–32), which are inactivated by deletion or mutation, and the Bcl-2 family of antiapoptotic proteins which is activated by poorly defined mechanisms (33). Interestingly, previous studies using DFMO in this model suggested that the compound reduced cell proliferation rates and mutation spectra as a means of preventing lymphomagenesis (11). To analyze what the consequence of long-term treatment of \textit{λ-Myc} mice with 4MCHA was on the mutation spectra, we performed Western blot and real-time PCR analyses on a panel of tumors from untreated and 4MCHA-treated animals. High levels or absent levels of p53 correlated with very strong expression of Arf, suggesting that the tumors contained nonfunctional p53 (and were therefore stable) or a deleted p53 gene so that Arf no longer could be repressed [ref. 34; see tumor nos. 1930, 2019, and 3484 of the untreated mice; and no. 1422 (high Arf RNA) and no. 2529 of the 4MCHA-treated mice in Supplementary Fig. S4A]. Very low or absent levels of Arf RNA was used to determine which tumors had lost the Arf gene (Supplementary Fig. S4B). Taken together, the analysis suggests that the rates of p53 mutation and the deletion rates in 4MCHA-treated animals (4 out of 10) are similar to that found in tumors from untreated animals (4 out of 10). The rate of Arf deletion (6 out of 10), in 4MCHA-treated animals was also similar to that found in untreated animals (6 out of 10).

Discussion

The Myc-to-polyamine synthesis axis is an attractive target for chemoprevention that has produced very promising results in recent clinical trials on colon cancer using doses of DFMO that were free of side effect (12, 35). Because Myc overexpression is a hallmark of cancer, it is likely that chemoprevention of a large number of tumors could be employed by inhibiting this pathway. However, the use of polyamine synthesis inhibitors, such as DFMO, against established tumors has been a large disappointment. Several explanations for the poor success have been proposed including rescue via transport by the tumor cells of dietary polyamines from the blood, poor uptake of the drug, and metabolism thereof (36). Some of these issues, we could observe here as well when using the Srm inhibitor 4MCHA, which inhibited the growth of lymphoma cells \textit{in vitro} but not \textit{in vivo}. If these issues could be handled without increasing the level of side effects associated with polyamine depletion, a place for Odc and/or Srm inhibitors in the arsenal against established tumors might still be possible.

A case for Srm has been neglected because Srm is constitutively expressed and inhibition has been proven inefficient in blocking the proliferation of several cell lines (36). As shown here, Srm is induced in every Myc-induced tumor and to a higher relative level than Odc. Despite this, Srm inhibition with 4MCHA did not affect tumor growth \textit{in vivo}, suggesting that it might complement rather than replace Odc as a target in the polyamine biosynthetic pathway. A concern about the specificity, efficacy, and bioavailability of 4MCHA could be raised but it seems sufficient to exert chemoprevention potential in \textit{λ-Myc} mice, and original studies showed specificity of 4MCHA to Srm over the highly related spermine synthase (25). The mice only tolerated weak concentrations of the drug, due to the taste, so alternative routes of administration or new drugs should be developed. Novel Srm inhibitors are being developed and these may provide additional complementation to the chemoprevention exerted by DFMO. If a patient will require life-long treatment with a chemopreventive agent, it is likely that some mode of resistance could develop involving, e.g., amplification of the \textit{Odc} gene (37), enhanced synthesis
of the protein (38), mutations in Odc or tumor suppressive pathways downstream of polyamine synthesis inhibition, or altered polyamine uptake. Changing from Odc to Srm inhibition in the midst of treatment may reduce the risk of resistance development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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