Molecular Pharmacology Fast Forward. Published on April 16, 2009 as doi:10.1124/mol.108.054411




# S-Adenosylmethionine and Methylthioadenosine Inhibit Cellular FLICE Inhibitory Protein Expression and Induce Apoptosis in Colon Cancer Cells

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Molecular Pharmacology Fast Forward. Published on April 16, 2009 as DOI: 10.1124/mol.108.054411
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***Running title***: SAMe and MTA lower cFLIP expression in colon cancer cells

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***Number of text pages:*** 22

***Number of tables:*** 1

***Number of figures:*** 9

***Number of references:*** 33

***Number of words in Abstract:*** 248

***Number of words in Introduction:*** 520

***Number of words in Discussion:*** 1600

***Abbreviations used*** (alphabetical order): 5-FU, 5-fluorouracil; BFAR, bifunctional apoptosis regulator; BIRC7, baculoviral inhibitor of apoptosis repeat-containing 7; cFLIP, cellular FLICE inhibitory protein; $cFLIP_L$, cFLIP-long; $cFLIP_S$, cFLIP-short; COX, Cytochrome *c* oxidase; DISC, death inducing signaling complex; EGF, epidermal growth factor; FBS, fetal bovine serum; IGF-1, insulin-like growth factor-1; MAT, methionine adenosyltransferase; MTA, methylthioadenosine; OXA, oxaliplatin; PCR, polymerase chain reaction; rTRAIL, recombinant TRAIL; SAMe, S-adenosylmethionine; TNFα, tumor necrosis factor α; TRAIL, TNFα-related apoptosis-inducing ligand; UTR, untranslated region.

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**ABSTRACT**

S-Adenosylmethionine (SAMe) and its metabolite methylthioadenosine (MTA) inhibit mitogen-induced proliferative response in liver and colon cancer cells. SAMe and MTA are also pro-apoptotic in liver cancer cells by selectively inducing Bcl-$x_S$ expression. The aims of this work were to assess whether these agents are pro-apoptotic in colon cancer cells and if so, elucidate the molecular mechanisms. We found both SAMe and MTA are pro-apoptotic in HT-29 and RKO cells in a dose- and time-dependent manner. Gene microarray uncovered down-regulation of cellular FLICE inhibitory protein (cFLIP). SAMe and MTA treatment led to a fall in the mRNA and protein levels of both the long and short cFLIP isoforms. This required de novo RNA synthesis and was associated with activation of procaspase-8, Bid cleavage, and release of cytochrome c from the mitochondria. Inhibiting caspase 8 activity or over-expression of cFLIP protected against apoptosis whereas supplementing with polyamines did not. SAMe and MTA treatment sensitized RKO cells to tumor necrosis factor α-related apoptosis-inducing ligand-induced apoptosis. While SAMe and MTA are pro-apoptotic in colon cancer cells, they have no toxic effects in NCM460 cells, a normal colon epithelial cell line. In contrast to liver cancer cells, SAMe and MTA had no effect on Bcl-$x_S$ expression in colon cancer cells. In conclusion, SAMe and MTA are pro-apoptotic in colon cancer cells but not normal colon epithelial cells. One molecular mechanism identified is inhibition of cFLIP expression. SAMe and MTA may be attractive agents in the chemoprevention and treatment of colon cancer.

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S-adenosylmethionine (SAMe) is the principal biological methyl donor and a precursor for polyamine biosynthesis in mammalian cells (Lu and Mato, 2005). SAMe is metabolized from methionine and ATP by methionine adenosyltransferase (MAT) (Lu and Mato, 2005). In mammals, there are three MAT enzymes MATI, MATII and MATIII (Kotb *et al.*, 1997). MAT1A and MAT2A are genes that encode for the catalytic subunits of MATI/MATIII and MATII, respectively (Kotb *et al.*, 1997). In the normal liver, where most of the SAMe is produced by MATI/MATIII, MAT1A is expressed, while MAT2A expression is absent (Lu and Mato, 2005). MAT2A is expressed in all other non-hepatic tissues, including colon (Horikawa and Tsukada, 1992; Kotb *et al.*, 1997).

Aberrant MAT2A expression in the liver has been linked to increased growth and dedifferentiation (Cai *et al.*, 1998; Cai *et al.*, 1996; Huang *et al.*, 1998; Huang *et al.*, 1999). In hepatocellular carcinoma, MAT2A is transcriptionally induced (Cai *et al.*, 1996; Lu and Mato, 2005). In addition, we recently showed that MAT2A expression is also increased in colon cancer cell lines, mouse intestinal polyps and human colon cancer tissue samples as compared to normal adjacent tissue (Chen *et al.*, 2007). Mitogens such as leptin, insulin-like growth factor-1 (IGF-1), and epidermal growth factor (EGF) induced MAT2A expression in colon cancer cell lines (Chen *et al.*, 2007). SAMe and 5'-methylthioadenosine (MTA), a byproduct of polyamine synthesis from SAMe catabolism as well a spontaneous breakdown product of SAMe, lowered MAT2A expression in colon cancer cell lines (Chen *et al.*, 2007). SAMe and MTA also prevented leptin, IGF-1 and EGF from inducing MAT2A expression and exerting their mitogenic effect in colon cancer cells (Chen *et al.*, 2007). SAMe and MTA also exerted an inhibitory effect on MAT2A expression in proximal small intestine when administered orally for six days (Chen *et al.*, 2007).

In addition to growth modulatory effects, SAMe and MTA regulate hepatocyte apoptotic response (Ansorena *et al.*, 2002; Yang *et al.*, 2004). Interestingly, while both agents are anti-apoptotic in normal hepatocytes, they are pro-apoptotic in liver cancer cells (Ansorena *et al.*, 2002). A key molecular mechanism for their differential pro-apoptotic effect in liver cancer cells is the selective induction of Bcl-$x_S$ expression (Yang *et al.*, 2004). This effect required the participation of protein phosphatase 1, which enhanced the alternative splicing of Bcl-x (Yang *et al.*, 2004). While we have shown that SAMe and MTA can inhibit mitogen-induced cell growth in colon cancer cells, whether they also modulate apoptosis in colon cancer cells remains unknown.

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The goals of this study were to first, examine the apoptotic potential of SAMe and MTA in colon cancer cells, second to identify novel gene targets that may be associated with the SAMe/MTA pro-apoptotic effect and third, to test the feasibility of SAMe and MTA as a potential therapeutic agent in colon cancer treatment. Our studies revealed that indeed, they are pro-apoptotic in colon cancer cells but not in normal colon epithelial cells. Importantly, the molecular mechanism is quite different from that in liver cancer cells. Our findings also support the notion that these agents may be useful in colon cancer chemotherapy.

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## MATERIALS AND METHODS

### *Materials*

Cell culture media for HT-29 and RKO cells were obtained from the USC Norris Cancer Center Cell Culture Core. Fetal bovine serum (FBS) was obtained from Omega Scientific (Tarzana, CA) and M3:Base culture media from INCELL Corporation (San Antonio, TX). SAMe in the form of disulfate p-toluenesulfonate dried powder was generously provided by Gnosis SRL (Cairate, Italy) and MTA was purchased from Sigma-Aldrich (St. Louis, MO). Caspase-8 and cFLIP antibodies were purchased from Alexis Biochemicals (San Diego, CA). $cFLIP_L$ and $cFLIP_S$ expression vectors were a kind gift from Dr. Jürg Tschopp (Dept of Biochemistry, University of Lausanne, Switzerland). All other reagents were of analytical grade and were obtained from commercial sources.

### *Cell Culture and SAMe, MTA, Caspase 8 Inhibitor, Polyamines, 5-FU, and rTRAIL Treatments*

HT-29 and RKO colon cancer cells were obtained from the Cell Culture Core of the USC Research Center for Liver Diseases. These cell lines were grown according to the conditions specified by the American Type Culture Collection (ATCC, Rockville, MD). NCM460 normal colon epithelial cells (Moyer *et al.*, 1996) were from INCELL Corporation (San Antonio, Texas) and grown in M3:Base cell culture medium supplemented with 10% FBS at 37ºC in a 5% $CO_2$ humidified incubator.

Cells were plated, grown to 50-60% confluence on 6 well plates and changed to media containing 1% FBS (RKO and HT-29) or 0% FBS (NCM460 cells) for 24 hours prior to treatment with either SAMe or MTA. Cells were treated with either SAMe (0.25mM to 5mM) or MTA (0.25mM to 2mM) in 0% FBS for 1 to 24 hours.

To test the effect of inhibiting caspase 8 on SAMe or MTA mediated apoptosis, RKO cells were treated with 2mM SAMe, 1mM MTA or vehicle in the presence or absence of 100μM Z-IETD-FMK, a caspase 8 inhibitor (Calbiochem, San Diego, CA). Cells were changed to 1% FBS for 24 hours and then to 0% FBS when treated with these agents.

In some experiments, RKO cells were treated with 1mM MTA or vehicle in the presence or absence of increasing amounts of polyamines (putrescine:spermidine:spermine) in a 1:18.5:86.8 ratio, for 24 hours. The ratios are to recapitulate intracellular polyamine ratios in control colon

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cancer cells (Chen *et al.*, 2007). Cells were changed to 1% FBS for 24 hours and then to 0% FBS when treated with these agents.

In other experiments, RKO cells were treated with 2mM SAMe, 1mM MTA, or 5μM of 5-FU (Sigma-Aldrich) alone or in combination with 100ng/mL of rTRAIL (EMD biosciences, San Diego, CA) for 24 hours. Cells were changed to 1% FBS for 24 hours prior to treatment and then to 0% FBS when treated with these agents.

***cFLIP Over-expression Studies***

RKO cells ($5x10^5$cells/well) were plated to 60-70% confluence in media containing 1% FBS. Two micrograms of $cFLIP_L$, $cFLIP_S$ or 1μg each of both isoforms were transfected using 3μL of Transmax (Generous gift from Dr. ZZ Wang, University of Southern California) transfection reagent for a total of 48 hours in 1% FBS. Twenty-four hours post-transfection, the media was replaced to 0% FBS and the cells were also treated with 2mM SAMe or 1mM MTA for 24 hours. Cells were either harvested to check for cFLIP protein expression or used for Hoechst staining to detect the level of apoptosis.

***Apoptosis Detection***

Apoptosis was measured using Hoechst staining and flow cytometry using the Apo-direct Kit (Pharmingen, San Diego, CA), which utilizes the TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) assay as we described (Yang *et al.*, 2008). Hoeschst staining and TUNEL yielded comparable results and were pooled for analysis.

The ApoAlert Cell Fractionation Kit (Clontech, Mountain View, CA) was used to isolate mitochondrial and cytosolic fractions of HT-29 cells treated with either 2mM SAMe or 1mM MTA for 24 hours. Western blot analysis was performed to detect cytochrome c release. Antibodies to Cytochrome *c* oxidase (COX) 4 and α tubulin (Cell Signaling Technology, Danvers, MA) were used as loading controls and to check the purity of the mitochrondrial and cytosolic fractions, respectively. The assay was performed according to the suggested manufacturer's protocol.

***RNA Isolation and Microarray Analysis***

HT-29 cells were treated with either 2mM SAMe or 1mM MTA for 6 hours and total RNA was extracted using the ArrayGrade Total RNA Isolation Kit (SABiosciences, Frederick, MD). One microgram of RNA was labeled and cRNA synthesized using TrueLabeling-AMP 2.0 kit (SABiosciences). cRNA cleanup was done using ArrayGrade cRNA Cleanup kit (SABiosciences).

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Four micrograms of cRNA was used to hybridize to an Oligo GEArray Human Apoptosis Array membrane containing apoptosis related genes (SABiosciences) at 60 ºC overnight. Chemiluminescent detection was done using Nonrad-GEArray Q series Detection Kit (SABiosciences) and exposed to autoradiographic film. Densitometric analysis was performed on the apoptosis Oligo GEArray using Bio-Rad's Quantity-One software (Hercules, CA).

***RNA Isolation and Quantitative Gene Expression Analysis***

RNA isolation, cDNA synthesis and real-time PCR analysis was done as previously described (Chen *et al*., 2007). Taqman probes to $cFLIP_L$, $cFLIP_S$, BFAR, TNF, IGF1R, Bcl-$x_S$, and the housekeeping genes (ubiquitin C or hypoxanthine phosphoribosyl-transferase-1) were purchased from Applied Biosystems (Foster City, CA).

***Effects of Actinomycin D and Cycloheximide on cFLIP Expression***

RKO cells were pretreated for 1 hour with 5μg/mL actinomycin D to inhibit de novo RNA synthesis or 10μg/mL cycloheximide to inhibit de novo protein synthesis followed by treatment with 1mM MTA or vehicle control for up to 4 hours. RNA was extracted for determination of cFLIP mRNA levels at different time points. Regression analysis using the best-fit line was used to calculate half lives of the cFLIP isoforms.

***Cell Viability Assay***

Cell viability was assayed using the 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based cell growth determination kit (Sigma-Aldrich). RKO or NCM460 cells were treated with 1mM SAMe or 1mM MTA for 24 hours in serum free media. The assay was performed according to the manufacturer's suggested protocol.

***Western Blot Analysis***

For cFLIP, Bid and caspase-8 expression, RKO cells were treated with either SAMe (0.5mM to 2mM) or MTA (0.25mM to 2mM) for 6 to 24 hours or transfected with cFLIP expression vectors for 48 hours. Western blot analysis was done as we previously described (Chen *et al.*, 2004) using anti-cFLIP, Bid (Cell Signaling Technology) and caspase-8 antibodies.

***Statistical analysis***

Data are given as mean±SEM. Statistical analysis was performed using ANOVA and Fisher's test. For mRNA and protein levels, ratios of genes and proteins to respective housekeeping densitometric values were compared. Significance was defined by $p<0.05$.

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## RESULTS

### *SAMe and MTA are Pro-apoptotic in Colon Cancer Cells*

Similar to their effects in liver cancer cells, SAMe and MTA exerted a pro-apoptotic effect in both HT-29 (Fig. 1A) and RKO (Fig. 1B) cells in a dose-dependent manner after 24 hours of treatment. The pro-apoptotic effect was noted after 16 hours (data not shown). Fig. 1C shows the typical appearance of RKO cells treated for 24 hours with 1mM MTA, with about a 3-fold increase in apoptotic cells.

### *Molecular Targets of SAMe and MTA*

Since SAMe and MTA can selectively induce Bcl-$x_S$ in liver cancer cells, we measured the mRNA levels of Bcl-$x_S$ following SAMe and MTA treatment in colon cancer cells. Unlike their effects in liver cancer cells, SAMe and MTA had no effect on the Bcl-$x_S$ mRNA level in colon cancer cells (not shown).

To elucidate the molecular targets of SAMe and MTA in their pro-apoptotic effects, we used a microarray approach to see their effects on the expression of pro-apoptotic or anti-apoptotic genes. No significant change in the mRNA level of pro-apoptotic genes was detected (not shown) but down-regulation in the expression of several anti-apoptotic genes was noted (Table 1). Of the anti-apoptotic genes included in the array, more than 40% reduced expression with either SAMe or MTA occurred with bifunctional apoptosis regulator (BFAR), baculoviral inhibitor of apoptosis repeat-containing 7 (BIRC7), CFLAR (same as cFLIP), and tumor necrosis factor (TNF), and 34% reduced expression was observed with IGF-1R (Table 1). Real-time polymerase chain reaction (PCR) analysis confirmed only CFLAR expression. Of note, only cFLIP-short ($cFLIP_S$) was included on the microarray. Using real-time PCR, we confirmed that in HT-29 cells, 2mM SAMe treatment lowered the mRNA levels of both cFLIP isoforms by 25-30% after 6 hours. One mM MTA was more potent, lowering the mRNA levels of the same genes by 50-60% after 6 hours (Fig. 2A). SAMe and MTA also exerted a dose-dependent (Fig. 2B), and time-dependent (Fig. 2C) inhibitory effect on the mRNA levels of both cFLIP isoforms in RKO cells. Western blot analysis performed after 6 hours of treatment with either SAMe or MTA showed lower protein levels of both cFLIP-long ($cFLIP_L$) and $cFLIP_S$ (Fig. 2D).

### *MTA's Inhibitory Effect on cFLIP Expression in Colon Cancer Cells Requires De Novo RNA But Not Protein Synthesis*

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To identify the molecular mechanism of MTA's inhibitory effect on cFLIP expression, RKO cells were pretreated with either actinomycin D or cycloheximide for 1 hour prior to 1mM MTA treatment for 1 to 4 hours. The half lives of $cFLIP_L$ and $cFLIP_S$ mRNAs are 4.8 hours and 5.2 hours, respectively. MTA treatment led to a more rapid fall in the mRNA levels of both cFLIP isoforms ($t_{1/2}$ for $cFLIP_L$ = 3.0 hours and $cFLIP_S$ = 3.6 hours) as compared to actinomycin D alone (Fig. 3A and 3B). However, in the presence of actinomycin D, MTA did not exert any additional inhibitory effect. Cycloheximide pretreatment increased the mRNA levels of both cFLIP isoforms but had no effect on the reduction of cFLIP mRNA levels in MTA treated RKO cells (Fig. 3C and 3D).

***Activation of Procaspase-8 and Release of Cytochrome c***

cFLIP is structurally related to procaspase-8 but lacks enzymatic activity (Sharp *et al.*, 2005). It is anti-apoptotic by preventing caspase-8 activation (Sharp *et al.*, 2005). Consistent with this, treatment of either SAMe or MTA for 6 hours led to the activation of procaspase-8 (Fig. 4A) and the release of cytochrome c from mitochondria (Fig. 5B). In addition, an inhibitor to caspase 8 was able to significantly block the SAMe or MTA-mediated apoptosis in RKO cells (Fig. 4B). Truncated Bid, cleaved by active caspase 8, can lead to the release of cytochrome c (Yin, 2006). Figure 5A shows that MTA treatment led to a clear increase in Bid cleavage.

***Role of cFLIP on SAMe and MTA-induced Apoptosis***

To see whether down-regulation of cFLIP is the cause of SAMe and MTA-induced apoptosis in colon cancer cells, cells were transfected with over-expression vector for $cFLIP_L$ and/or $cFLIP_S$ for 24 hours prior to treatment with SAMe or MTA. Figure 6 shows that over-expression of either $cFLIP_L$ or $cFLIP_S$ alone or together prevented the ability of SAMe and MTA to induce apoptosis.

***Role of Polyamines in MTA-Induced Apoptosis in Colon Cancer Cells***

We have shown previously that 6 hour MTA treatment of colon cancer cells reduced polyamine levels (Chen *et al.*, 2007). To determine whether MTA's pro-apoptotic effect is due to a reduction of polyamine levels, we added polyamines to MTA treated RKO cells for 24 hours. There is a dose-dependent significant pro-apoptotic effect of the polyamines in RKO cells but lower doses of polyamines (50 or 100 pmol/L of polyamines with respect to putrescine) had a tendency (not statistically significant) to reduce the level of apoptosis in MTA-treated RKO cells (Figure 7).

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***SAMe and MTA Sensitize RKO Cells to tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL)-induced Apoptosis***

Colon cancer is known to have over-expression of cFLIP (Zhou *et al.*, 2005) and $cFLIP_L$ inhibited chemotherapy-induced colorectal cancer cell death (Longley *et al.*, 2006). To see whether SAMe and MTA can enhance the ability of recombinant TRAIL (rTRAIL) to induce apoptosis, we compared the effect of SAMe and MTA to that of 5-fluorouracil (5-FU) on rTRAIL-induced apoptosis. Figure 8A shows that while rTRAIL by itself had a small inductive effect on apoptosis, co-treatment with SAMe, MTA or 5-FU all enhanced the pro-apoptotic effect. This enhanced pro-apoptotic effect correlates with increased activation of procaspase 8, especially with MTA (Fig. 8B). Of the three agents, MTA in particular had the most dramatic effect on promoting the apoptotic effect.

***SAMe and MTA are Not Toxic to NCM460 cells***

NCM460 cells are normal colon epithelial cells (Moyer *et al.*, 1996). We examined the effect of SAMe and MTA at doses that are toxic to colon cancer cells in this cell line. Figures 9A, 9B and 1 show that SAMe and MTA are selectively toxic to colon cancer cells but have no harmful effect on NCM460 cells. Both agents had no effect on Bcl-$x_S$ expression but interestingly, MTA but not SAMe, reduced cFLIP expression (Fig. 9C).

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## DISCUSSION

SAMe is essential for life as the principal biological methyl donor and precursor for polyamines (Lu and Mato, 2005; Mato *et al.*, 2002). In contrast to SAMe, MTA inhibits transmethylation and polyamine biosynthesis (Clarke, 2006). SAMe is highly unstable with significant conversion to MTA when delivered exogenously (Chen *et al.*, 2007). Given the ready availability of SAMe as a nutritional supplement in the United States and as a therapeutic agent elsewhere, it is important to juxtapose actions of SAMe and MTA. MTA has thus far recapitulated SAMe's effect on MAT2A and TNFα expression (Chen *et al.*, 2007; Veal *et al.*, 2004), growth and apoptosis (Ansorena *et al.*, 2002; Ramani *et al.*, 2008; Yang *et al.*, 2004), at doses lower than SAMe. Our speculation is that many pharmacologic actions of SAMe may actually be mediated by MTA.

In this work we have examined the actions of SAMe and MTA on apoptosis in colon cancer cell lines RKO and HT-29 as compared to the normal colon epithelial cell line NCM460. This work was prompted by our findings that these agents modulate growth in liver and colon cancer cells and are selectively pro-apoptotic in liver cancer cells (Ansorena *et al.*, 2002; Chen *et al.*, 2007; Ramani *et al.*, 2008; Yang *et al.*, 2004). If they are also selectively pro-apoptotic in colon cancer cells, then they might be of great value in either chemoprevention or treatment of colon neoplasia.

Both SAMe and MTA exerted a pro-apoptotic effect in HT-29 and RKO cells in a dose- and time-dependent manner. HT-29 and RKO differ in p53 status - HT-29 cells express inactive p53, while RKO cells express wild type p53 (ATCC). Thus, the pro-apoptotic effects of SAMe and MTA are independent of p53. SAMe and MTA treatment increased procaspase 8 cleavage and MTA in particular increased Bid cleavage. Treatment with SAMe and MTA also resulted in cytochrome c release, implicating mitochondrial involvement in the process. Importantly, both agents are not toxic to NCM460 cells, which was originally established in 1996 as the first non-malignant colon epithelial cell line derived from the transverse colon (Moyer *et al.*, 1996). NCM460 cells are non-tumorigenic, have similar features as normal human colonocytes in primary culture and have served as an excellent in vitro model of normal colon epithelial cells (Moyer *et al.*, 1996). The fact that SAMe is non-toxic to normal colon epithelial cells is consistent with its excellent safety profile and lack of significant side effects (Mato JM, 2005). Although MTA reduced cFLIP mRNA expression, it did not induce apoptosis. This suggests that NCM460 cells

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may have protective mechanism(s) to prevent the induction of apoptosis with reduced cFLIP expression.

SAMe and MTA's selective pro-apoptotic effects in liver cancer cells is a result of inducing Bcl-$x_S$ in cancerous but not normal hepatocytes (Yang *et al.*, 2004). However, these agents had no effect on Bcl-$x_S$ expression in colon cancer cell lines, suggesting the molecular mechanisms differ in liver versus colon cancer cells.

To gain insight into the mechanism of exogenous SAMe and MTA-induced apoptosis, we used a microarray approach focusing on apoptosis related genes. Of the several genes altered, the one that intrigued us is cFLIP, which was down regulated particularly by MTA. Only 3 of the 11 splice variants of the cFLIP gene are translated ($cFLIP_L$, $cFLIP_S$, and $cFLIP_R$) (Golks *et al.*, 2005; Tschopp *et al.*, 1998). $cFLIP_R$ was recently found to be expressed exclusively in the Raji B cell line (Golks *et al.*, 2005), whereas $cFLIP_L$ and $cFLIP_S$ are widely expressed. $cFLIP_L$ is a catalytically inactive version of procaspase-8 (Budd *et al.*, 2006). $cFLIP_S$ is a truncated form that lacks the caspase-like domain (Budd *et al.*, 2006). cFLIP proteins are important in lymphocyte activation and development, as wells as interact with caspase-8 to modulate the death receptor mediated apoptotic signal (Budd *et al.*, 2006; Sharp *et al.*, 2005). $cFLIP_S$ can act as a dominant negative to compete with procaspase-8 for recruitment to the death inducing signaling complex (DISC) (Budd *et al.*, 2006). $cFLIP_L$ can also compete with procaspase-8 for binding to the DISC, but it also form heterodimers with procaspase-8 at the DISC (Budd *et al.*, 2006). This interaction allows for partial cleavage of procaspase-8, but prevents its full activation thus limiting its pro-apoptotic activity in the cell (Budd *et al.*, 2006). Therefore, the balance between caspase-8 and cFLIP is crucial in determining the apoptotic potential of a cell.

Both SAMe and MTA treatment down-regulated the expression of both cFLIP isoforms at the mRNA and protein levels in a dose- and time-dependent manner with MTA exerting a much more potent effect at comparable doses. The effect requires de novo synthesis of RNA (but not protein) as in the presence of actinomycin D, MTA no longer exhibited an inhibitory effect. Although this may signal a transcriptional mechanism, it is interesting to note that cFLIP isoform mRNA levels fell more rapidly following MTA treatment than actinomycin D alone. This suggests MTA also accelerated the cFLIP mRNA degradation but this requires de novo RNA synthesis. The exact molecular mechanism of this effect will require additional work to elucidate. Cycloheximide

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treatment increased the mRNA levels of both cFLIP isoforms. This is consistent with the ability of cycloheximide to prevent degradation of labile mRNAs (Ohh and Takei, 1995).

Consistent with cFLIP's known role in preventing procaspase-8 activation, treatment with SAMe or MTA led to procaspase-8 activation. Importantly, inhibition of caspase 8 attenuated SAMe and MTA's pro-apoptotic effect on colon cancer cells, thus confirming the importance of caspase 8 in SAMe/MTA-mediated apoptosis in these cells. In addition, over-expression with either cFLIP isoform prevented apoptosis induced by either SAMe or MTA, supporting an important role for cFLIP down-regulation in the apoptotic event.

We previously reported that 6 hour MTA treatment in colon cancer cells depleted intracellular polyamine levels (Chen *et al.*, 2007), which can lead to apoptosis (Seiler and Raul, 2005). We co-treated RKO cells with MTA and varying doses of polyamines to see if the pro-apoptotic effect of MTA can be blocked. Polyamines increased apoptosis in a dose-dependent manner. While the role of polyamines in growth is well accepted, their role in apoptosis is often contradictory. Polyamine depletion has been shown to both enhance and protect against apoptosis, in a cell line and apoptotic stimulus-dependent manner (Seiler and Raul, 2005). Complicating this further, excessive polyamines can also activate apoptosis (Seiler and Raul, 2005). Taken together, although polyamine depletion may have contributed to MTA's pro-apoptotic effect, it is unlikely to be a dominant mechanism.

cFLIP is overexpressed in many cancers including colon cancer (Longley *et al.*, 2006; Ryu *et al.*, 2001; Zhou *et al.*, 2005). A number of studies have demonstrated that over-expression of cFLIP protects cancer cells from apoptosis induced by a wide variety of chemotherapeutic drugs (Kruyt, 2008). In one study, $cFLIP_L$ over-expression in HCT116 cells reduced the apoptotic potential of 5-FU, oxaliplatin (OXA), and irinotecan (CPT-11); whereas siRNA knockdown of cFLIP in RKO and HCT116 cells increased the potency of these same chemotherapeutic drugs (Longley *et al.*, 2006). Recently, many studies have focused on the potential use of TRAIL as a chemotherapeutic agent in treating cancer. TRAIL, a ligand for the TNF superfamily and a potent activator of the extrinsic apoptosis pathway, is currently in phase I clinical trials (Kruyt, 2008; Zhang and Fang, 2005). TRAIL is attractive as a cancer therapy drug because it causes apoptosis in cancer cells but has minimal toxicity in most normal cells (Pitti *et al.*, 1996; Wiley *et al.*, 1995). However, a number of cancer cell lines are chemoresistant to TRAIL, which might also translate to human

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tumors (Kruyt, 2008; Zhang and Fang, 2005). Increased expression of cFLIP may be a major factor in chemoresistance to TRAIL (Kruyt, 2008; Zhang and Fang, 2005). 5-FU, OXA and CPT-11 have been shown to enhance sensitivity and potency of TRAIL in HCT116 cells (Longley *et al.*, 2006) by reducing the expression of both cFLIP isoforms (Galligan *et al.*, 2005). This makes cFLIP an increasingly attractive target for improving TRAIL mediated apoptosis in resistant cancers.

To examine whether SAMe and MTA can also sensitize TRAIL resistant colon cancer cell lines to apoptosis, we used TRAIL resistant RKO cells (Vasilevskaya and O'Dwyer, 2005). Simultaneous treatment of either 2mM SAMe or 1mM MTA with 100ng/mL of TRAIL increased cellular apoptosis by 60% and 160%, respectively, as compared to TRAIL alone. MTA appears to have a greater effect on TRAIL mediated apoptosis in RKO cells, which may be due to the greater effect MTA has on cFLIP expression and procaspase 8 activation. The magnitude of apoptosis was even higher than combination treatment of 5-FU and TRAIL. However, 5-FU is indiscriminate in killing either cancer or normal cells, while SAMe and MTA, like TRAIL, only target cancer cells. In addition, SAMe and MTA are naturally occurring molecules found in every person, thus increasing the likelihood of having fewer toxic side effects.

In summary, SAMe or MTA was able to induce apoptosis in colon cancer cell lines but not in normal colon epithelial cells. This mimics the effect of SAMe and MTA on liver cancer cells but with different molecular mechanism(s). The apoptotic signal may be due to reduced cFLIP expression in the treated cells as cFLIP over-expression was able to prevent SAMe and MTA-induced apoptosis. Finally, our results suggest that SAMe and MTA can enhance the ability of TRAIL to induce apoptosis in colon cancer cells. Overall, the results suggests that SAMe and MTA via the down-regulation of cFLIP can be potentially effective and specific chemopreventive and chemotherapeutic agents in the treatment of colonic neoplasia.

Molecular Pharmacology Fast Forward. Published on April 16, 2009 as DOI: 10.1124/mol.108.054411
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Molecular Pharmacology Fast Forward. Published on April 16, 2009 as DOI: 10.1124/mol.108.054411
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***Grant Support****:* This work was supported by NIH grants AT004896 (S. C. Lu, J. M. Mato, and T. K. Aw) and DK44510 (T. K. Aw). RKO and HT-29 cells were provided by the Cell Culture Core and confocal microscope was provided by the Cell Imaging Core of the USC Research Center for Liver Diseases (DK48522).

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Table 1

Effects of SAMe and MTA Treatment on Expression of Anti-Apoptotic Genes in HT-29 Cells

| Genes | 2 mM SAMe | 1 mM MTA |
|---|---|---|
| | Fold of untreated control | Fold of untreated control |
| BAG1 | 1.05 | 0.90 |
| BAG3 | 1.214 | 1.08 |
| BAG4 | 1.03 | 1.02 |
| BCL2L2 | 0.97 | 0.91 |
| BCL2L10 | 1.38 | 0.93 |
| BFAR | 0.55 | 0.60 |
| BIRC1 | 0.97 | 0.96 |
| BIRC6 | 0.95 | 0.86 |
| BIRC7 | 0.82 | 0.56 |
| BRAF | 0.99 | 0.72 |
| CFLAR | 1.19 | 0.45 |
| IGF1R | 0.74 | 0.66 |
| MCL1 | 1.12 | 0.98 |
| TNF | 0.80 | 0.36 |

HT-29 cells were treated with 2mM SAMe or 1mM MTA for 6 hours and subjected to microarray analysis as described in Methods. See list of genes in Apoptotic Array (SABiosciences, Bioscience).

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**FIGURE LEGENDS**

**Fig. 1** SAMe and MTA are pro-apoptotic in HT-29 and RKO cells. HT-29 and RKO cells were treated with SAMe and MTA at varying doses for 24 hours and apoptosis was measured as described in Methods. **Part A)** effect in HT-29 cells, **part B)** effect in RKO cells, **part C)** Hoechst staining after 24 hrs of MTA treatment in representative RKO cells. *$p<0.05$ vs. control, †$p<0.01$ vs. control from 3-4 experiments.

**Fig. 2** SAMe and MTA inhibit cFLIP expression in colon cancer cells. **Part A)** HT-29 cells were treated with 2mM SAMe or 1mM MTA and cFLIP isoform mRNA levels were determined by real-time PCR as described in Methods. *$p<0.01$, †$p<0.001$ vs. control from 3 to 4 experiments. **Part B)** RKO cells were treated with SAMe (0.25-5mM) (left panel) or MTA (0.25-2mM) (right panel) for 6 hrs and cFLIP isoform mRNA levels were measured using real-time PCR. **Part C)** RKO cells were treated with 1mM MTA and cFLIP isoform mRNA levels were determined after one to 16 hours. **Part D)** RKO cells were treated with SAMe or MTA at the given doses and protein levels of cFLIP isoforms were determined by Western blots analysis using 80µg protein per lane. Numbers below each blot are densitometric values expressed as % of control after normalizing to actin. Representative blots from 3 separate experiments are shown.

**Fig. 3** MTA's effect on cFLIP mRNA expression in colon cancer cells requires de novo RNA synthesis but not de novo protein synthesis. **Parts A and B)** RKO cells were pretreated with either 5µg/mL actinomycin D (Act D) or vehicle (ETOH) for 1 hour prior to either 1mM MTA treatment or vehicle control (DMSO) for 1 to 4 hours. RNA was extracted at each time point and cFLIP expression assayed by quantitative real-time PCR analysis **Part A** for $cFLIP_L$, **Part B** for $cFLIP_S$). Results are expressed as the average ± SE of 5 independent experiments. Linear regression analysis with best-fit lines provided formulas for calculation of $t_{1/2}$. *$p<0.01$ vs. Act D+MTA. **Part C and D)** De novo protein synthesis is not required for cFLIP mRNA down-regulation by MTA in colon cancer cells. RKO cells were pretreated with either 10µg/mL cycloheximide (CHX) or vehicle (ETOH) for 1 hour before treatment with either 1mM MTA or vehicle control (DMSO) for 1 to 4 hours.

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RNA was extracted for each time point and cFLIP expression levels determined by quantitative real-time PCR (**Part C.** for $cFLIP_L$, **Part D.** for $cFLIP_S$). Results are expressed as the average ± SE of 3 to 5 experiments.

**Fig. 4** SAMe and MTA activates procaspase-8 in colon cancer cells and is required for SAMe or MTA-mediated apoptosis of colon cancer cells. **Part A)** RKO cells were treated with SAMe or MTA for 6 hours and Western blot analysis was performed for various forms of caspase-8 using 80µg protein per lane. Densitometric values (mean±SE) are % of control, which are normalized to actin for loading control (bar graph below the Western blot). Blot is representative of 3 independent experiments. *p<0.005 vs. control. **Part B)** RKO cells were co-treated with either 2mM SAMe or 1mM MTA with 100µM caspase 8 inhibitor for 24 hours. Apoptosis was determined as described in Methods. Results are mean±SE from 3 to 6 experiments. *p<0.05 vs. control, **p<0.001 vs. control, †p<0.05 vs. SAMe, ††p<0.05 vs. MTA.

**Fig. 5** SAMe or MTA can affect downstream components of the apoptotic pathway. **Part A)** RKO cells were treated with either SAMe or MTA at the indicated doses for 24 hours. Western blot analysis against Bid was done with 80µg of protein per lane. The graph below represent mean±SE densitometric values from 3 experiments. *p<0.05 vs. control, †p<0.005 vs. control. **Part B)** HT-29 cells were treated with 2mM SAMe or 1mM MTA for 24 hours and cytoplasmic and mitochondrial cytochrome c levels were measured by Western blot analysis using 10µg protein per lane as described in Methods. The bar graph below represents mean±SE densitometric values expressed as % of control cytosolic fraction cytochrome c levels from 3 experiments. *p<0.05 vs. control.

**Fig. 6** cFLIP over-expression protects against SAMe and MTA-induced apoptosis. RKO cells were transfected with empty vector (control), $cFLIP_L$, $cFLIP_S$ or $cFLIP_{L/S}$ vectors for 48 hours as described in Methods. **Part A)** shows the effect of over-expression on cFLIP protein levels on Western blot analysis using 20µg protein per lane. **Part B)** Treatment with 2mM SAMe or 1mM MTA was started 24 hours after transfection for another 24

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hours. *p<0.001 vs. control+empty vector, †p<0.001 vs. SAMe+empty vector, ††p<0.001 vs. MTA+empty vector from 6 to 9 experiments.

**Fig. 7** Effects of polyamine addition on MTA's pro-apoptotic effect in colon cancer cells. RKO cells were treated with either 1mM MTA or vehicle control with increasing amounts of polyamines concurrently for 24 hours as described in Methods. Apoptosis levels were determined by Hoechst staining. Results represent an average ± SE of 6 independent experiments. *p<0.01 vs. untreated control, †p<0.05 vs. untreated control.

**Fig. 8** SAMe and MTA sensitize RKO cells to TRAIL-induced apoptosis. **Part A)** RKO cells were treated with 2mM SAMe, 1mM MTA, 100ng/mL TRAIL, 5μM 5-FU, alone or in combination for 24 hours and apoptosis was determined as described in Methods. Results are expressed as mean ± SE from 4 to 7 experiments. *p<0.05 vs. control, ** p<0.001 vs. control, †p<0.05 vs. TRAIL, ††p<0.05 vs. MTA or TRAIL, †*p<0.05 vs. 5-FU or TRAIL. **Part B)** Western blot analysis of caspase 8 using 80μg of total protein of the various treatments as above. The bar graph below the blot represent densitometric results shown as mean±SE from 3 experiments. *p<0.01 vs. control or TRAIL, **p<0.05 vs. control or TRAIL, †p<0.05 vs. MTA.

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**Fig. 9** SAMe and MTA are selectively toxic to colon cancer cells. **Part A)** NCM460 and RKO cells were treated with 1mM SAMe or MTA for 24 hours and MTT assay was used to measure cell viability as described in Methods. Results are mean±SE from 4 experiments. *p<0.05 vs. control, †p<0.01 vs. control. **Part B)** NCM460 cells were treated with varying doses of SAMe or MTA for 24 hours. Apoptosis levels were determined as mean±SE from 3 experiments as described in Methods. **Part C)** NCM460 cells were treated with either 2mM SAMe or 1mM MTA for 4 hours and total RNA isolated as described in Methods. Real-time PCR analysis was done using Taqman probes to Bcl-$x_S$ and both cFLIP isoforms. Results are mean±SE from 3 experiments. *p<0.001 vs. control.

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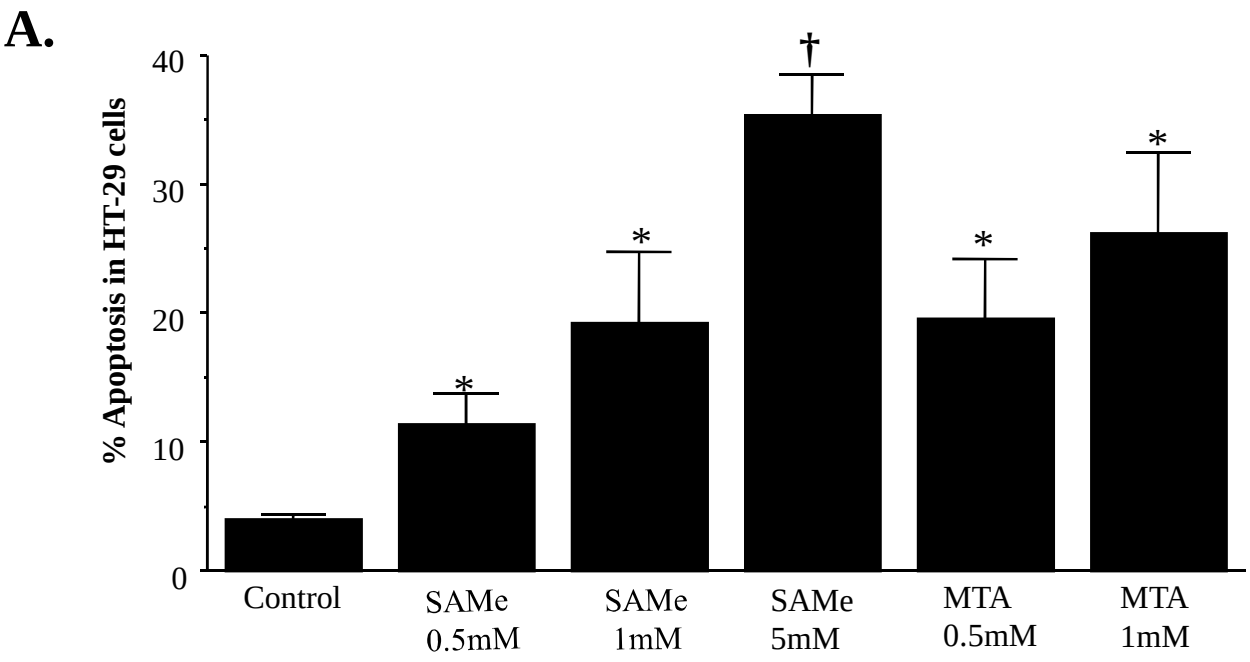


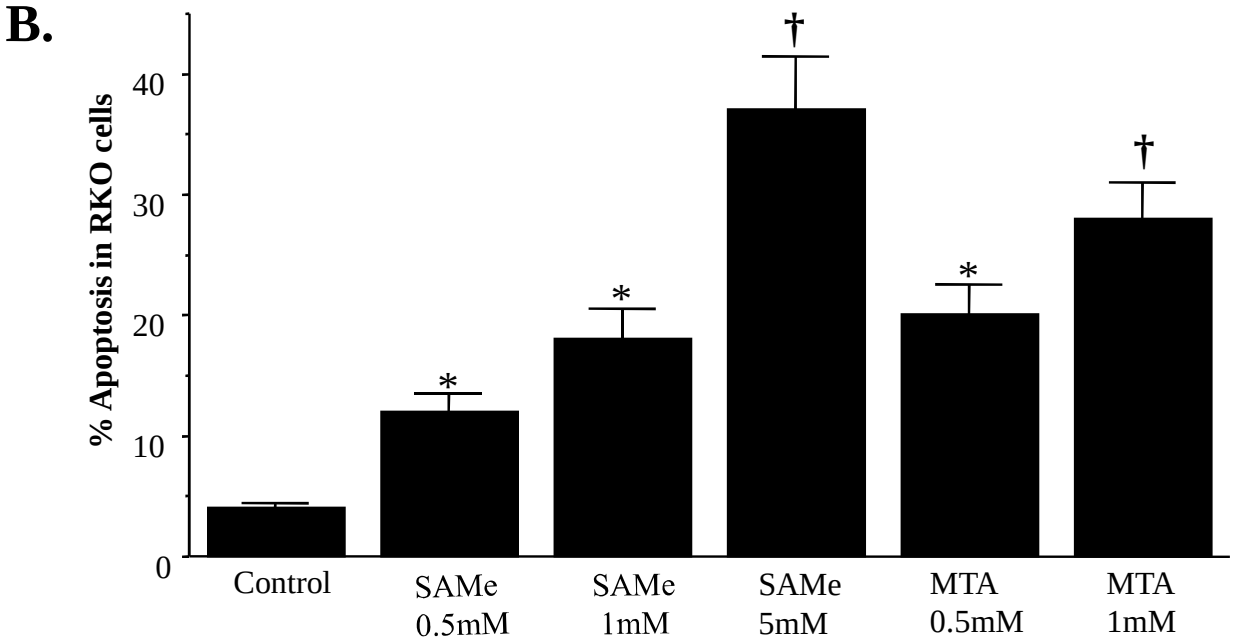


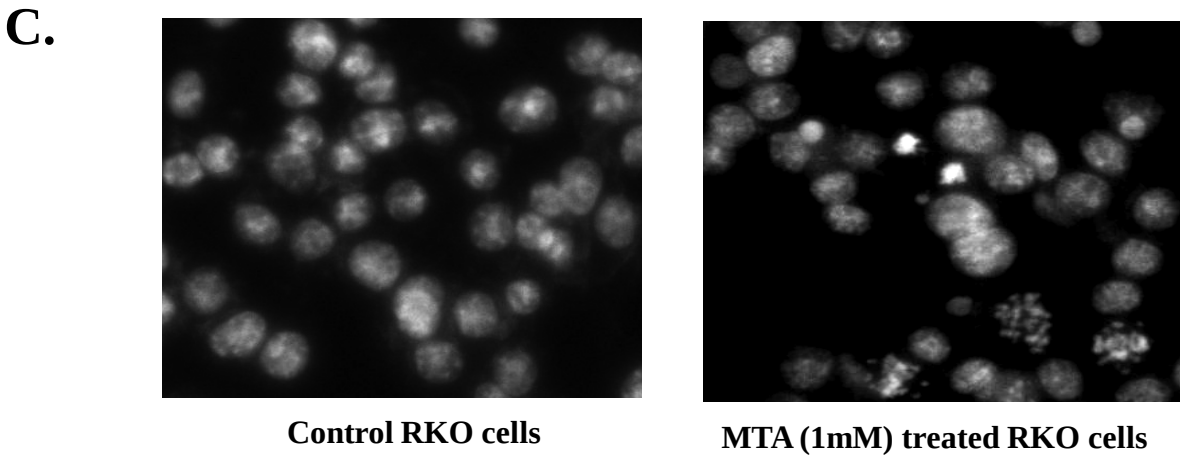


**Figure 1**

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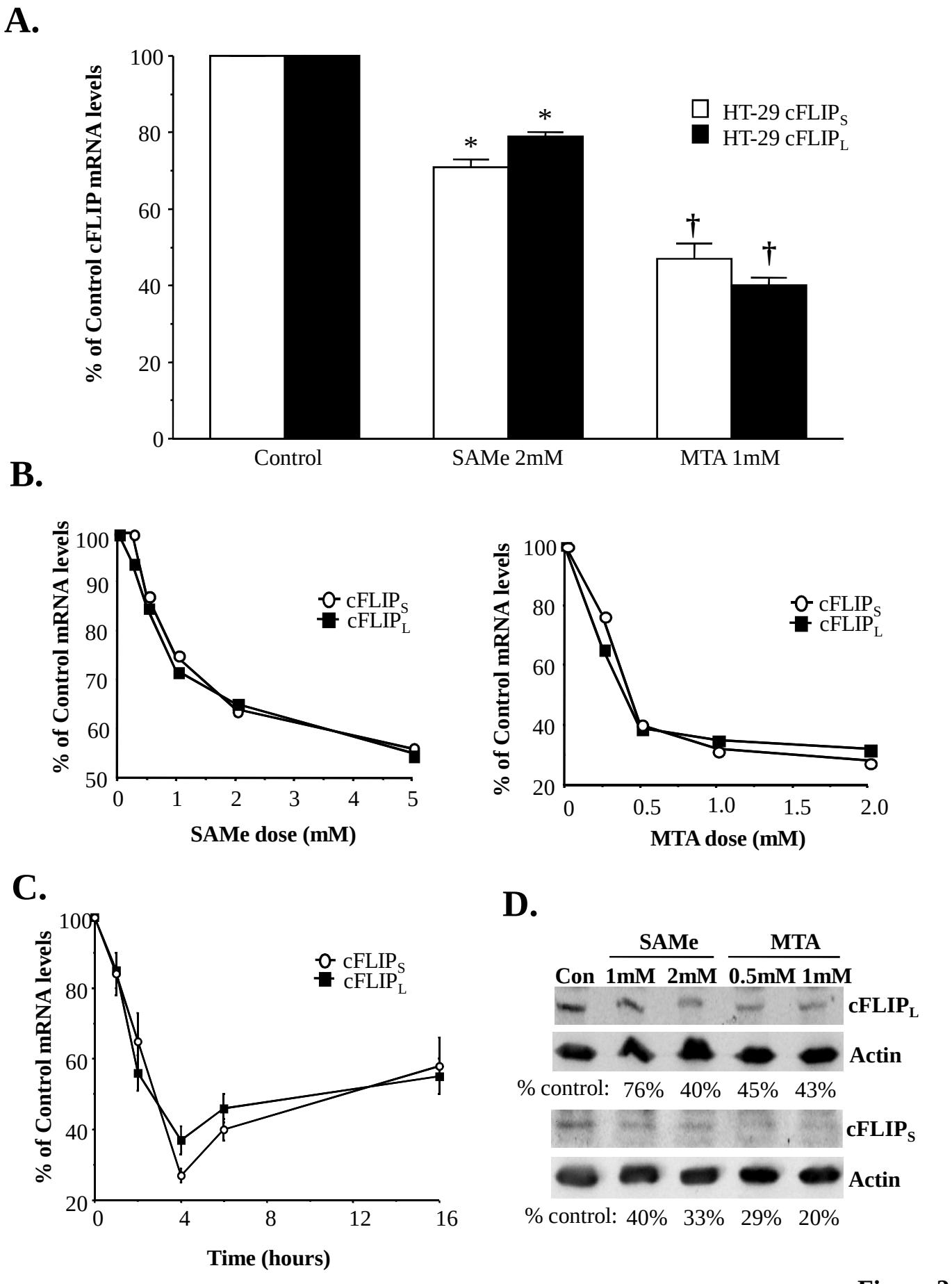


**Figure 2**

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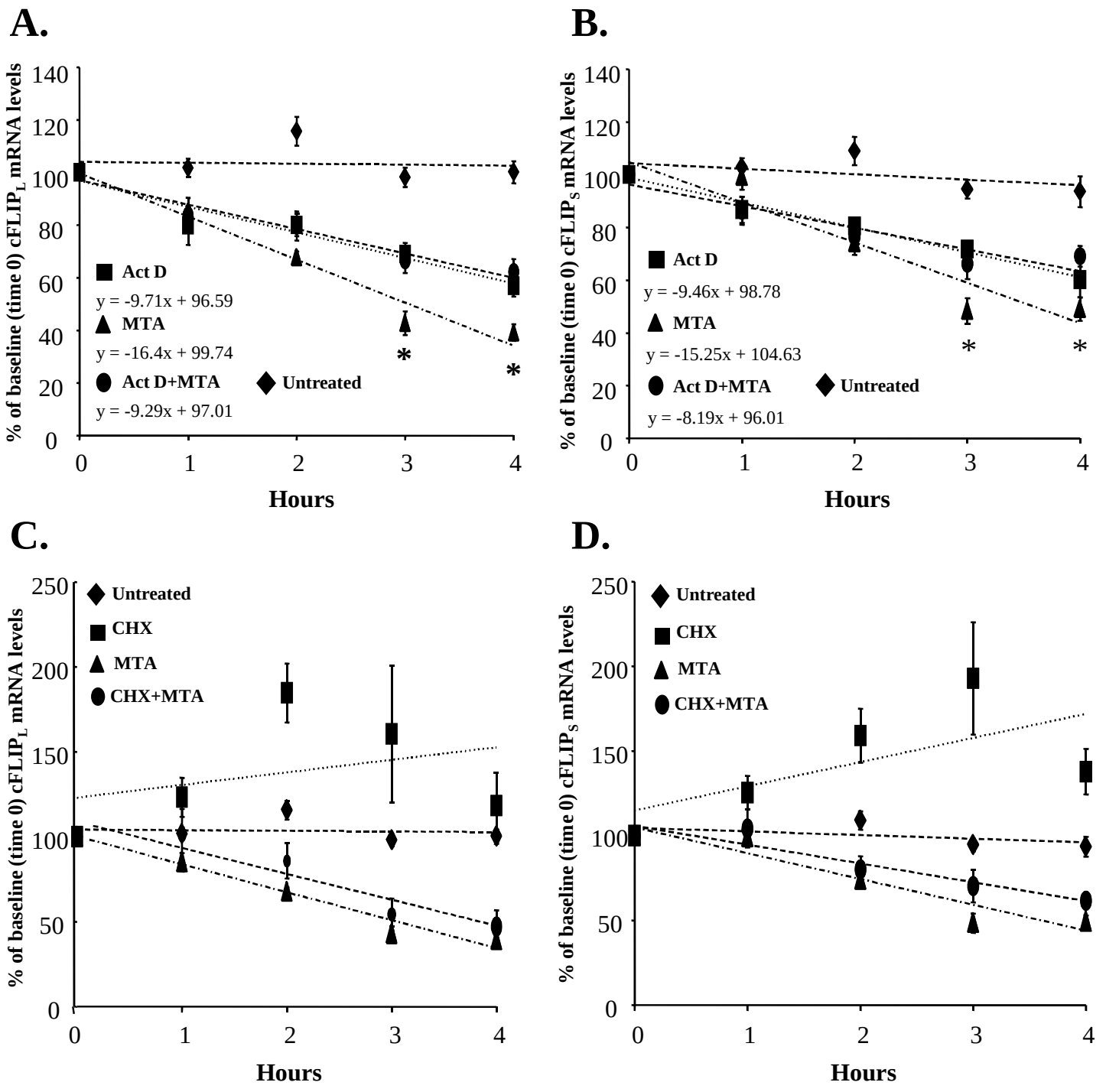


**Figure 3**

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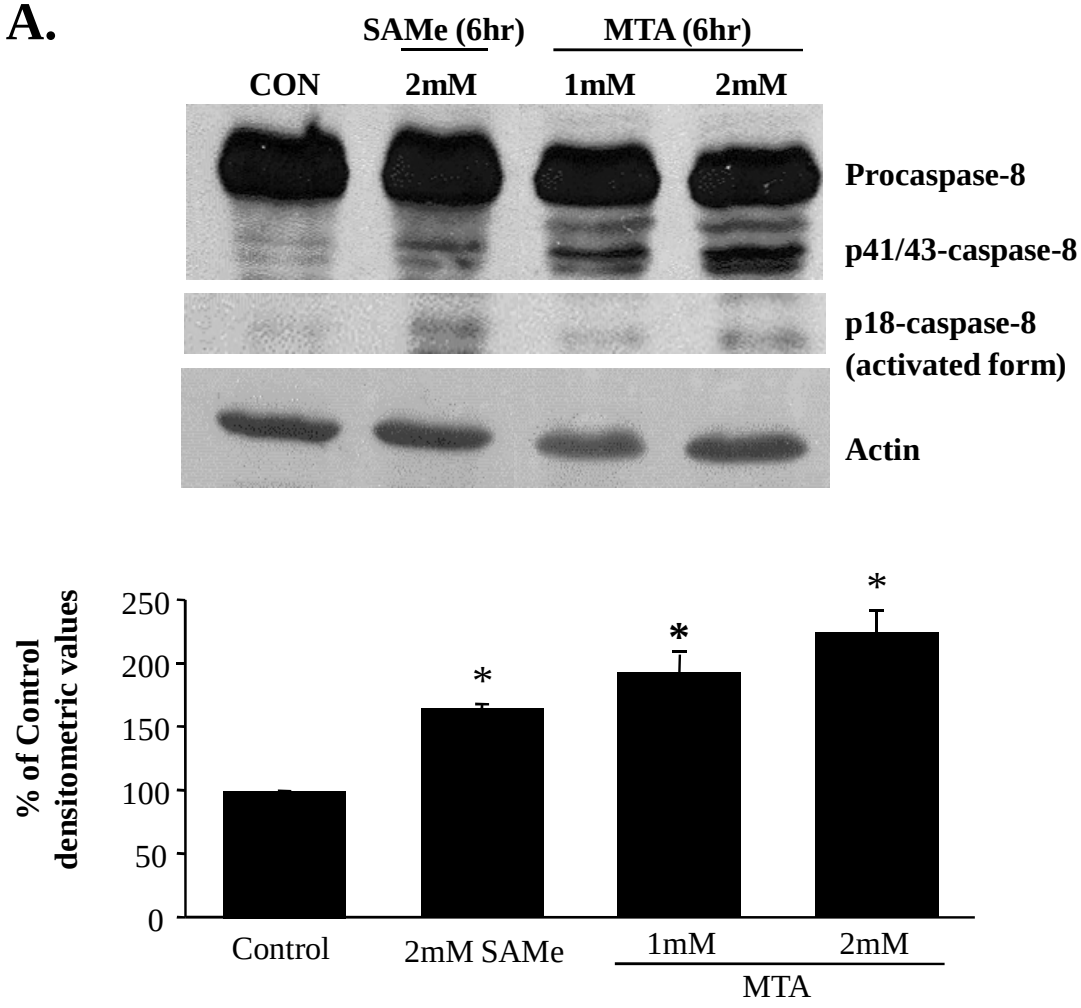
**A.**




**B.**

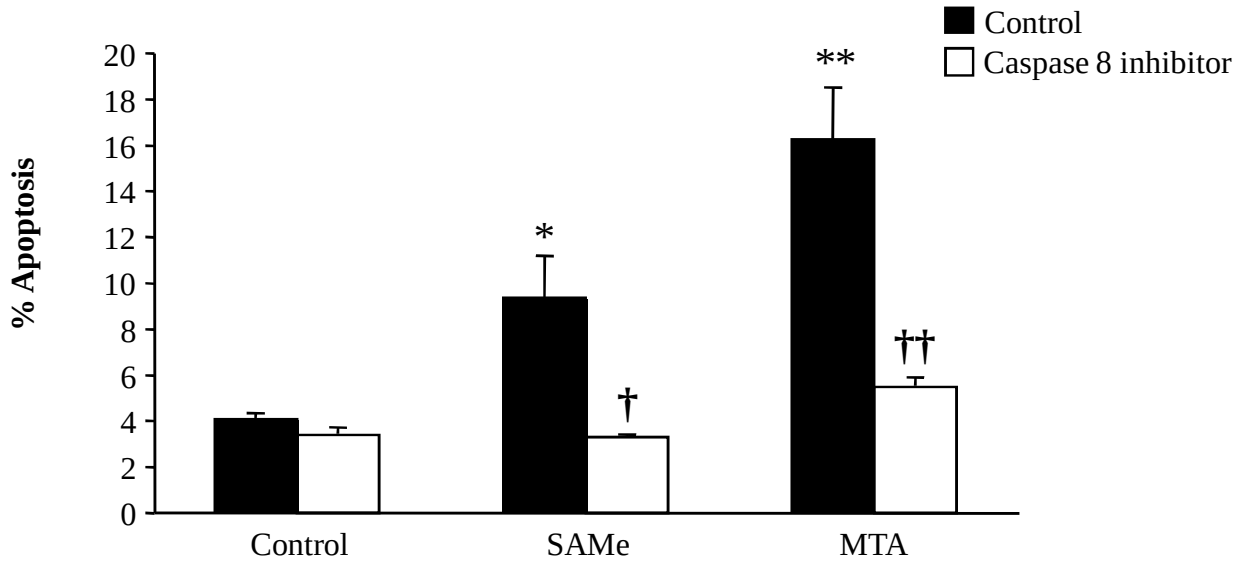



**Figure 4**

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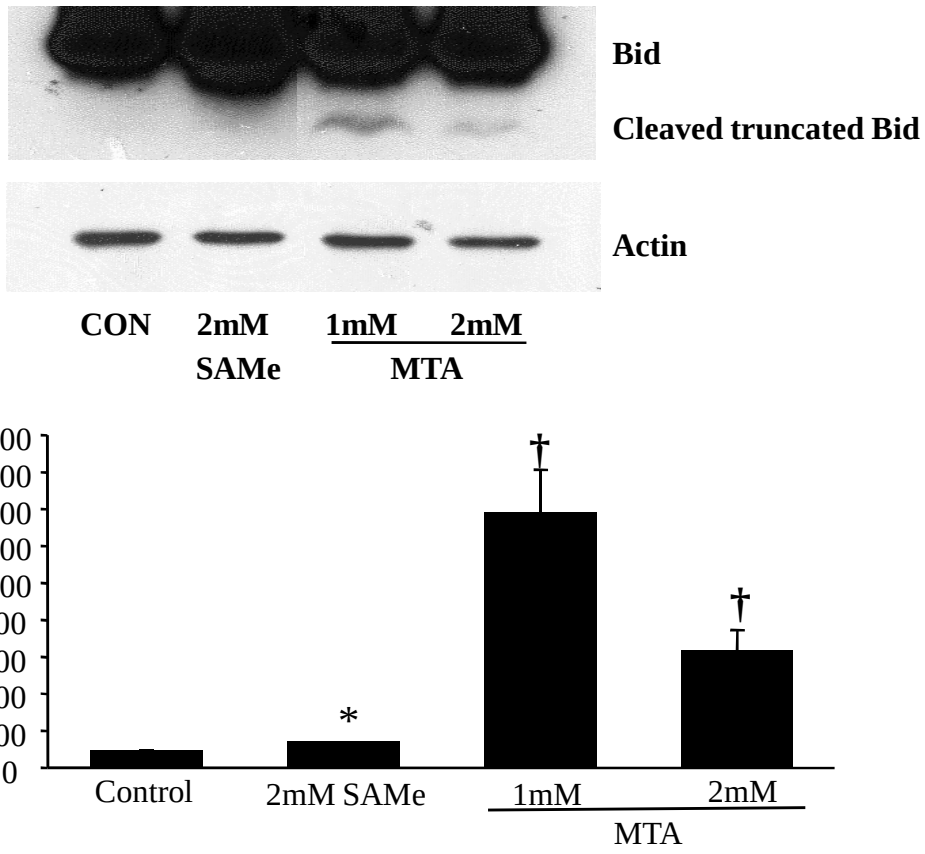
**A.**

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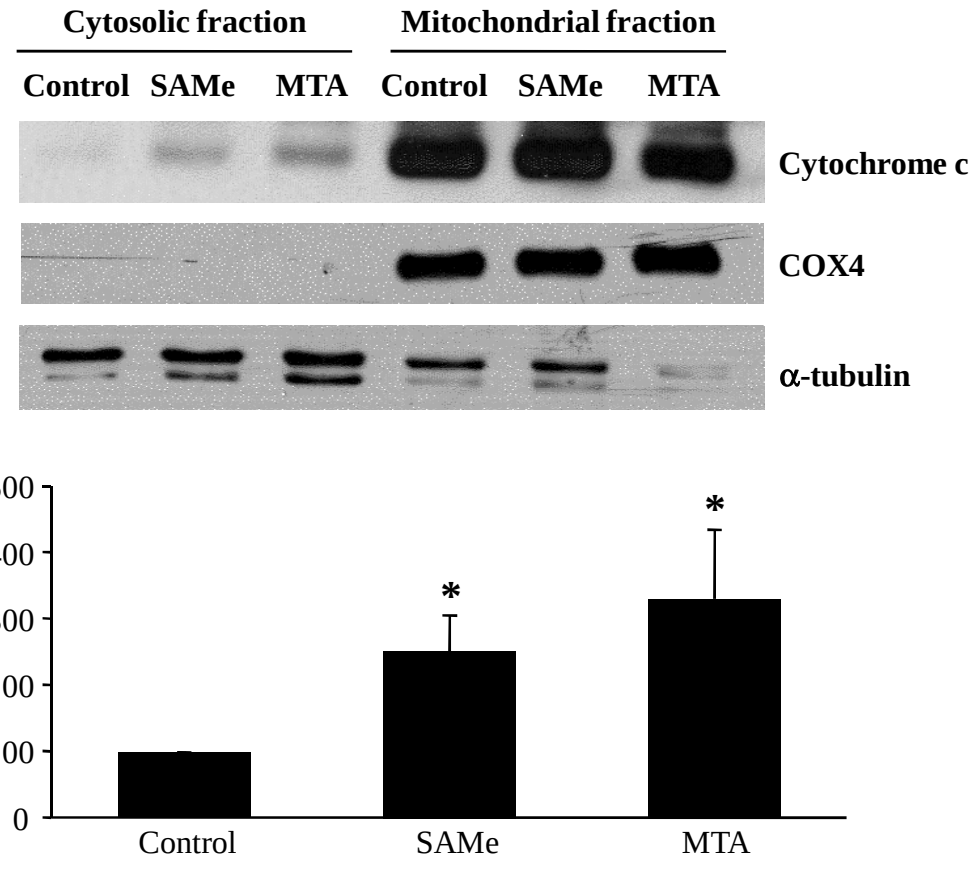


**Figure 5**

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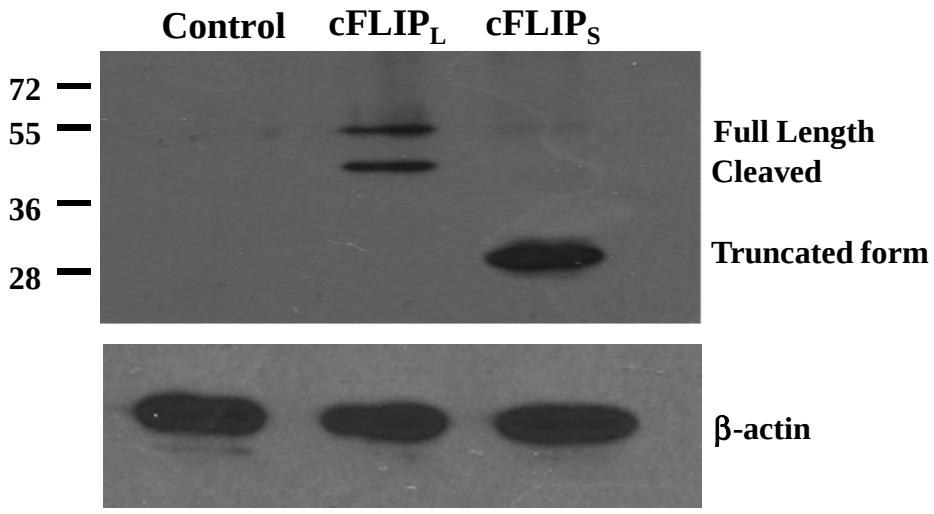
**A.**




**B.**

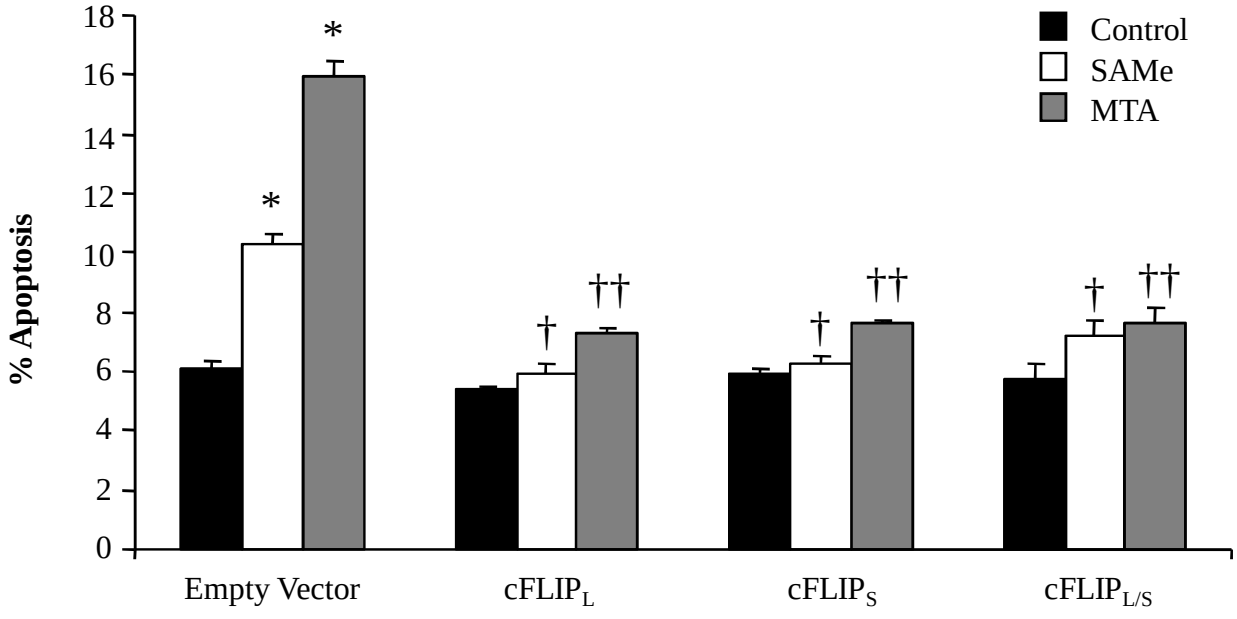



**Figure 6**

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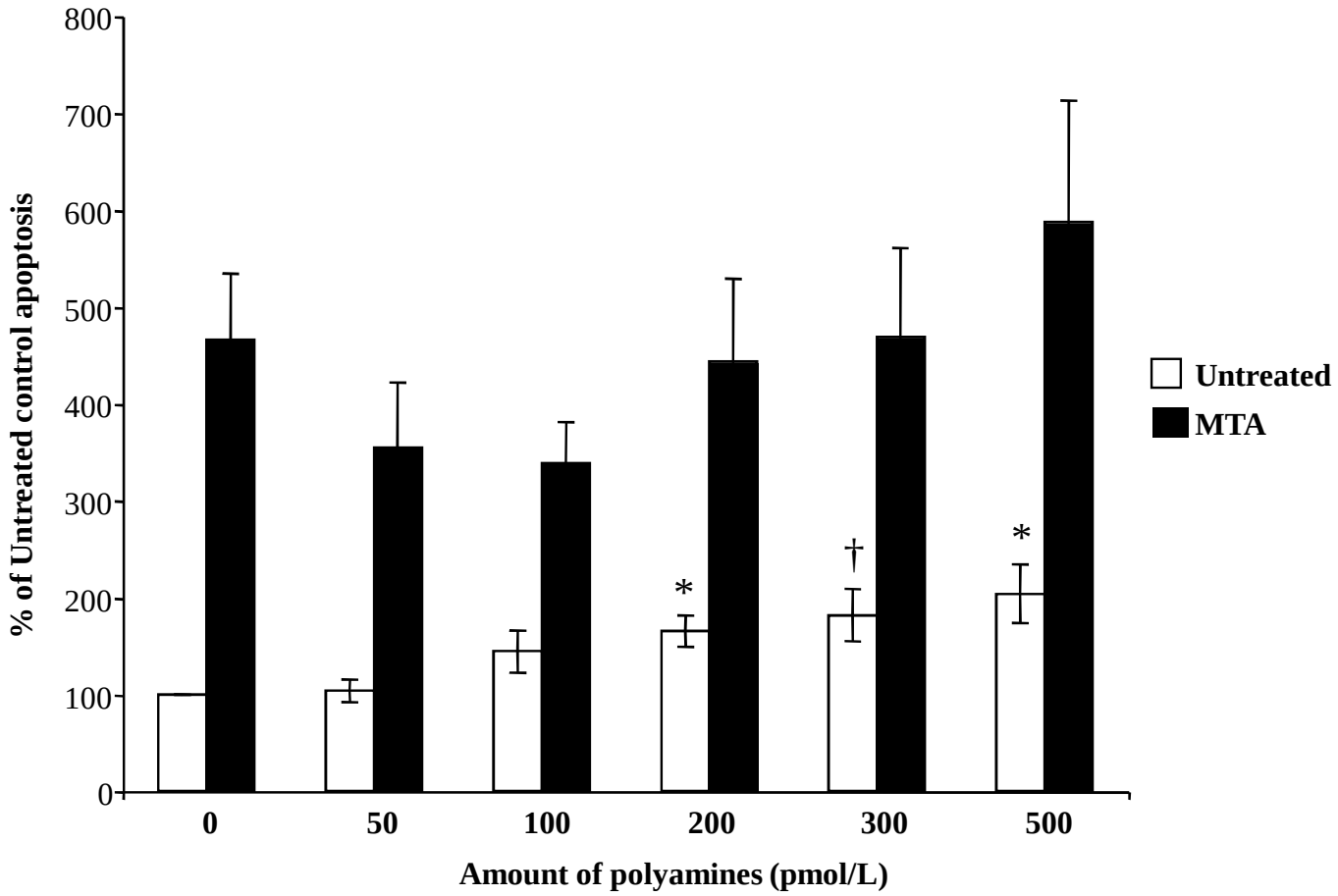




**Figure 7**

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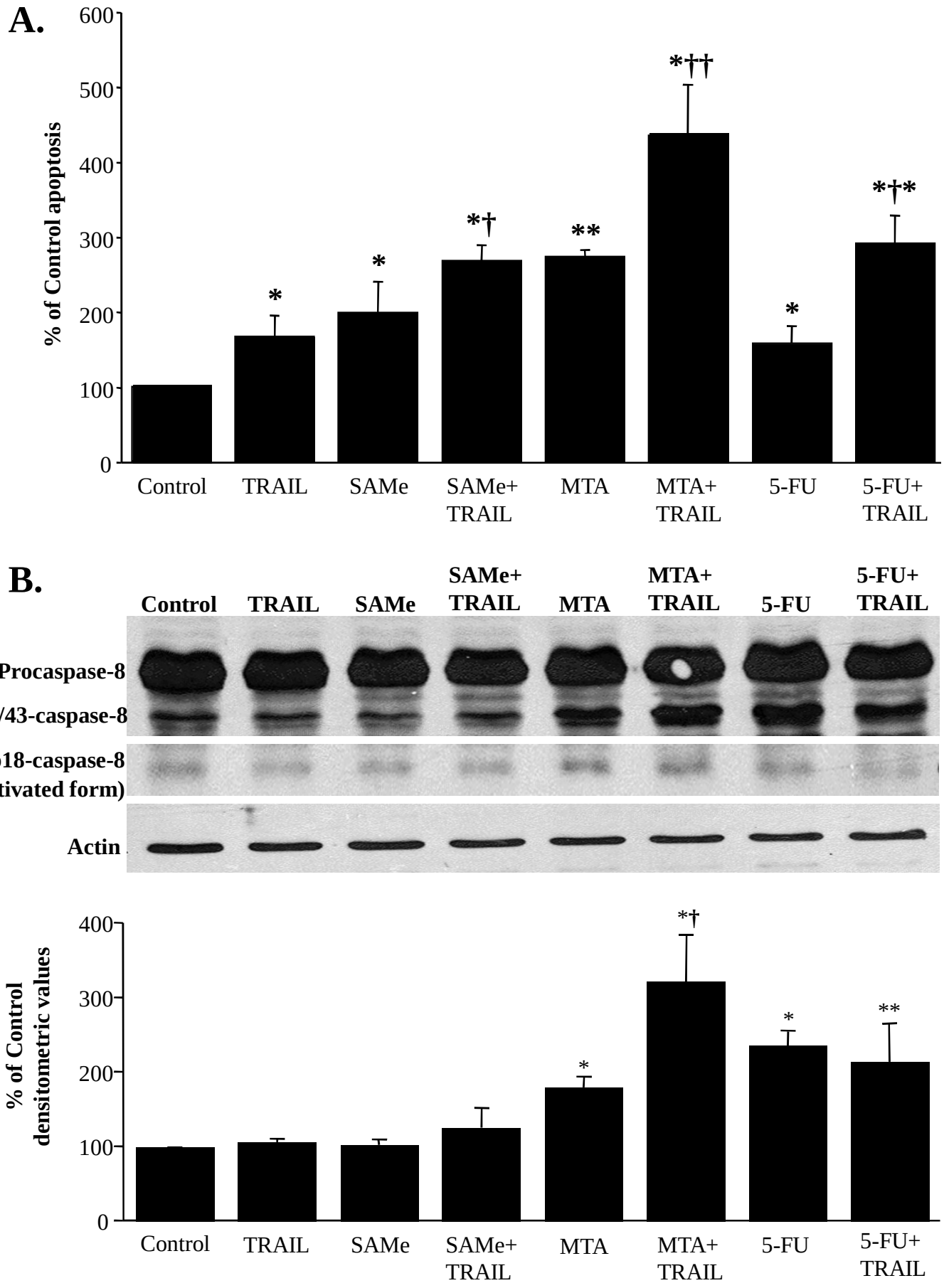


**Figure 8**

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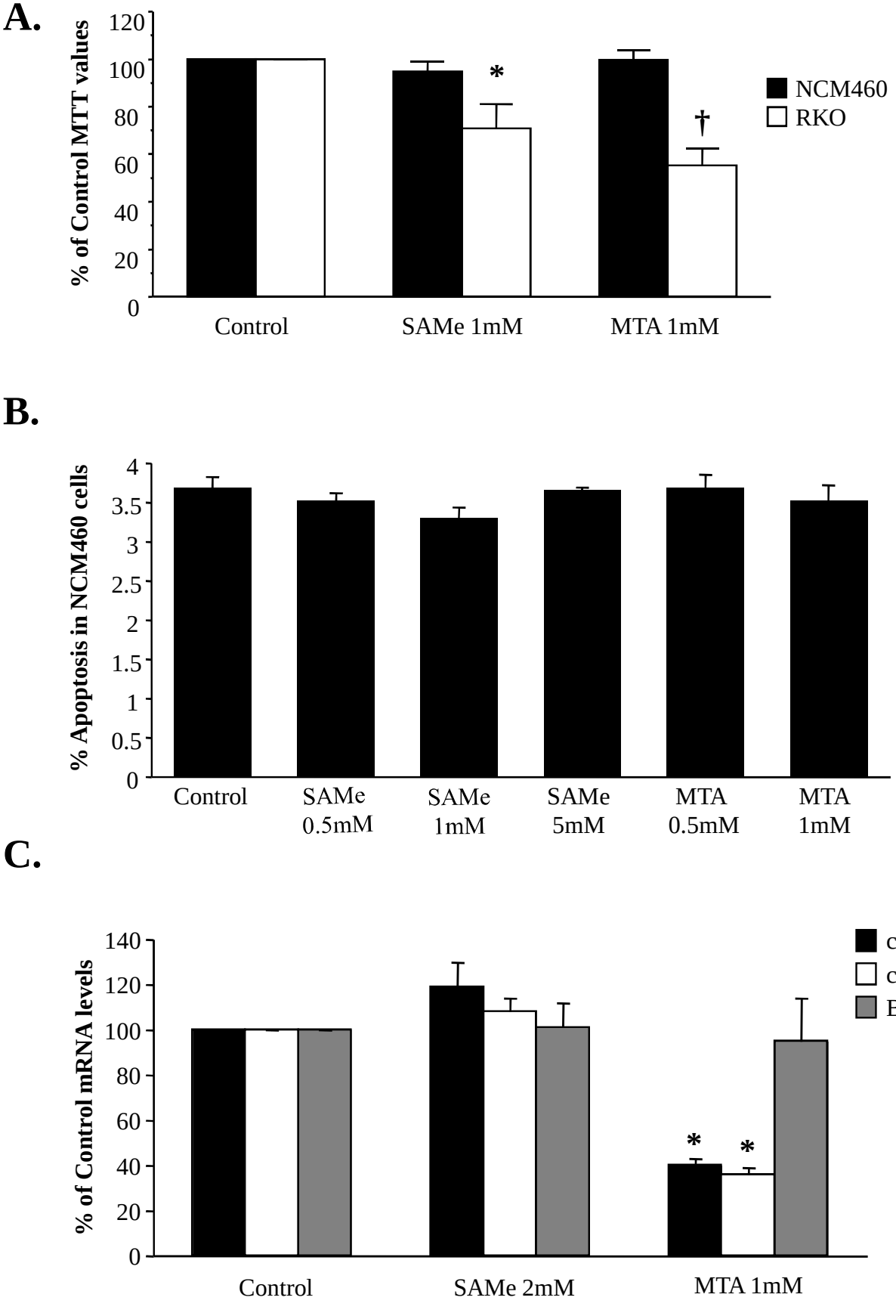


**Figure 9**