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Thioredoxin reductase 1 ablation sensitizes colon cancer cells to methylseleninate-mediated cytotoxicity

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Abstract

The relationship between selenium and cancer is complex because individuals with low serum selenium levels benefit from selenium supplementation, but those with high serum selenium levels are at increased risk for other diseases. This suggests that the use of selenocompounds might be limited to particular circumstances, such as adjuvant therapy. A contributor to this dichotomy may be the activity of certain selenium containing enzymes like the cytosolic thioredoxin reductase (TR1). We evaluated the cellular response to select selenocompounds that have anticancer activity when TR1 was attenuated by siRNA in RKO colon cancer cells. Methylseleninic acid (MSA), which is a substrate for TR1, enhanced cytotoxicity to colon cancer cells when TR1 was attenuated. MSA induced stress in the endoplasmic reticulum, as measured by GRP78 protein levels. However, this pathway did not appear to account for the change in cytotoxicity when TR1 was attenuated. Instead, knockdown of the cytosolic TR plus incubation with MSA increased autophagy, as measured by LC3B cleavage, and apoptosis, as measured by Annexin V and mitochondrial dysfunction. Therefore, the use of selenocompounds with anticancer activity, like MSA, might be utilized most effectively with agents that targets TR1 in chemotherapeutic applications.

Introduction

Historically, selenium levels in forage crops have inversely correlated with mortality from several types of cancer in the United States (Shamberger and Willis, 1971; Clark *et al.*, 1991); however, translating selenium-mediated cancer prevention to the clinical remains a challenge. Indeed, the relationship between selenium supplementation and cancer susceptibility has been the focus of two cancer prevention trials; a smaller scale trial (Nutritional Prevention of Cancer trial, NPC) suggested supplementation reduced cancer mortality as well as certain cancer risk (Clark *et al.*, 1996) while a recent large scale Selenium and Vitamin E Cancer Prevention Trial (SELECT) did not confirm the reduction in prostate cancer risk expected, based on the NPC trial with selenomethionine supplementation (Lippman *et al.*, 2009). Indeed, these supplementation trials suggest that high levels of serum selenium correlate with increased risk of type II diabetes (Stranges *et al.*, 2007), and serum selenium levels have a non-linear relationship with all-cause mortality, reducing mortality when supplemented at low levels but perhaps increasing mortality at the highest selenium levels (Bleys *et al.*, 2008). At a minimum, these studies have raised questions regarding the utility of selenomethionine supplementation as a long term prevention agent. Whether selenomethionine

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is the most appropriate or efficacious selenocompound for anticancer therapy or preventions is still a matter of debate (Hatfield and Gladyshev, 2009). Therefore, selenium compounds might be better utilized for anti-cancer activity in conjunction with other therapeutics (Hu et al., 2008; Li et al., 2008) rather than long term supplementation for cancer prevention.

In spite of the results in the clinical trials, serum selenium levels continue to be inversely correlated with colon cancer in epidemiology studies (Peters *et al.*, 2006; Marshall, 2008; Peters and Takata, 2008; Connelly-Frost *et al.*, 2009). One potential mechanism of selenium-mediated cancer modulation is its utilization in the synthesis of selenoproteins (Lobanov *et al.*, 2009). The role of certain selenoenzymes, like cytosolic thioredoxin reductase (TR1), is complex as it can display both anticancer and pro-carcinogenesis functions (Hatfield *et al.*, 2009). On the genomic level, a human single nucleotide polymorphism has been identified within the TXNRD1 sequence that reduces the risk of distal colon adenocarcinoma; however, this polymorphism may not only affect the TXNRD1 gene (Peters *et al.*, 2008).

One additional complication is that there may be interactions between selenocompounds and TR1 in anti-cancer activities. Initially, certain selenocompounds were thought to be inhibitors of TR1, but instead, some selenocompounds are substrates of TR1 (Gromer and Gross, 2002; Zhao and Holmgren, 2002; Zhao *et al.*, 2002). Therefore, our aim was to evaluate the TR1 dependence of the cytotoxicity of selenomethionine, 1,4-phenylenebis(methylene) selenocyanate (p-XSC), or methylseleninic acid (MSA) in RKO colon cancer cells where we attenuated the endogenous TR1 with siRNA. These compounds were chosen because they represent distinct selenocompound classes. Selenomethionine is a selenium-containing amino acid that can provide selenium to multiple cellular pools (Suzuki *et al.*, 2006a), p-XSC is an organic selenocompound that reacts with glutathione but does not release selenium to cellular pools (Sohn *et al.*, 2005), and MSA is readily converted to methylselenol, the selenocompound thought to be responsible for selenium-mediated anticancer activity (Ip et al., 2000). Selenomethionine has been used in clinical studies while p-XSC and MSA have displayed promising anticancer activity in preclinical studies. Since MSA is a substrate for reduction to methylselenol by TR1 (Gromer and Gross, 2002), we anticipated that this selenocompound might demonstrate TR1-dependence in its cytotoxic anticancer activities. Indeed, we found that MSA was the only selenocompound of this group that displayed differential activity dependent on TR1 status. In addition, the targeting of TR1 with siRNA plus MSA accelerated autophagy and the pro-apoptotic activity of this selenocompound.

Materials and Methods

Materials

Advanced DMEM, Glutamax, MitoProbe JC-1 Assay Kit, and Hank's balanced salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Hyclone (Logan, UT). The RKO cell line was purchased from American Tissue Type Culture Collection (Manassas, VA). CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega (Madison WI). Selenocompounds used included L-selenomethionine from Acros Organics (Morris Plains, NJ), p-XSC was from LKT Laboratories, Inc (St. Paul, MN) and methylseleninic acid (MSA) was from PharmaSe, Inc. (Lubbock, TX). Antibodies directed at BiP/GRP78 and LC3B were purchased from Becton Dickinson and Company (Franklin Lakes, NJ) and Sigma-Aldrich (St. Louis, MO), respectively. TR1 polycolonal antibodies and secondary, donkey polyclonal anti-mouse and anti-rabbit antibodies conjugated with horseradish peroxidase, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Western Lighting chemiluminescence reagents were from PerkinElmer Life Sciences (Boston, MA). Dithiothreitol and 3-methyladenine are purchased from Sigma-Aldrich.

Cell culture

RKO colon cancer cells were used as a representative colon cell line and were maintained in Advanced DMEM supplemented with 1% Glutamax and 2% fetal bovine serum. Advanced DMEM is a medium with a low serum requirement that is supplemented with 5 μg/l sodium selenite, and the serum used contained 37 ng/ml selenium, resulting in a selenium media containing ~37 nM selenium. This selenium content is similar to standard media supplemented with 10% serum, and the selenite content alone is sufficient to support maximal glutathione peroxidase 1 activity (a measure of selenoenzyme synthesis), and is ~10× above the levels of selenium required to abrogate selenium-deficient cell death (Leist *et al.*, 1996; Baker *et al.*, 1998; Saito *et al.*, 2003). RKO cells were used in this study since serum selenium levels and colon cancer have been previously linked and the knockdown of thioredoxin reductase 1 was accomplished with siRNA as previously described in this same cell line (Cassidy *et al.*, 2006).

ATP content analysis

Cellular viability was determined using the CellTiter-Glo Luminescent Cell Viability assay which measures ATP content in cells (Crouch *et al.*, 1993). ATP-dependent luciferase activity was measured using a Perkin-Elmer VictorV^3 Multimode Microplate Reader.

Cytometric Assay for Free Thiols

Cells were seeded into 6-well plates at a density of ~ 2×10^5 cells/well and allowed to grow overnight. Culture medium was refreshed at the time of treatment. Cells were trypsinized, centrifuged at 250×*g* for 5 minutes, and resuspended in 1 ml phosphate buffered saline (PBS). Cells were then centrifuged at 250×*g* for 5 minutes and resuspended in a fresh 1 ml of 1× PBS. 40 μM monobromobimane (mBBr) was added and samples were incubated at room temperature in the dark for 5 minutes. mBBr fluorescence concentration was then determined. Cellular fluorescence concentrations were determined 24 hrs after treatment using a Beckman Cell Lab Quanta SC flow cytometer by dividing the fluorescence for each cell by its measured electronic volume. For each assay, a minimum of 10,000 events per sample were recorded.

Immunochemical analysis

Cells in 6-well plates were placed on ice. Media was aspirated and cells were then washed with 1ml of cold 1× PBS, the PBS aspirated, and protein lysates were prepared as previously described (Cassidy *et al.*, 2006). Protein concentrations were determined using Bradford reagents. Absorbance at 595 nm was measured using a Perkin-Elmer VictorV3. Proteins were separated by NuPAGE 10% or 4-12% Bis-Tris gels, and transferred to a polyvinyl difluoride membrane. Membranes were blocked with 5% nonfat dry milk in TBS-T and then probed for TR1 (1:300), BiP/GRP78 (1:1000) or GAPDH (1:500) with specific antibodies. Peroxidase conjugated secondary antibodies (1:5000) were used in conjunction with chemiluminescence detection to evaluate protein expression. Protein was detected using Western Lightning Western Blot Chemiluminescence reagent, visualized, and quantified using a Kodak Image Station 440.

Apoptotic assays

Two cytometric assays were used to evaluate markers of apoptosis and mitochondrial status using a CellLab Quanta SC flow cytometer.

Mitochondrial potential evaluation

Mitochondrial potential was measured using the MitoProbe JC-1 Assay Kit where 1 μM JC-1 was added to medium of cells attached to 6-well plates after MSA treatment. Cells were

incubated at 37°C with 5% CO_2 and 95% air for 30 minutes and then trypsinized, washed and resuspended in HBSS. JC-1 fluorescence at 525 nm (JC-1 "green") and 575 nm (JC-1 "red" or "J-aggregates") was determined for each sample. JC-1 fluoresces green when the mitochondrial potential has been depolarized and forms aggregates that fluoresce red when the mitochondrion is polarized (Salvioli *et al.*, 1997). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used at 25 μM to disrupt the mitochondrial membrane potential as a control. Cellular fluorescence was determined 24 hrs after treatment by flow cytometry and for each sample, a minimum of 10,000 events per sample was recorded.

Annexin V/PI staining

The Vybrant Apoptotic Assay Kit #2 was used in a dual color cytometric assay to evaluate Annexin V as an early marker of cell death along with PI exclusion using a CellLab Quanta SC flow cytometer. Cells were trypsinized, washed, and resuspended in 100 μl annexin-binding buffer, 5 μl Alexa-488 conjugated Annexin V and 1 μl of 100 μg/ml PI was for a 15 min incubation, and then the cells were diluted to 500 μl prior to cytometric evaluation. Cellular fluorescence was determined following 16 or 24 hrs of MSA exposure and a minimum of 20,000 events per sample was recorded. The percentage of the cells that were not stained, representing viable cells; Annexin V positive, representing early apoptotic cells; and Annexin V plus PI double positive, representing late apoptotic cells, were quantified by flow cytometry.

Statistical Analysis

GraphPad Instat, version 3.06, was used to evaluate the statistical significance of the results. Statistical significance was determined by one-way ANOVA with Bonferroni multiple comparison post hoc tests and differences were considered significant for $p<0.05$.

Results

The role of TR1 in the cytotoxic response of these selenocompounds was examined in RKO cells with endogenous levels of TR1 or with TR1 knocked-down by siRNA. We evaluated the cytotoxic response following a 24 hr incubation of SEM, p-XSC, and MSA by measuring cellular ATP content (figure 1). The attenuation of TR1 did not markedly alter SEM- and p-XSC-mediated responses. SEM was only minimally toxic within 24 hr over the highest concentration range used (figure 1A), while p-XSC's toxicity was independent of TR1-status (figure 1B). However, the toxicity of MSA was TR1-dependent (figure 1C). Cells with reduced TR1 levels due to siRNA treatment displayed a significant reduction in ATP levels at MSA concentrations of ~5 μM. The effect on TR1 protein expression following selenocompound incubation was evaluated when the cells had ~80% of ATP content of the control. The level of TR1 protein was evaluated by immunochemical analysis as shown in figure 1D. The TR1 protein remaining, following the knockdown of TR1 compared to the scrambled siRNA control, was 8 ± 4%. In cells treated with SEM, the remaining TR1 protein following knockdown was 26 ± 7% compared to the control siRNA. In cells treated with p-XSC the remaining TR1 protein following knockdown was 25 ± 5% compared to the control siRNA. In the cells treated with MSA, the remaining TR1 protein following knockdown was 11 ± 4% compared to the control siRNA.

To determine if the mechanism of enhanced cytotoxicity following MSA treatment in RKO cells with reduced TR1 was related to endoplasmic reticulum (ER) stress (Wu *et al.*, 2005; Zu *et al.*, 2006), ER chaperonins and cellular thiol status were evaluated and compared to cells treated with the known ER disrupter dithiothreitol (DTT). The rationale for using DTT as a positive control was from studies where DTT was shown to produce an ER stress response (Thomas *et al.*, 2007). It has also been demonstrated in yeast that DTT-mediated cytotoxicity is dependent on the thioredoxin system (Trotter and Grant, 2002). The protein expression of

BiP/GRP78, a marker of ER stress, was increased significantly ($p<0.003$) when cells were incubated with MSA and DTT. MSA at either 15 or 25 μM increased Bip/GRP78 expression by ~80% over the control ($p<0.01$). No significant differences were observed between the doses of 15 and 25 μM MSA. DTT increased Bip/GRP78 expression by ~60% over the control ($p<0.05$). The difference between MSA and DTT treatment was not found to be statistically significant. Examples of the immunoblot data are displayed in figure 2A. Since we have previously demonstrated that MSA alters free thiol status in other cancer cell lines as measured by monobromobimane (mBBr) fluorescence (Poerschke *et al.*, 2008), we compared cells with endogenous TR1 or those where TR1 was attenuated, due to siRNA knockdown, to cell populations also treated with MSA. TR1 knockdown decreased cellular free thiols as measured by mBBr fluorescence however MSA increased mBBr fluorescence to comparable levels, regardless of TR1 status (figure 2B). Incubation of the RKO cells with DTT at concentrations that demonstrate ER stress showed a similar increase in mBBr fluorescence to that observed in MSA-treated RKO cells (figure 2C). However, when we evaluated cellular ATP content following TR1-knockdown, we observed no significant difference in viability that was dependent on TR1 status (figure 2D). Therefore, it appears that the enhanced cytotoxicity observed following MSA treatment of TR1-knockdown cells was due to other factors besides enhanced ER stress.

Cells treated with si-TR1 and MSA seemed to round or appear smaller, so we measured the autophagic response. Since autophagy is a mechanism activated in many stress situations, we evaluated the cleavage of the LC3B protein as a robust biochemical marker of this process, and there was a clear increase in the LC3B protein cleavage product, LC3B II, in cells with reduced TR1 and treated with MSA compared to treatments with MSA or the siRNA alone (figure 3A). The compound 3-methyladenine is commonly used to inhibit autophagy (Seglen and Gordon, 1982). We measured the dose-dependent cytotoxicity of 3-methyladenine in RKO cells and found that 1 mM was the highest dose we could use with minimal toxicity (~80% viable as measured by ATP content). This dose was insufficient to inhibit LC3B cleavage or alter cell viability when the cells were treated with MSA and TR1-knockdown (data not shown). However, the observed increase in autophagy appears distinct from the ER stress as DTT does not promote LC3B cleavage (figure 3B), and therefore, this process may contribute to the enhanced cytotoxicity we have observed with MSA and TR1 attenuation.

Since our measure of cellular viability was ATP production, we next examined the mitochondrial membrane potential as a common marker of apoptosis in the primary organelle that generates ATP. Cytometric evaluation of JC-1 stained cells show that TR1-knockdown and MSA treatment resulted in mitochondrial dysfunction, as measured by a decrease in mitochondrial potential, but that either MSA treatment or TR1-knockdown alone did not alter the mitochondrial potential (figure 4). Since mitochondrial dysfunction can be a consequence of autophagy or apoptosis, we next determined that the TR1-knockdown also promotes apoptosis. We examined the Annexin V and PI double staining as a measure of apoptosis progression. Annexin V positivity is a marker of early apoptosis when PI is excluded, but both stain late apoptotic cells. Therefore, we measured the population of cells that were positive for Annexin V conjugated to Alexa-488 and PI at 16 and 24 hrs after treating with 15 μM MSA (a representative dataset is shown in figure 5, and triplicate datasets are combined in table 1). There were low levels of Annexin V staining in the MSA and siRNA control samples but there was an increase in Annexin V positive and increasing double positive cells over time (figure 5E & F) that show statistically significant changes with MSA concentration and time (table 1).

Discussion

Several selenocompounds have demonstrated anticancer activity in preclinical experiments. In particular, MSA and p-XSC induce cell cycle arrest and apoptosis (Ip *et al.*, 2002; Swede

et al., 2003; El-Bayoumy *et al.*, 2006). However, the interaction of selenocompounds and selenoproteins in cell growth and apoptotic related activities is less clear. Some selenocompounds likely contribute to selenoprotein expression. For example, TR protein expression and activity has been demonstrated to be dependent on selenium availability (Berggren *et al.*, 1997; Gallegos *et al.*, 1997; Berggren *et al.*, 1999). However, the selenium available for selenoprotein synthesis is dependent on the metabolism of the particular selenocompounds (Suzuki *et al.*, 2006a; Suzuki *et al.*, 2006b; Suzuki *et al.*, 2006c; Suzuki *et al.*, 2006d). Small molecule selenocompounds have been hypothesized to be required for the anti-cancer activities of selenium (Ip *et al.*, 2000), and therefore, the requirement for selenoproteins has been questioned. Experimental systems have been generated that can address these concerns more directly. For example, in mice with disrupted selenocysteine tRNA have reduced selenoprotein synthesis, but increased colon cancer susceptibility following induction with the carcinogen, azoxymethane, as measured by increased tumor incidence and pre-malignant lesions compared to wild-type mice (Irons *et al.*, 2006). However, selenium supplementation resulted in accumulation of non-proteinacious selenium and protection in both groups of mice. Therefore, the authors interpreted these results as indicating that both selenoproteins and low molecular weight selenocompounds protect against cancer. In another study, bigenic mice were engineered to be selenoprotein deficient, again due to disruption of the selenocysteine tRNA, but also contained targeted expression of SV40 tumor antigens to promote the development of prostate cancer. These mice were maintained on selenium sufficient diets but the mice without selenoprotein synthesis demonstrated accelerated lesion development with enhanced disease progression compared to transgenic mice with only the SV40 tumor antigens (Diwadkar-Navsariwala *et al.*, 2006). Therefore, these studies strongly support the hypothesis that selenoproteins, and not just small molecule selenocompounds, have roles in the cancer prevention activities of selenium. However, TR1 may be unique among selenoproteins in that attenuation of TR1 expression also mitigates tumor phenotypes in transformed cells (Yoo *et al.*, 2006; Yoo *et al.*, 2007).

MSA functions by increasing stress to the ER and activating the unfolded protein response (Wu *et al.*, 2005; Zu *et al.*, 2006). Moreover, the TR-Trx system was required to protect against reductive stress (i.e. by treatment of these cells with DTT) mediated through the ER in yeast (Trotter and Grant, 2002), so we anticipated that without TR1, we may have even a more dramatic ER stress following incubation with MSA. However, functional differences may exist between the mammalian and yeast thioredoxin system. Indeed, the yeast TR is not a selenoprotein but it nevertheless provides many of the same reducing functions within the yeast cell. Still, we observed increased free thiols with cells treated with MSA, and we observed increases in expression of the ER chaperon protein, BiP/GRP78, which we interpret as consistent with ER stress (figure 2). However, MSA certainly has a broader spectrum of activity than simply inducing ER stress since DTT gave comparable results but in the context of the TR1 knocked-down, MSA displays enhanced cytotoxicity while DTT does not.

The autophagic response observed with MSA appears to be novel for an organoselenocompound; however, autophagy has been associated with inorganic selenium-mediated cell death (Kim *et al.*, 2007; Kim and Choi, 2008). Autophagy is generally associated with promoting survival by recycling cellular components under nutrient limiting conditions (Levine and Kroemer, 2008), and can have a pro-survival activity in cancer. However, the same ability to remove damaged cellular components may mitigate tumor promotion (White and DiPaola, 2009). Therefore, the autophagic response to MSA and TR1 knockdown is suggestive of increased metabolic stress, but may not be the proximal mechanism of the enhanced cytotoxicity.

Perhaps the best use for selenocompounds in cancer prevention and treatment could be their utilization during cancer therapy along with other anticancer agents. Even selenomethionine,

which has not demonstrated efficacy in the SELECT clinical studies, has demonstrated enhancement of cell killing when lung cancer cell lines, but not normal lung cancer cells, were subject to ionizing radiation (Shin *et al.*, 2007). Selenomethionine may not be the most appropriate selenocompounds for cancer prevention or neoadjuvant therapy since it can be incorporated in cellular proteins in the place of methionine rather than generate functional small molecule selenocompounds (Reid *et al.*, 2002) and we did not observe any alterations in cytotoxicity when TR1 was knocked-down. However, MSA has demonstrated improved efficacy of conventional therapeutics in model systems (Hu *et al.*, 2008; Li *et al.*, 2008) and we clearly observe multiple activities when TR1 is targeted simultaneously.

In this study, we demonstrate that MSA displays increased cytotoxicity when TR1 levels are reduced using siRNA. Our data confirm that MSA induces ER stress; however, ER stress by DTT did not result in cell death when TR1 was knocked-down. The combination of MSA and the TR1 knockdown generate additional cellular stress as demonstrated by LC3B cleavage as a biochemical marker of autophagy that was not observed by either treatment alone within the time frame of the experiment. In addition, the knockdown of TR1 and treatment MSA promoted apoptosis. In sum, these data suggest that MSA might be particularly effective in cancer therapeutics in combination with agents that target TR1.

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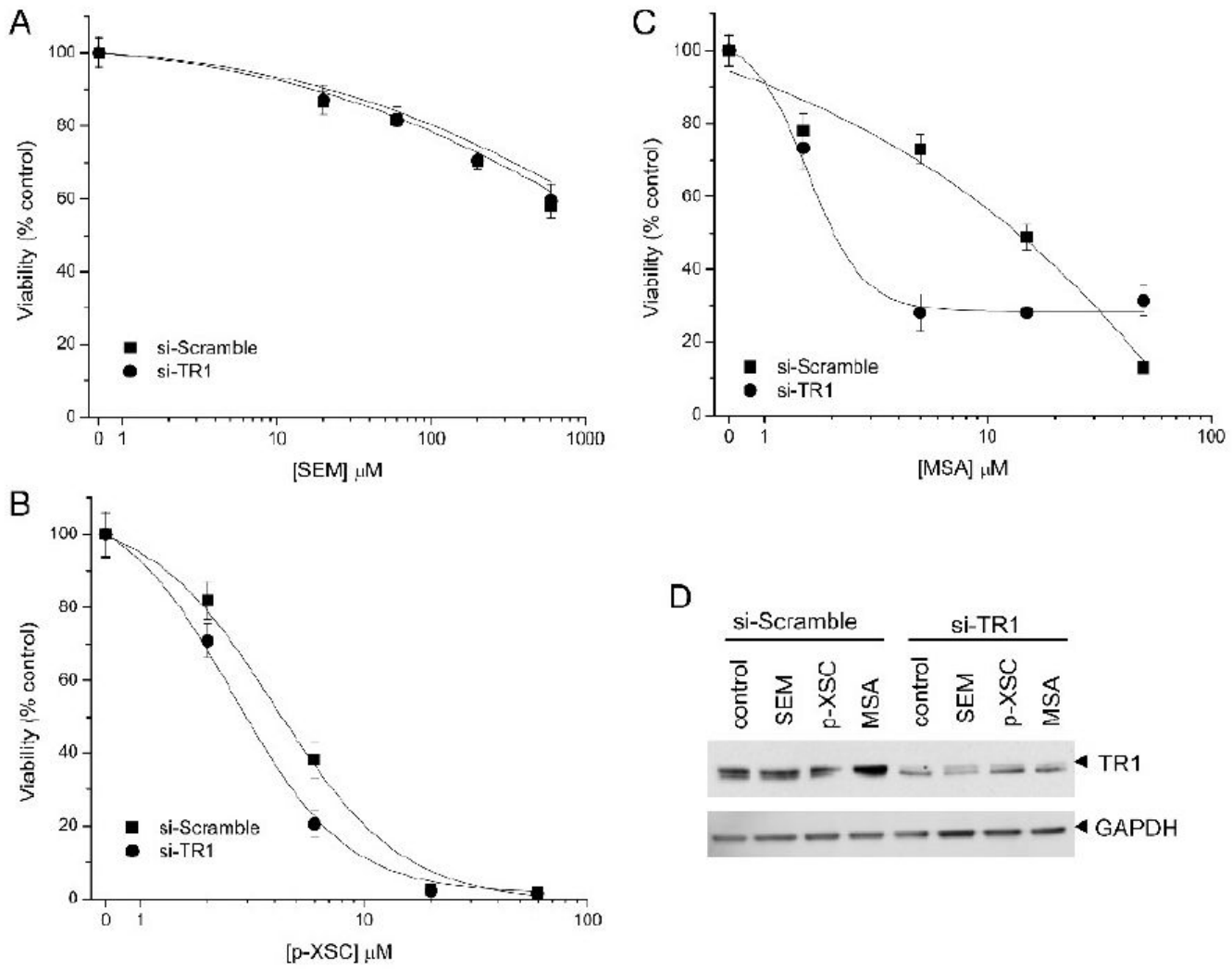

Figure 1.

Cell viability as measured by ATP content of RKO cells treated with siRNA directed at TR1 (si-TR1, filled circle) or a control siRNA (si-Scramble, filled square) and then incubated with increasing doses of A) SEM, B) p-XSC, and C) MSA. (D) Immunochemical analysis of TR1 protein levels in cells incubated with 60 μM SEM, 2 μM p-XSC, or 1.5 μM MSA.

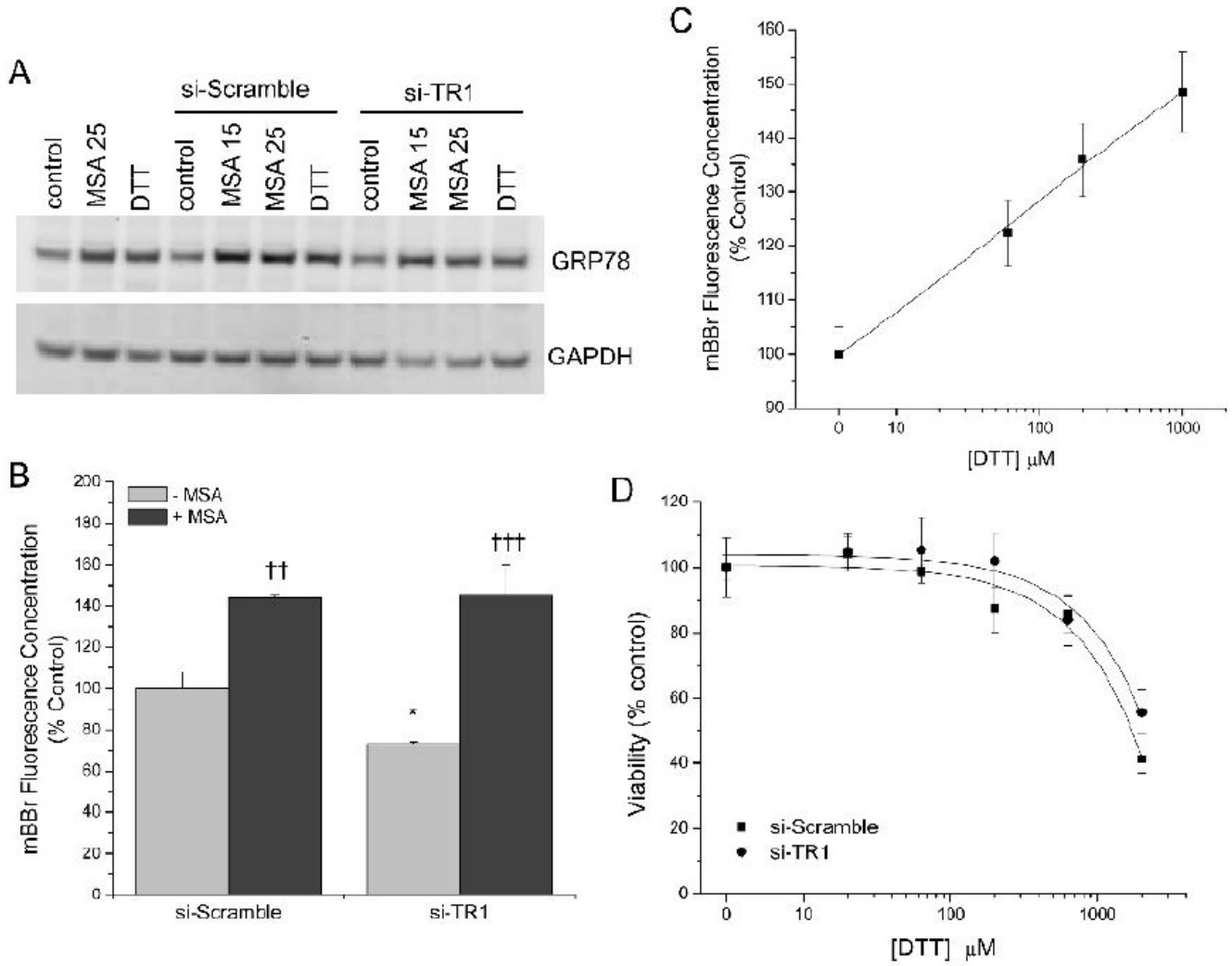

Figure 2.

Evaluation of ER stress and cellular free thiol status following treatment of RKO cells with MSA or DTT along with TR1 attenuation via siRNA. (A) Immunochemical analysis of the ER chaperone BiP/GRP78 following DTT or MSA incubation. (B) mBBr evaluation of cellular free thiols following 15 μM MSA treated RKO cells. Cells with attenuated TR1 display decreased free thiols compared to the control si-Scramble (*, $p<0.05$). When cells are incubated with MSA, significant increases mBBr fluorescence were observed compared to the samples without MSA incubation (††, $p<0.01$; †††, $p<0.001$). (C) DTT treatments result in similar levels of free thiols as measured by mBBr. (D) Cell viability as measured by ATP content of RKO cells treated with siRNA directed at TR1 (si-TR1, filled circle) or a control siRNA (si-Scramble, filled square) and incubated with increasing amounts of DTT.

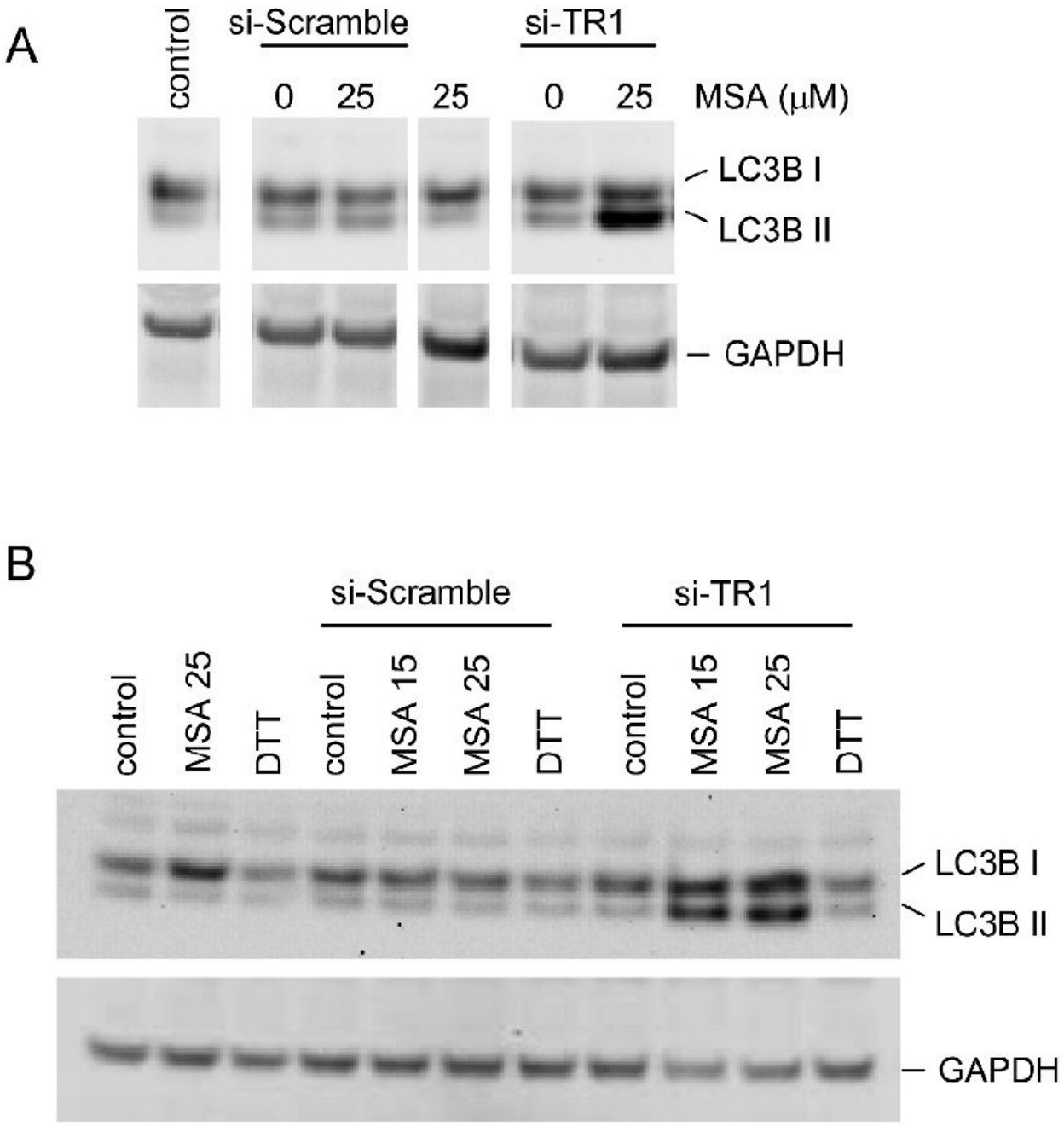

Figure 3.
Biochemical assessment of autophagic response to MSA and TR1 knockdown. (A) Immunochemical analysis of LC3B demonstrates increased cleavage to the LC3B II form with the MSA treatment and TR1 knockdown compared to the control siRNA condition. (B) Increased LC3B cleavage is observed in TR1 knockdown cells treated with MSA but not DTT.

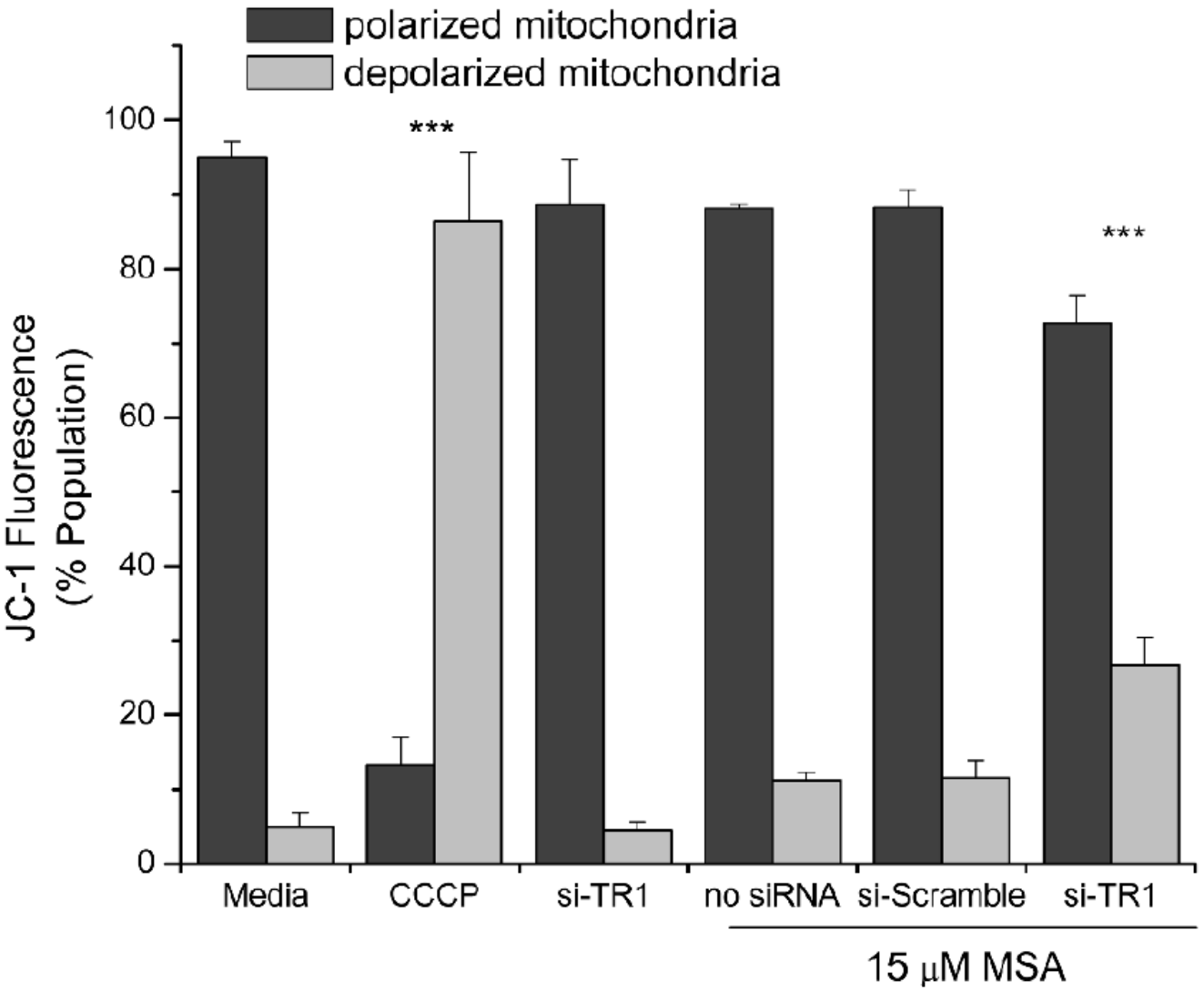

Figure 4.

Mitochondrial dysfunction as a measure of apoptosis. Cytometric analysis of RKO cells incubated with MSA (15 μM) and TR1 knockdown using the mitochondrial fluorophore JC-1 to cytometrically measure the mitochondrial membrane potential. CCCP (25 μM), a recognized mitochondrial membrane potential disrupter, was utilized as a control. Bars represent the percentage of the cellular population with polarized mitochondria as indicated by red fluorescence (JC-1 red, 525 nm) or depolarized mitochondria as indicated by green fluorescence (JC-1 green, 575 nm) with standard deviations. An ANOVA indicated significance ($p<0.0001$) and the CCCP and si-TR1 MSA treated samples were significantly different from the other samples (***, $p<0.001$).

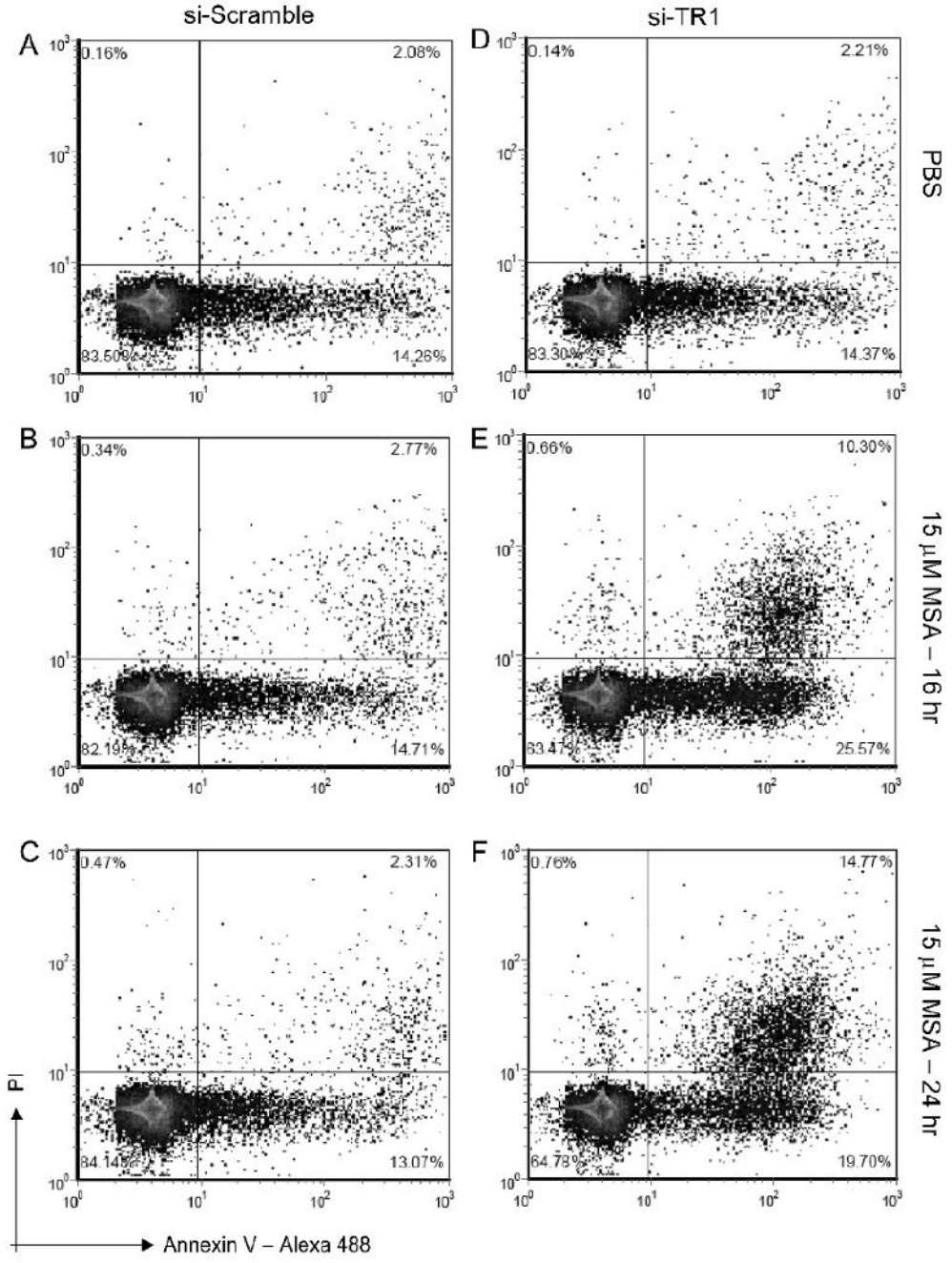

Figure 5.

Annexin V and PI cytometric analysis as a measure of apoptosis. Panels A-C and D-F display control, and MSA treated cells for 16 and 24 hrs, for cells with endogenous TR1 and with TR1 knockdown, respectively. The lower left quadrant displays the percentage of cells that are intact (PI negative) and Annexin V negative. The lower right quadrant displays the cells that are Annexin V positive, or are undergoing early phases of apoptosis. The upper right quadrant displays cells that are both positive for PI and Annexin V and represents late apoptotic cells.

Table 1
Apoptosis Measured by Annexin V and PI Staining

		si-Scramble	si-TR1
PBS	Intact Cells	84±4.8%	86±5.4%
	Early Apoptotic	14±3.6%	12±4.7%
	Late Apoptotic	2.1±1%	2.4±1.1%
16 hr, 15 μM MSA	Intact Cells	82±8.9%	67±3.3%
	Early Apoptotic	15±8.3%	23±3%
	Late Apoptotic	2.7±0.9%	9.3±1%
16 hr, 25 μM MSA	Intact Cells	81±4.5%	56±3% **
	Early Apoptotic	16±4%	29±3.5%
	Late Apoptotic	3.2±1%	15±1% ***
24 hr, 15 μM MSA	Intact Cells	85±3.4%	60±11% **
	Early Apoptotic	12±3.6%	25±8%
	Late Apoptotic	2.2±1%	15±3.4% ***
24 hr, 25 μM MSA	Intact Cells	81±3.3%	36±8.8% ***
	Early Apoptotic	15±6.3%	34±3% **
	Late Apoptotic	4.3±3%	29±6.4% ***

Values represent percent of the population that represent intact cells (Annexin V and PI negative), early apoptotic (Annexin V positive), or late apoptotic (Annexin V and PI positive). An ANOVA suggested a significant change ($p<0.0001$) in the cellular populations and Bonferroni multiple comparisons tests indicate that statistically significant changes from the vehicle control samples in the Annexin V positive and double positive populations occur in the si-TR1, 16 hr, 25 μM MSA (**, $p<0.01$) as well as in the si-TR1, 24 hr, 15 and 25 MSA treatment samples (**, $p<0.01$, and ***, $p<0.001$, respectively).