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Transcriptional Regulation of Activating Transcription Factor 3 (ATF3) Involves the Early Growth Response-1 (Egr-1) Gene

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Non-standard Abbreviation. (ATF3) Activating transcription factor 3; (Egr-1) early growth response gene-1; (RT-PCR) reverse transcription-polymerase chain reaction; (NSAIDs) non-steroidal anti-inflammatory drugs; (NAG-1) NSAID Activated Gene-1; (Cox) cyclooxygenase; (CHX) cyclohexamide; (siRNA) small interfering RNA; (Erk) extracellular regulated kinase; (MAPK) mitogen-activated protein kinase; (JNK) c-Jun N-terminal kinase/stress-activated protein kinases; (MMP-2) matrix metalloproteinase-2; (p-Erk1/2) phospho-Erk1/2; (AP-1) activator protein-1; (PD98059) 2'-amino-3'-methoxyflavone; (PPARγ) peroxisome proliferator-activated receptor-γ.

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ABSTRACT:

Previously, our laboratory identified Activating Transcription Factor 3 (ATF3) as up-regulated by non-steroidal anti-inflammatory drugs (NSAIDs) using microarray analysis of mRNA from human colorectal cancer cells treated with sulindac sulfide. ATF3 is a transcription factor involved in cell growth, apoptosis, and invasion, and is induced by a variety of anti-cancer and dietary compounds. However, the regulation of ATF3 by anti-cancer agents is not known. The promoter of ATF3 contains several transcription factor binding sites. We identified three putative Egr-1 binding sites in the promoter of ATF3 and report for the first time that the molecular mechanism responsible for the transcriptional regulation of ATF3 by two divergent pharmaceutical compounds sulindac sulfide and troglitazone, involved the early growth response gene-1 (Egr-1). For example, overexpression of Egr-1 protein induced ATF3 mRNA 3.5-fold and transcriptional activity of an ATF3 promoter construct more than 20-fold. ATF3 and Egr-1 mRNA and protein and ATF3 promoter activity were induced by these compounds, whereas induction of ATF3 by these compounds was blocked by Egr-1 small interfering RNA. Sulindac sulfide and troglitazone regulated ATF3 promoter activity, which was suppressed when the two Egr-1 sites were mutated. These compounds induced phosphorylation of extracellular regulated kinase1/2 (Erk1/2) while a dominant negative inhibitor of MEK1 blocked the induction of ATF3. The MEK1/2 inhibitor 2'-amino-3'-methoxyflavone (PD98059) blocked the induction of ATF3 and Egr-1 mRNA expression and ATF3 promoter activity by these compounds. Therefore, this is a novel, first report demonstrating that the expression of ATF3 occurs via Egr-1 downstream of Erk1/2.

INTRODUCTION:

Numerous animal studies, population-based studies, and *in vitro* studies provide evidence that traditional non-steroidal anti-inflammatory drugs (NSAIDs) have chemopreventive activity directed against colorectal cancer as illustrated in a recent review (Thun et al., 2002). Until recently NSAIDs mode of action was thought to be solely through the inhibition of Cox-2, which along with its products such as prostaglandin E_2, are up-regulated in tumors resulting in a variety of biological activities such as enhanced tumor cell invasion, increased angiogenesis, and a reduction in apoptosis. However, recent investigations illustrate that Cox inhibitors may elicit chemopreventive activity by regulating gene modulation in addition to their Cox-inhibitory activity.

With microarray analysis of colorectal cancer cells treated with sulindac sulfide, we previously identified several candidate genes potentially involved in the chemopreventive activity of NSAIDs independent of their Cox inhibitory activity (Bottone et al., 2003). One gene, Activating Transcription Factor 3 (ATF3) was considered for further study because it is a transcription factor involved in cell proliferation (Fan et al., 2002), apoptosis (Mashima et al., 2001), and invasion (Stearns et al., 2004; Bottone, 2005). We demonstrated that ATF3 expression is down-regulated in colorectal tumors and is induced by a variety of cancer chemopreventive compounds including the traditional NSAIDs sulindac sulfide and indomethacin (Bottone et al., 2003), Cox-1 and Cox-2 selective inhibitors (Bottone et al., 2004), dietary compounds, and troglitazone (TGZ) (Bottone et al., 2005). ATF3 has anti-tumorigenic and anti-invasive properties evident by its ability to repress the promoter of the pro-invasive gene MMP-2 (Yan et al., 2002; Chen and Wang, 2004; Stearns et al., 2004) and inhibit invasion in colorectal cancer cells (Bottone et al., 2005). ATF3 is a member of the ATF/cyclic adenosine

monophosphate responsive element binding protein (ATF/CREB) family of transcription factors historically referred to as a stress response gene. More recently, ATF3 is linked to the carcinogenic process. For example, as a transcription factor, ATF3 modulates the expression of genes linked to cancer including gadd153/Chop10 (Fawcett et al., 1999), MMP-2 (Yan et al., 2002; Chen and Wang, 2004; Stearns et al., 2004), and the anti-tumor gene p53 (Yan et al., 2002). The promoter of ATF3 is regulated by a variety of molecular mechanisms including MEKK1, which is upstream of Erk1/2 (Fan et al., 2002), p53 (Kannan et al., 2001), and c-Jun N-terminal kinase/stress-activated protein kinases (JNK) mitogen-activated protein kinase (MAPK) pathway (Fan et al., 2002). The promoter of ATF3 contains a variety of response elements including numerous activator protein-1 (AP-1), Myc/Max, NFκB, and E2F sites (Liang et al., 1996), plus several uncharacterized sites. The promoter of ATF3 is regulated by a variety of mechanisms including the tumor suppressor p53 (Zhang et al., 2002) following treatment with ultra violet radiation and the proteasome inhibitor MG132 and via JNK following treatment with the growth factor tumor necrosis factor-α and various stress signals such as ionizing radiation (Kool et al., 2003) and homocystine treatment (Cai et al., 2000). However, the molecular mechanisms responsible for the induction of ATF3 mRNA or protein expression by NSAIDs or other anti-cancer compounds have yet to be determined.

Our laboratory previously identified NSAID Activated Gene-1 (NAG-1), which is a transforming growth factor-β super-family member with pro-apoptotic and anti-tumorigenic activity (Baek et al., 2002). NAG-1 is induced following treatment with NSAIDs (Baek et al., 2005) and peroxisome proliferator-activated receptor-γ (PPARγ) ligands (Baek et al., 2004), two compounds with reported anti-cancer and gene regulatory ability. This occurred independent of Cox inhibition and PPARγ, respectively. The molecular mechanism for the induction of NAG-1

by these compounds occurs via an early growth response-1 gene (Egr-1) dependent mechanism involving Erk1/2. Sulindac sulfide and TGZ induce Egr-1 expression at the transcriptional and post-transcriptional level involving promoter regulation and message stability. Egr-1 overexpression induces ATF3 (Fu et al., 2003) and both ATF3 and Egr-1 are regulated by NSAIDs and TGZ. Therefore, to increase the knowledge about the regulation of ATF3 by these and potentially other chemopreventive compounds we chose to test the hypothesis that Egr-1 regulates the induction of ATF3 by sulindac sulfide and TGZ. We report for the first time that the induction of ATF3 by two divergent compounds with similar biological activities, sulindac sulfide and TGZ, requires the expression of Egr-1 and involves the Erk MAPK pathway.

METHODS:

Cell lines and reagents.

Human colorectal cancer cells were purchased from ATCC (Manassas, VA) and were maintained at 37ºC/5 % CO2. Cell culture reagents were from Life Technologies (Rockville, MD) unless otherwise indicated. HCT-116 cells were maintained in McCoy's 5A medium supplemented with 10 % fetal bovine serum and 10 mg/L gentamicin (complete media). Sulindac sulfide was from Sigma Chemical Company (St. Louis, MO). TGZ, cyclohexamide, and 2'-amino-3'-methoxyflavone (PD98059) were from EMD Biosciences (San Diego, CA).

Cell culture treatments.

Cells were grown overnight to 60-70% confluency in complete media and treated in SFM for the times indicated. For MAPK inhibitor studies, cells were serum starved for 1-h in the presence of MAPK inhibitors (pre-treatment) and subsequently co-treated as indicated. Vehicle consisted of dimethyl sulfoxide. For protein assays, cells were treated for 6-h unless otherwise indicated. For RNA experiments, cells were treated for 4-h unless otherwise indicated.

Human ATF3 promoter constructs.

The ATF3 promoter constructs Luc-110 and Luc-1850 were a generous gift from Dr. Shigetaka Kitajima, Department of Biochemical Genetics, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo, 113-8510, Japan and were generated as previously described (Liang et al., 1996; Cai et al., 2000). Other ATF3 luciferase promoter constructs were generated by PCR of human genomic DNA using the primers indicated below purchased from Invitrogen (Carlsbad, CA). Primer design was based on the published sequence of the human ATF3 promoter region (Liang et al., 1996) and clones were subsequently sequence verified. The promoter regions cloned are indicated in parenthesis relative to the transcriptional

start site (-2073 to +45) Luc-2073 For: TCACGTGTTCTCCCTCCTCTC and Luc-2073 Rev: GCGAGAGAAGAGAGCTGTGC. Luc-41 (-41 to +45) consists of the following sequence For: TGAGGGCTATAAAAGGGGTGATGCAACGCTCTCCAAGCCAC AGTCGCACGCAGCCAGGCGCGCACTGCACAGCTCTCTTCTCTCGC. Products were cloned into pCR2.1 (Invitrogen) then transferred by restriction digestion in the appropriate direction using the enzymes HindIII/Xho1 (NEB, Beverly, MA) into the pGL3 luciferase reporter vector (Promega, Madison, WI), ligated with T4 DNA ligase (Gibco BRL, Rockville, MD), and sequenced verified. The Egr-1 luciferase promoter construct, pEGR-1260Luc (+34 to –1260) was designed as previously described (Baek et al., 2003).

The ATF3 promoter contains three putative Egr-1 sites. The two Egr-1 sites that were more critical according to luciferase reporter assays were mutated. The Egr-1 sites are indicated in bold from the published ATF3 promoter sequence are located between -273 and +45 relative to the start codon. The QuickChange II site directed mutagenesis kit (Stratagene, La Jolla, CA) was used to incorporate the mutations according to the manufacturer's instructions. Each of the two GG pairs (indicated in capital letters) illustrated below were mutated in the ATF3 Luc-2073 promoter construct using unique primer pairs into two TT's at the two putative Egr-1 sites as indicated (in **bold**). The -273 to +45 region of the human promoter and ATF3 Luc-2073 promoter construct is illustrated below and contains both of the putative Egr-1 binding sites and regions that were mutated:

gaGGgcgggctggtgtgtgtctcagtgagc**gaGGgcggg**ggaacgcgcctgggctggctcctccccgaacttgcatcaccagtgcc ccctctctccacccccttcggccccgccttggcccctcctccacccccccttcctccgctccgttcggccggttctcccgggaagctattaatagc attacgtcagcctgggactggcaacacggagtaaacgaccgcgccgccagcctgagggctataaaaggggtgatgcaacgctctccaag ccacagtcgcacgcagccaggcgcgcactgcacagctctcttctctcgc. The following HPLC purified primers

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(Sigma) were used to incorporate two point mutations in the putative Egr-1 sites in the ATF3 promoter region. Mutation primers were designed for incorporated into the ATF3 Luc-2073 promoter construct (from GG to TT) are indicated (in **bold**) and the sequence locations are indicated in parentheses relative to the transcriptional start site. Egr-1 Mut1 (-270/271) For: CCTGATATGGAGAGAGA**TT**GCGGGCTGGTGTGTGTC; Rev: GACACACACCAGCCCGC**AA**TCTCTCTCCATATCAGG

Egr-1 Mut2 (-241/242) For: TGTCTCAGTGAGCGA**TT**GCGGGGGAACGCG; Rev: CGCGTTCCCCCGC**AA**TCGCTCACTGAGACA. The Egr-1 Mut1+2 construct is a combination of the resulting constructs mutated with the apposing primer.

Luciferase reporter assays.

HCT-116 cells were transiently transfected in 12-well dishes at 150,000 cells/well for 5-h in SFM containing 0.7 µg of the reporter plasmid containing an ATF3 promoter construct or vector DNA and 0.05 µg of the control Renillia luciferase plasmid pRLnull (Promega) using Lipofectamine/Plus reagent according to the manufacture's instructions. Cells were recovered overnight in complete media. Subsequently, cells were pre-treated with PD98059 (only as indicated) and\or treated in SFM containing vehicle and\or the compounds indicated for 24-h. Cells were washed with PBS then protein isolated using 0.25 ml of 1X passive lysis buffer per well of a 12-well dish followed by shaking at room temperature for 5-min. Lysates were routinely stored, concentrated, and used for Western blotting to confirm simultaneous induction of endogenous ATF3 protein expression by the treatments indicated. Values shown are mean ± STD of three independent transfections.

Sequence Confirmation

The cloned human ATF3 luciferase reporter constructs were sequenced using the ABI Prism dRhodamine terminator cycle sequencing ready reaction kit (Applied Biosystems). Dye incorporated cDNA was purified by centrifugation using Qiagen's DyeEx spin columns according to the manufacturer's instructions. Sequences were determined following gel electrophoresis by the DNA sequencing facility at NIEHS (RTP, NC). Results were verified using a nucleotide-nucleotide Blast search on the NCBI website.

Egr-1 post-translational gene silencing, MEK1 dominant negative inhibitor, and Erk1/2 inhibitor studies.

Egr-1 oligo small interfering RNA (siRNA) (AAGTTACTACCTCTTATCCAT) and scrambled RNA (ATTGTATGCGATCGCAGAC) were designed from various regions of the Egr-1 mRNA which resulted in a significant suppressive effect. Fifty μM stocks were used and the ability of the Egr-1 siRNA to knock-down Egr-1 expression was confirmed by Western and reverse transcription-polymerase chain reaction (RT-PCR) analyses. HCT-116 cells were transfected with 50 nM siRNA or scrambled RNA in 12-well dishes using lipofectamine 2000 and Opti-MEM I medium according to the manufacture's instructions. Cells were recovered overnight in complete media and subsequently treated for 24-h. The human MEK1 dominant negative inhibitor (pMEV-MEK1-DN) and vector (pMEV) are under control of the CMV promoter and were from Biomyx Technology (San Diego, CA). The MEK1 dominant negative inhibitor contains the following mutations: K97R, S218E, and S222A and is neither activated nor can it phosphorylate its downstream effectors. The MEK1 dominant negative inhibitor or vector pDNA (0.7 μg) were co-transfected with various luciferase constructs as described above. For inhibitor assays using the Erk1/2 specific inhibitor PD98059 (20 μM), cells were pre-treated for 1-h in SFM followed by treatment with sulindac sulfide or TGZ as indicated.

Overexpression of Egr-1.

For Egr-1 pcDNA 3.1 vector and an Egr-1 full length protein expression plasmid were described previously (Baek et al., 2003). Briefly, transfection experiments were carried out using 0.7 µg of the expression plasmid using Lipofectamine/Plus reagent (Invitrogen) according to the manufacture's instructions in 12-well dishes. Transfection experiments were carried out using 1 µg of the expression plasmid using Lipofectamine/Plus reagent (Invitrogen) according to the manufacture's instructions.

RNA isolation.

Following treatments, cells were rinsed twice with PBS then RNA was isolated using the Qiagen (Valencia, CA) RNeasy MINI kit according to the manufacturer's instructions. Quantitation was performed by dissolving a small aliquot of RNA in 10 mM Tris-pH 8.0 using a Beckman DU7400 spectrophotometer (Beckman Coulter, Fullerton, CA). RNA was stored at -80ºC until use.

Reverse transcription.

RNA was treated with 1 unit of amplification grade Deoxyribonuclease I (Life Technologies) per µg of RNA at room temperature for 15-min to remove genomic DNA followed by inactivation of the Deoxyribonuclease I with 2.5 mM EDTA (pH 8.0) followed by incubation at 65ºC for 5-min then RNA was stored on ice. Reverse transcription (RT) was performed using Qiagen's Omniscript reverse transcription kit according to the manufacturer's instructions. A negative control containing all of the RT reagents in the absence of RT enzyme (no RT control) was routinely performed. After RT, cDNA was treated with 1 unit RNase H (Life Technologies) per µg RNA at 37°C for 20-min.

Real-time RT-PCR using sybergreen detection.

Real-time RT-PCR was performed in triplicate two or more times with individual time-matched vehicle-treated controls for each gene tested or relative to vector expressing cells for overexpression assays. Real-time RT-PCR primer design, deoxyribonuclease treatment, reverse transcription, and real-time RT-PCR assays using an ABI Prism 7700 (Applied Biosystems, Foster City, CA) were performed as previously described by this laboratory (Bottone et al., 2003). Egr-1 endogenous primers were designed from an untranslated region of mRNA using the following primers: Egr-1 For: TTTCACGTCTTGGTGCCTTTG; Rev: CCCTCACAATTGCACATGTCA (66bp) and the resulting product/template is not necessary for full-length expression when cloned into an expression vector as previously reported (Baek et al., 2003) by this laboratory. Primers for the Egr-1"b" exogenous primer pair recognize both endogenous and exogenous Egr-1 and were designed from a region of Egr-1 which can be found in the translated region of Egr-1 and in the overexpression plasmid and are as follows: Egr-1b For: GCCTGCGACATCTGTGGAA; Rev: CGCAAGTGGATCTTGGTATGC (71bp). ATF3 For: AAGAACGAGAAGCAGCATTTGAT; Rev: TTCTGAGCCCGGACAATACAC (71bp). Actin: CCTGGCACCCAGCACAAT; GCCGATCCACACGGAGTACT (70). Product size was routinely confirmed by running a fraction of the product on a 1 % agarose, 0.5X tris-borate EDTA (pH 8.0) gel in the presence of 0.1 µg/ml ethidium bromide, and visualized under UV illumination.

Western blot analysis.

Protein was isolated in 1X RIPA buffer including one Complete-Mini® protease inhibitor tablet from Roche Diagnostics (Indianapolis, IN). DNA was sheared using a 23-gauge needle then cell lysates were stored at 4˚C for 30-minutes followed by centrifugation at 12,000 X g at 4ºC for 20-minutes to remove cellular debris. Quantitation of protein was performed by BCA (Pierce,

Rockford, IL) using BSA as a standard using a Beckman DU7400 spectrophotometer. Proteins (20 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoreses and transferred onto nitrocellulose membranes as previously reported in this laboratory (Bottone et al., 2004). Blots were blocked for 1-h with 10% skim milk in TBS containing 0.1% Tween-20 (TBS-T), then probed overnight at 4ºC in TBS-T with 5% milk containing the primary antibodies ATF3, actin, Egr-1, or Erk1/2 from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Erk1/2 (p-Erk1/2) was from Cell Signaling Technology (Beverly, MA). Blots were washed in TBS-T then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature in TBS-T containing 5% milk and washed several times in TBS-T. Where necessary, blots were stripped of antibody before reuse while sealed in a plastic bag containing a solution of 62.5 mmol/L Tris-HCl, 2% SDS, and 100 mmol/L beta-mercaptoethanol for 30 minutes with constant agitation in a 50ºC water bath.

p-Erk1/2 enzyme immunoassay.

The Erk1/2 phospho-specific TiterZyme® enzyme immunometric assay kit was from Assay Designs, Inc. (Ann Arbor, MI) and was used per the manufacture's instructions. Briefly, 1.5 x 10^6 cells were plated in 60-mm dishes overnight. Cells were serum starved overnight then treated with vehicle, sulindac sulfide, or TGZ for 0, 5, 10, or 30-min. Cells were washed in PBS then immediately isolated in 0.25 ml lysis buffer plus protease inhibitors (Sigma Chemical Co., St. Louis, MO), diluted 1:4 in assay buffer, then stored on ice until use. Aliquots were stored to determine protein concentrations and for Western blot analysis to confirm the results.

Densitometry measurements.

Autoradiograms from blots were scanned using an Umax® Powerlook III® scanner equipped with a transparency adapter and scanning software on a PC. Subsequently, blots were quantitated using Scion Image® beta version 4.0.2, cut to size for publication, and labeled using Adobe® Photoshop® 5.0.

Statistical analyses.

For MEK1 dominant negative inhibitor studies, values are relative to vehicle-treated cells and statistical significance is relative to similarly-treated, vector-transfected cells. For p-Erk1/2 immunoassay studies, values and statistical significance is relative to time-matched vehicle-treated cells. For mRNA and luciferase studies, values and statistical significance are expressed relative to vehicle-treated and similarly-transfected cells, respectively. Values from PD-treated cells are shown for completeness but were not included in the statistical analyses. Values were analyzed using ANOVA with Bonferroni t-test for multiple comparisons at the $p<0.05$ level of significance unless otherwise stated. *denotes statistical significance at the $p<0.05$ level whereas **denotes the $p<0.01$ level.

RESULTS:

Sulindac sulfide and TGZ induce ATF3 and Egr-1 protein expression.

HCT-116 cells serve as a model system for studying human colorectal cancer therefore they were selected to study the regulation of ATF3. Physiological concentrations of sulindac sulfide are in the 10-20 μM range while those of TGZ are in the 0.1-1.0 μM range based upon reported plasma levels in human subjects (American Society of Health-System Pharmacists., 2003). Therefore we attempted to approach these concentrations when possible. To evaluate if Egr-1 is involved in the induction of ATF3 by sulindac sulfide and TGZ, the expression of ATF3 and Egr-1 were determined by Western blot analysis. Following treatment with vehicle, sulindac sulfide, or TGZ at various time points and concentrations, the expression of ATF3 and Egr-1 protein expression was measured. These compounds increased ATF3 and Egr-1 protein expression in a time-dependent manner, relative to vehicle treated cells with significant induction of both genes at 4 to 6-h range (Fig 1A) and was maintained until 24-h (data not shown). This induction was concentration-dependent relative to vehicle-treated cells with a significant expression in the 20-30 μM range for sulindac sulfide and a 5-10 μM range for TGZ following 6-h of treatment (Fig 1B). To confirm the finding that TGZ induced ATF3, and to ensure that the observed increase in ATF3 expression was not cell-line dependent, HCT-15 colorectal cancer cells were treated with these compounds then protein expression was measured. Both sulindac sulfide and TGZ treatment resulted in significant induction of ATF3 protein expression (data not shown).

Sulindac sulfide and TGZ induced ATF3 and Egr-1 mRNA.

To determine if the induction of ATF3 and Egr-1 occur at the mRNA level, ATF3 and Egr-1 mRNA expression was measured using real-time RT-PCR. The induction of ATF3 and Egr-1

mRNA by these compounds occurs in a time- and concentration-dependent manner (Fig 2) relative to vehicle-treated cells with significant expression following a 2-4-h treatment for Egr-1 and 4-6-h for ATF3 indicating the induction of Egr-1 mRNA precedes that of ATF3. The induction of Egr-1 at the mRNA level by these compounds is significant (20 to 60-fold), particularly at higher concentrations. The large induction of Egr-1 mRNA may, in part, be due to mRNA stability which is important to the induction of Egr-1 by TGZ as documented previously by this laboratory (Baek et al., 2003). However, induction of Egr-1 luciferase promoter activity occurred following treatment with these compounds (data not shown) as previously reported indicating Egr-1 mRNA expression is also induced by these compounds (Baek et al., 2003).

Sulindac sulfide and TGZ regulate the promoter of ATF3.

To determine if the induction of ATF3 occurred at the transcriptional level the promoter region of ATF3 was evaluated. Egr-1 mRNA and protein expression is induced by NSAIDs and TGZ at relatively early time points and three putative Egr-1 sites were found in a 2-kb region of the ATF3 promoter located at -1875, -273, and -243 relative to the transcriptional start site. Therefore, we wanted to test the hypothesis that Egr-1 might be involved in the transcriptional regulation of ATF3 by these compounds. Various ATF3 luciferase reporter constructs were used to determine if the regions of the ATF3 promoter containing these three putative Egr-1 sites are crucial to its induction by these compounds (Fig 3). HCT-116 cells were transiently transfected with various ATF3 luciferase reporter constructs. Cells were subsequently treated with vehicle, sulindac sulfide, or TGZ for 24-h in SFM. The two larger constructs (Luc-1850 and Luc-2073) were most significantly induced indicating the presence of binding sites between the shorter and longer promoter regions is significant. Furthermore, the two largest promoter constructs contain

two and three putative Egr-1 sites, respectively. The Luc-41 and Luc-110 promoter constructs, which do not contain putative Egr-1 sites, were only minimally activated relative to the larger constructs following treatment with these compounds. The greatest relative activation occurred using the Luc-1850 followed by the Luc-2073 ATF3 promoter constructs, which are the largest two constructs used in this study indicating the possibility of a *cis*-acing elements in these regions (Fig 4A). TGZ resulted in a similar pattern of induction in luciferase activity indicating it likely works via a similar mechanism (Fig 4B). The values are expressed as relative luciferase units so that the different sized constructs can be compared. The largest construct, Luc-2073, was not induced as dramatically by these compounds as the Luc-1850 construct, suggesting the possibility of a repressor in this region. Because the two putative Egr-1 sites appeared significant, these sites were mutated and labeled Mut1 and Mut2, respectively. Mutation of these two Egr-1 sites suppressed the induction of the ATF3-2073 luciferase promoter construct by sulindac sulfide (Fig 4C) and TGZ (Fig 4D). Mutation at the Mut2 site resulted in the most significant suppression of ATF3 luciferase construct, resulting in a 38% and 55% suppression following treatment with sulindac sulfide and TGZ, respectively. Mutation of both sites (Mut1+2) did not add to this suppression, indicating that both sites are equally as important or the Mut2 site is modestly more important for the induction of ATF3 by Egr-1 following treatment with these compounds.

Induction of ATF3 requires protein synthesis and the Egr-1 gene.

To further evaluate the significance of Egr-1 to the induction of ATF3, we confirmed that the induction of ATF3 requires protein synthesis. Cyclohexamide (CHX) is an inhibitor of *de novo* protein synthesis therefore should suppress the induction of ATF3 mRNA by sulindac sulfide or TGZ treatment if protein synthesis of Egr-1 or another gene is required for its induction. In fact,

CHX blocked the induction of ATF3 mRNA by sulindac sulfide and TGZ (Fig 5A). Because ATF3 induction by these drugs appeared to require Egr-1, we overexpressed full-length Egr-1 protein in HCT-116 cells by transiently transfection of an Egr-1 expression plasmid as previously reported (Baek et al., 2003). Egr-1 mRNA was induced 8.4-fold by this construct relative to vector after incubation for 24-h, indicating it serves as a good model for Egr-1 overexpression (Table 1). Egr-1 overexpression resulted in the induction of ATF3 by 3.5-fold at the mRNA level relative to vector expressing cells according to real-time RT-PCR indicating ATF3 is induced by Egr-1, which also occurred at the protein level (data not shown). Egr-1 expression was also measured using primers to an untranslated region of Egr-1 mRNA not found in the mRNA of the overexpression plasmid but found in Egr-1 endogenous mRNA. Endogenous Egr-1 mRNA expression was slightly repressed by Egr-1 overexpression at the mRNA level, which has been previously reported (Cao et al., 1993).

To determine if Egr-1 overexpression induced ATF3 at the transcriptional level, vector or Egr-1 overexpression plasmids were used in conjunction with various ATF3 luciferase promoter constructs and luciferase assays were performed. Induction of ATF3 luciferase promoter activity was observed at the promoter level following overexpression of Egr-1 relative to vector transfected cells. Significant induction of the Luc-1850 and Luc-2073 promoter constructs, which contain two and three, putative Egr-1 sites, respectively, occurred in the presence of Egr-1 overexpression relative to vector (Fig 5B). Induction of the Luc-110 luciferase promoter construct did not occur, supporting the conclusion that Egr-1 is likely involved. This is in general agreement with the results using various luciferase reporter constructs in cells treated with sulindac sulfide and TGZ as seen in Figure 4 A-B. Post-transcriptional gene silencing is another method that can be used to test the hypothesis that Egr-1 is critical to the induction of

ATF3. The suppression of Egr-1 by Egr-1 siRNA was confirmed as indicated in the materials and methods. To evaluate the significance of Egr-1 to the induction of ATF3, cells were transiently transfected with these constructs and subsequently treated with vehicle, sulindac sulfide, or TGZ. Egr-1 siRNA significantly blocked the induction of ATF3 protein expression by sulindac sulfide (Fig 5C) or TGZ (Fig 5D) relative to scrambled RNA according to Western blot analysis. In each instance, at least a fifty-percent reduction in ATF3 and Egr-1 protein expression was observed. Because complete suppression of ATF3 was not seen with Egr-1 siRNA, the possibility that multiple mechanisms may be involved was considered. Furthermore, a longer incubation with the siRNA may be required however this time point was chosen to minimize toxicity to the cells.

The MAPK pathway is involved in the induction of ATF3 by sulindac sulfide and TGZ.

Egr-1 is regulated by various MAPK pathways, in particular Erk1/2 (Wong et al., 2002). Therefore we tested the involvement of various MAPK pathways in the induction of ATF3. Sulindac sulfide and TGZ induced Erk1/2 phosphorylation more than 4-fold in the 1-4-hr time range (Fig 6A), which precedes the induction of ATF3 and Egr-1 protein and mRNA expression as illustrated above. The induction of Erk1/2 phosphorylation was confirmed using an enzyme immunometric assay kit specific for phospho-Erk1/2. Phosphorylation was between 1.5 and 2-fold and was greater for TGZ with significant phosphorylation detected in as early as 30-min by this assay (Fig 6B). MEK1 is an upstream activator of Erk1/2 and subsequently Egr-1. Therefore, HCT-116 cells were pre-treated for 1-h with the MEK1 inhibitor PD98059 (20 μM) based on previous assays and subsequently treated with vehicle, sulindac sulfide, or TGZ for 4-h and ATF3 and Egr-1 mRNA expression was determined. The Erk1/2 specific inhibitor PD98059 almost completely blocked the induction of Egr-1 mRNA by sulindac sulfide and TGZ as

reported elsewhere. The induction of ATF3 mRNA following treatment with sulindac sulfide was blocked by ~33% and almost completely blocked following treatment with TGZ in the presence of PD89059 according to real-time RT-PCR following a 4-h treatment (Table 2). This was confirmed for ATF3 at the promoter level in cells transiently transfected with the ATF3 Luc-2073 construct (data not shown) and at the protein level with similar results (Fig 6C). Therefore, the Erk1/2 pathway appeared to be involved in the induction of ATF3 by these compounds.

ATF3 is induced by MEKK1 (Fan et al., 2002), which is an upstream activator of MEK1. Meanwhile, Egr-1 is induced by MEK1, which is upstream of Erk and downstream of MEKK1. Therefore, to further evaluate the induction of ATF3 by this pathway, we used a dominant negative inhibitor of MEK1, which can neither be phosphorylated by its activators nor phosphorylate its downstream effectors in this pathway. Following transient transfection of HCT-116 cells with vector or a dominant negative inhibitor of MEK1 and an ATF3 luciferase construct, the MEK1 dominant negative inhibitor partially but significantly blocked the induction of ATF3 luciferase activity by sulindac sulfide and TGZ at the promoter level relative to vector DNA indicating the induction of ATF3 by these compounds is downstream of and requires MEK1 (Fig 6D). Thus it is clear that Egr-1 is critical to the induction of ATF3 by NSAIDs and TGZ and that this occurs at least in part via activation of Erk1/2 likely downstream of MEK1 as illustrated diagrammatically in Fig 7.

DISCUSSION:

ATF3 is a transcription factor known to regulate several downstream genes related to cell growth (Fan et al., 2002), apoptosis (Fawcett et al., 1999), and invasion (Yan et al., 2002; Chen and Wang, 2004; Stearns et al., 2004). The expression of ATF3 is modulated by a wide variety of pharmaceutical and dietary compounds with gene regulatory ability. However, no data on the molecular mechanisms responsible for the regulation of ATF3 by NSAIDs, PPARγ ligands, or other anti-cancer compounds exist. Egr-1 is induced by a variety of compounds that are independently reported to induce ATF3 such as anti-cancer agents (Quinones et al., 2003), growth factors (Hjoberg et al., 2004), and PPARγ ligands (Baek et al., 2003). Furthermore, Egr-1 overexpression regulates ATF3 according to microarray analysis (Fu et al., 2003), while the promoters of Egr-1 (Wong et al., 2002) and ATF3 (Fan et al., 2002) are regulated by MEKK1, which is an upstream mediator of Erk1/2. Our laboratory previously demonstrated that sulindac sulfide (Baek et al., 2005) and TGZ (Baek et al., 2004) increased the expression of Egr-1 independent of PPAR. However no reports on the regulation of ATF3 by Egr-1 following treatment with anti-cancer or other compounds exist. Sulindac sulfide and TGZ are independently reported to regulate the expression of ATF3 and Egr-1. Therefore, we chose to test the hypothesis that the tumor suppressor gene Egr-1 regulates the induction of ATF3 following treatment with these two compounds with divergent chemical structures and activities.

This is the first report to demonstrate that Egr-1 is required for the induction of ATF3 by sulindac sulfide and TGZ based on a wide variety of molecular data. Sulindac sulfide and TGZ significantly induced ATF3 and Egr-1 protein and mRNA expression and ATF3 required *de novo* protein synthesis for its induction by these compounds. ATF3 is induced by Egr-1 overexpression in these cells and the induction of ATF3 was blocked, in part, by Egr-1 siRNA.

The ATF3 promoter contains three putative Egr-1 sites in the first 2-kb of its promoter, and the two 5′ Egr-1 sites appeared most significant to the induction of ATF3 by these compounds according to luciferase reporter assays. Mutation of the two Egr-1 sites in the ATF3 Luc-2073 luciferase promoter construct suppressed activity of this construct following treatment with sulindac sulfide or TGZ. However, Mutation of both sites (Mut1+2) did not add to this suppression following treatment with these compounds, indicating that both sites are equally necessary for the induction of ATF3 by Egr-1 or the Mut2 site is modestly more significant. Thus the expression of ATF3, at least in part, requires Egr-1, but other known and potential binding sites are present in the promoter of ATF3. Therefore, the possibility that other regulatory binding sites are also involved in the induction of ATF3 can not be excluded.

The induction of ATF3 appears to involve the Erk1/2 MAPK cascade, which is upstream of Egr-1. For example, the phosphorylation of Erk1/2 was increased by sulindac sulfide and TGZ in these experiments and the induction of ATF3 and Egr-1 protein, mRNA, and luciferase reporter activity following treatment with sulindac sulfide or TGZ were blocked by the Erk1/2 inhibitor PD98059. The involvement of the Erk1/2 MAPK cascade was confirmed using a dominant negative inhibitor of MEK1, which is an upstream activator of Erk1/2. The MEK1 dominant negative inhibitor significantly attenuated the induction of ATF3 luciferase activity by sulindac sulfide and TGZ. However, the complete mechanisms for the induction of ATF3 or Egr-1 by these compounds such as potential upstream steps of MEK1 require further evaluation beyond the scope of this report. Nevertheless, taken together it is clear that Egr-1 is critical to the induction of ATF3 by these compounds and that this likely occurs downstream of MEK1 involving the Erk1/2 MAPK signal transduction pathway. The involvement of various MAPK pathways in the downstream effects of NSAIDs are reported elsewhere as illustrated in a recent

review (Tegeder et al., 2001). For example, Erk and p38 are activated by sulindac sulfide and NS-398 in colorectal cancer cells, which, along with the induction of apoptosis, are blocked by Erk and p38 selective inhibitors (Sun and Sinicrope, 2005). Importantly, the concentrations used in this investigation are relatively low and our results were observed with two distinctly different chemical compounds that are documented to regulate Erk1/2 and Egr-1.

The Cox-inhibitory action of NSAIDs plays an important role in the inhibition of tumorigenicity, but the global pattern of genes altered following exposure to these compounds need to be considered particularly in light of recent concerns that the use of selective Cox inhibitors in humans over long periods of time may results in dangerous cardiovascular events (Bresalier et al., 2005). In fact, we previously reported that NSAID regulate gene expression independent of their Cox-inhibitory activity based, in part, on the observation that the Cox-1 specific inhibitor SC-560, which has anti-tumorigenic activity (Daikoku et al., 2005), was more potent an inducer of gene regulation than SC-58125, a Cox-2 selective inhibitor (Bottone et al., 2004). Sulindac sulfide was chosen in this study because it is a potent inducer of ATF3. However, other NSAIDs such as SC-560 likely regulate the expression of ATF3 via Egr-1. In addition to ATF3, NSAIDs and other chemo-therapeutic compounds alter a number of genes associated with apoptosis, invasion, angiogenesis, and adhesion, some of which are regulated by Egr-1. NAG-1 is induced by a variety of natural and synthetic compounds, has pro-apoptotic and anti-tumorigenic activity, and is regulated by Egr-1. ATF3 is also modulated by a wide variety of cancer chemopreventive compounds including several dietary compounds and ATF3 has anti-tumorigenic activity in mouse tumor xenograft models *in vivo* and anti-invasive activity *in vitro* (Bottone et al., 2005). Therefore, we propose that NSAIDs and other potential anti-cancer agents may act, in part, by increasing the expression of Egr-1, a reported tumor

suppressor, which in turn regulates the expression of ATF3 or other downstream gene targets with anti-cancer activity or other biologically relevant activities. However, further work is needed to determine the biological significance of the gene regulatory ability of these compounds. The expression of anti-cancer genes such as ATF3, Egr-1, and NAG-1, are often lost during the carcinogenic process and compounds that induce their expression may act to restore this expression under these circumstances with beneficial effects. Thus, the downstream targets of Egr-1 could inhibit tumorigenicity via both apoptosis (Baek et al., 2001) and invasion (Bottone et al., 2005), respectively.

While this work provides significant insight into the promoter regulation of ATF3 by sulindac sulfide and TGZ, further work is needed to completely understand the mechanism or potentially overlapping mechanisms involved. This is a novel, first report to demonstrate that ATF3 is regulated at the transcriptional level by two pharmaceutical agents, sulindac sulfide and TGZ, with known gene regulatory and reported chemopreventive activity. It is possible that the induction of other anti-cancer genes by these agents occurs via the transcription factors Egr-1 or ATF3, thereby contributing to the biological activity of these compounds. Future studies in the area of chemoprevention should consider the diverse gene regulatory role of pharmaceutical or other compounds. Observations from animal experiments linking alteration in tumor growth to drug induced changes in Egr-1 and its downstream target genes are currently in progress.

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LEGENDS FOR FIGURES:

Figure 1. Sulindac sulfide and TGZ modulate ATF3 and Egr-1 protein expression in a time- and concentration-dependent manner. HCT-116 cells were treated with (A) vehicle, 20 μM sulindac sulfide, or 10 μM TGZ in SFM for the times indicated and ATF3, Egr-1, and actin protein expression determined by Western blot analysis. (B) HCT-116 cells were treated in SFM for 6-h with (left) vehicle (0, Lane-1), 10, 20, or 30 μM sulindac sulfide (Lanes 2-4) or (right) vehicle (Lane-1), 0.1, 1, 5, 10 μM TGZ and protein expression determined by Western blotting.

Figure 2. Sulindac sulfide and TGZ modulate ATF3 and Egr-1 mRNA expression in a time- and concentration-dependent manner. (A-B) Cells were treated with vehicle or (A) 10 μM sulindac sulfide or (B) 5 μM TGZ in SFM for the times indicated and mRNA expression measured by real-time RT-PCR relative to time-matched vehicle-treated controls adjusted for actin. (C-D) Cells were treated with vehicle or various concentrations of (C) sulindac sulfide or (D) TGZ for 4-h with the concentrations indicated in SFM and mRNA expression measured by Real-Time RT-PCR relative to vehicle-treated controls adjusted for actin. Values are means (± SEM) and are expressed as fold induction relative to time-matched vehicle treated cells.

Figure 3. Various luciferase reporter constructs used in these experiments illustrating the putative Egr-1 binding sites (black arrows), which were mutated in these experiments (gray dashed arrows). Several other known promoter binding sites are reported but are not shown.

Figure 4. Sulindac sulfide and TGZ induce various regions of the wild-type ATF3 promoter, which is suppressed following mutation of the two critical Egr-1 sites. HCT-116 cells were

transiently transfected with equal amounts of the ATF3 luciferase reporter construct indicated and control Renillia luciferase plasmid (pRLnull) then recovered overnight in complete media then treated with vehicle, (A and C) sulindac sulfide (10 μM), or (B and D) TGZ (5 μM) as indicated for 24-h in SFM. After 24-h cells were assayed for luciferase activity. Values are mean (± STD) and are expressed as (A-B) relative luciferase activity or (C-D) fold increase relative to similarly transfected, vehicle-treated controls. Statistical significance (C-D) is by ANOVA with Bonferroni t-test for pair-wise comparisons relative to sulindac sulfide-treated Luc-2073 transfected cells at the $p<0.05$ level of significance. *denotes statistical significance at the $p<0.05$ level whereas **denotes the $p<0.01$ level.

Figure 5. Transcriptional regulation of ATF3 requires *de novo* protein synthesis and Egr-1. (A) HCT-116 cells were pre-treated in SFM for 0.5-h with 5 μg/ml cyclohexamide (CHX) then treated for 4-h with vehicle, 20 μM sulindac sulfide, or 10 μM TGZ ATF3 mRNA expression determined by real-time RT-PCR relative to vehicle-treated controls and adjusted for actin. Values are means (± SEM) and are expressed as fold induction relative to vehicle treated cells. (B) HCT-116 cells were transiently transfected with equal amounts of pcDNA3.1 or Egr-1 plasmid DNA plus the ATF3 luciferase construct indicated as described above then assayed for luciferase activity. Values are means (± STD) and are expressed as fold induction relative to vector transfected cells. (C-D) Egr-1 siRNA blocks the induction of ATF3 by sulindac sulfide and TGZ. HCT-116 cells were transiently transfected for 5-h in SFM with scrambled RNA or Egr-1 siRNA as indicated, allowed to recover overnight in complete media, then treated for 24-h in SFM with vehicle or (C) 20 μM sulindac sulfide or (D) 10 μM TGZ. Cells were isolated for protein followed by Western blot analysis for ATF3, Egr-1, and actin.

Figure 6. Sulindac sulfide and TGZ induce Erk1/2 phosphorylation and the MAPK pathway is required for induction of ATF3 by these compounds. (A-B) HCT-116 cells were treated with vehicle, 20 μM sulindac sulfide, or 10 μM TGZ in SFM after overnight serum-starvation for the times indicated. (A) Western blot analysis of p-Erk1/2, total Erk, and actin. Fold induction [in parenthesis] is relative to time-matched vehicle-treated controls adjusted for actin. (B) Cells were treated for 0, 5, 10, or 30 minutes as indicated then p-Erk1/2 levels were determined using an Erk1/2 phospho-specific enzyme immunometric assay kit as described in the materials and methods. Values are expressed as fold induction over time-matched vehicle-treated cells. (C) Left panel: HCT-116 cells were treated with vehicle, 10, or 20 μM sulindac sulfide (lanes 1-3) or 20 μM PD98059 plus vehicle, 10, or 20 μM sulindac sulfide (lanes 4-6). Right panel: HCT-116 cells were treated with vehicle, 5, or 10 μM TGZ for 6-h followed by protein isolation and Western blot analysis of ATF3 and actin. (D) HCT-116 cells were transiently transfected as previously described with the ATF3 Luc-2073 reporter construct plus vector DNA or a MEK1 dominant negative inhibitor (DNI) as indicated. Cells were treated for 24-h in SFM with vehicle, 20 μM sulindac sulfide, or 10 μM TGZ and ATF3 luciferase activity determined by luciferase reporter assays. Values are relative to vehicle treated cells and statistical significance is versus vector-transfected cells exposed to the same drug treatment. Statistical significance (B, D) is by ANOVA with Bonferroni t-test for pair-wise comparisons at the $p<0.05$ level of significance. *denotes statistical significance at the $p<0.05$ level whereas **denotes the $p<0.01$ level.

Figure 7. Proposed molecular mechanism and evidence for the induction of ATF3 by sulindac sulfide and TGZ. Erk1/2 and Egr-1 are involved in the induction of ATF3 by sulindac sulfide

and TGZ. Bars with arrowheads (↓) indicate a potential downstream activation while lines with a bars (⊥) indicate steps in the pathway that were inhibited.

TABLE 1

Overexpression of Egr-1 Induces ATF3 mRNA gene expression.

HCT-116 cells were transiently transfected as described in the materials and methods using vector or an Egr-1 plasmid expressing full-length protein. Primers for exogenous Egr-1 recognize the Egr-1 sequence used in the overexpression plasmid while primers for endogenous Egr-1 are from an untranslated mRNA region not found nor required in the overexpression plasmid and is shown for comparison. Values are expressed as mean (± SEM) relative to vector-transfected cells according to real-time RT-PCR adjusted for actin.

Egr-1 Overexpression	Fold mRNA
ATF3	3.5 ± 0.12
Exogenous Egr-1	8.4 ± 0.16
Endogenous Egr-1	0.88 ± 0.03

TABLE 2

The induction of ATF3 and Egr-1 mRNA gene expression by sulindac sulfide and TGZ occurs via the Erk1/2 MAPK pathway.

Cells were treated for 4-h with vehicle (DMSO), sulindac sulfide (20 μM), or TGZ (10 μM) followed by mRNA expression for ATF3, Egr-1, and actin. Values are expressed as mean fold change (± SEM) relative to vehicle-treated cells adjusted for actin. The inhibitor PD98059 was used at 20 μM. Values from PD-treated cells are shown for completeness. Statistical significance is according to Bonferroni t-test for multiple comparisons at the $p<0.01$ level of significance on Ct values adjusted for actin from vehicle, sulindac sulfide (or TGZ), and sulindac sulfide (or TGZ) + PD98059 treated cells. [a-b] Values sharing a letter are not significantly different, while values with a unique letter are significantly different than vehicle-treated cells.

Treatment:	ATF3 mRNA	Egr-1 mRNA
sulindac sulfide	7.1 ± 0.14 [a]	21.6 ± 0.6 [a]
sulindac sulfide + PD98059	3.4 ± 0.05 [b]	0.5 ± 0.03 [b]
TGZ	37.1 ± 0.45 [a]	32.2 ± 0.8 [a]
TGZ + PD98059	1.5 ± 0.02 [b]	0.1 ± 0.01 [b]
PD98059	1.2 ± 0.05	0.1 ± 0.01

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Fig. 1

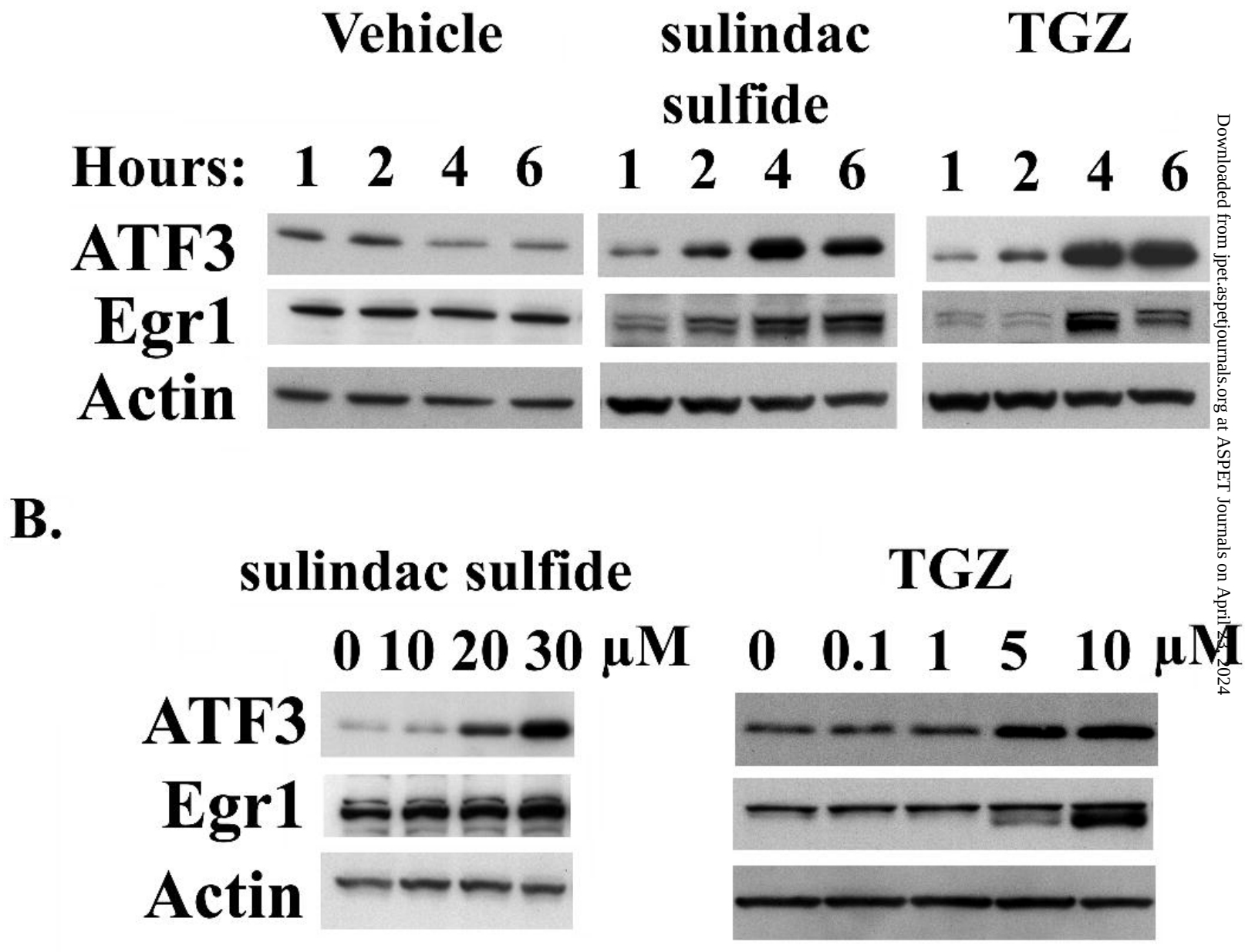

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Fig. 2

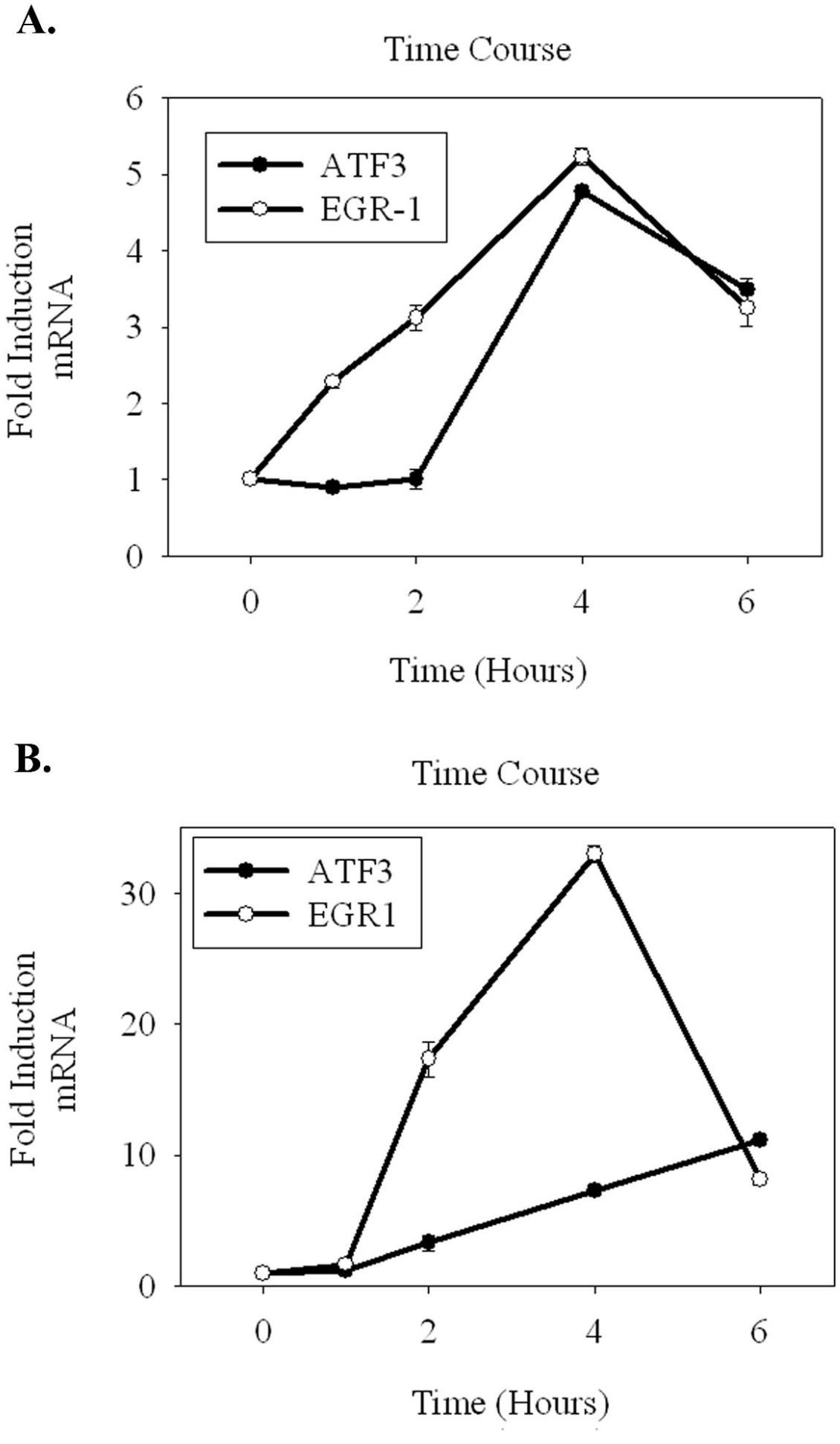

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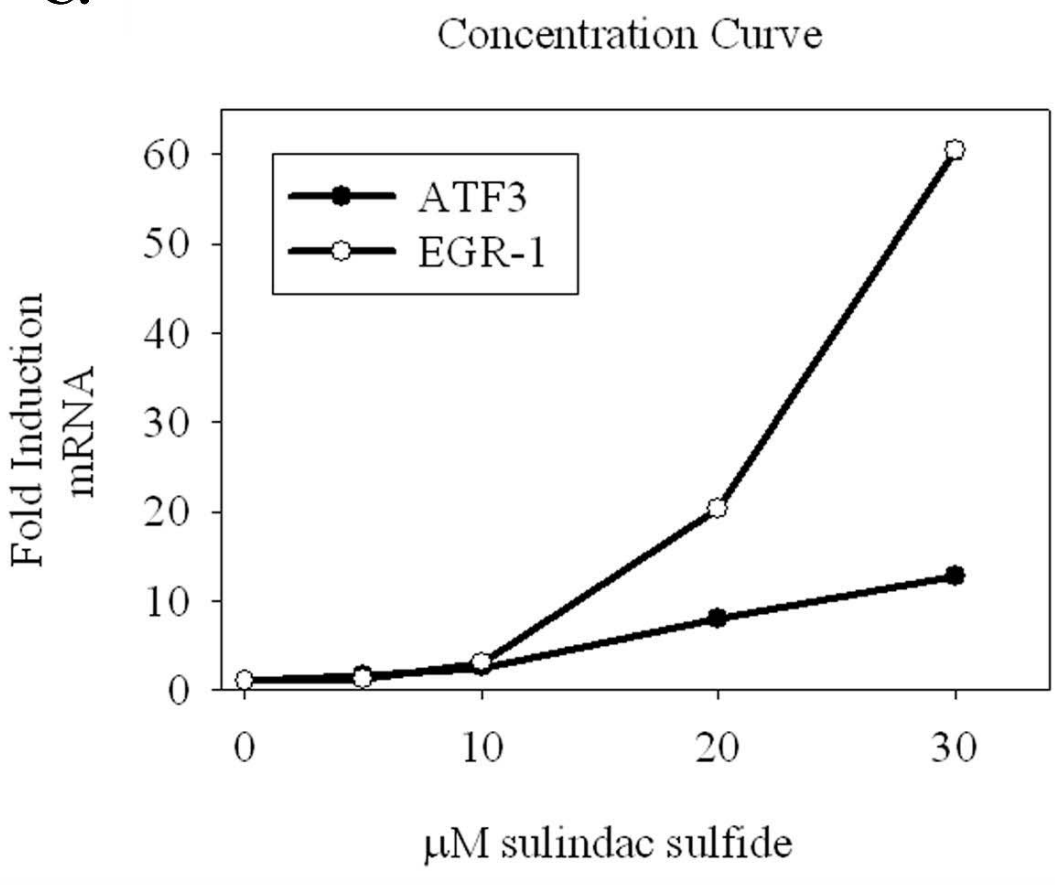
Fig. 2

C.



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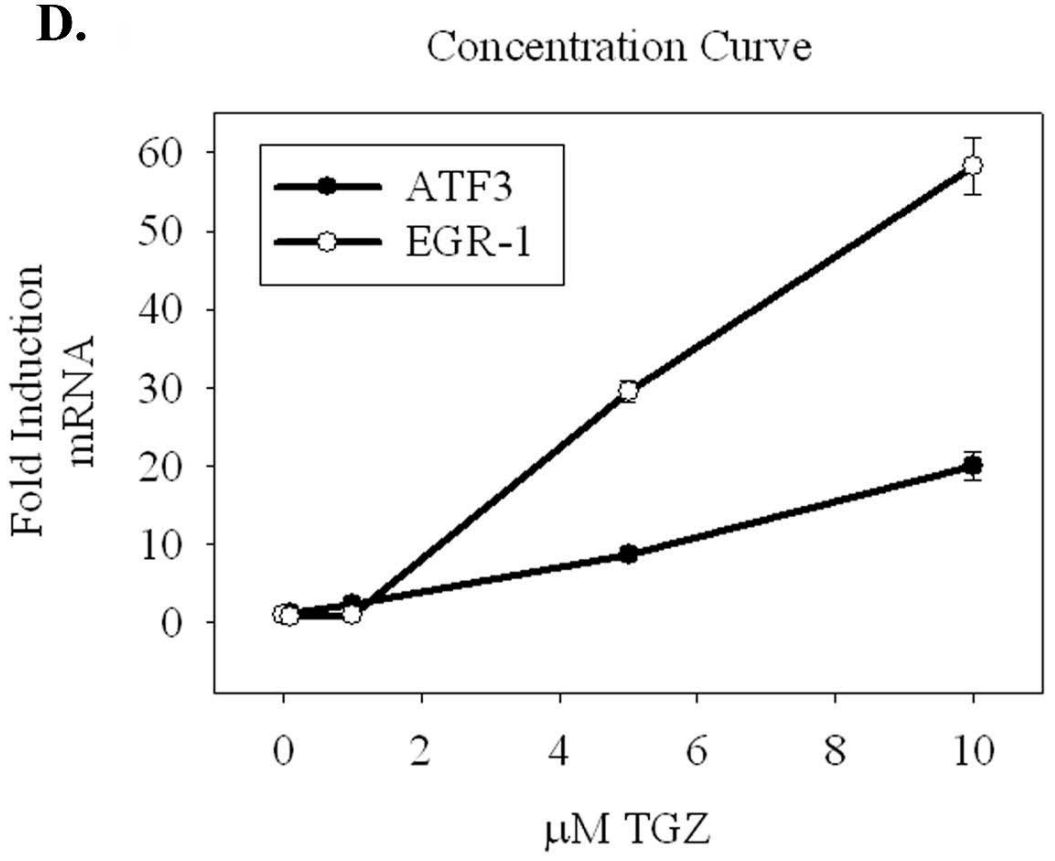

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Fig. 3

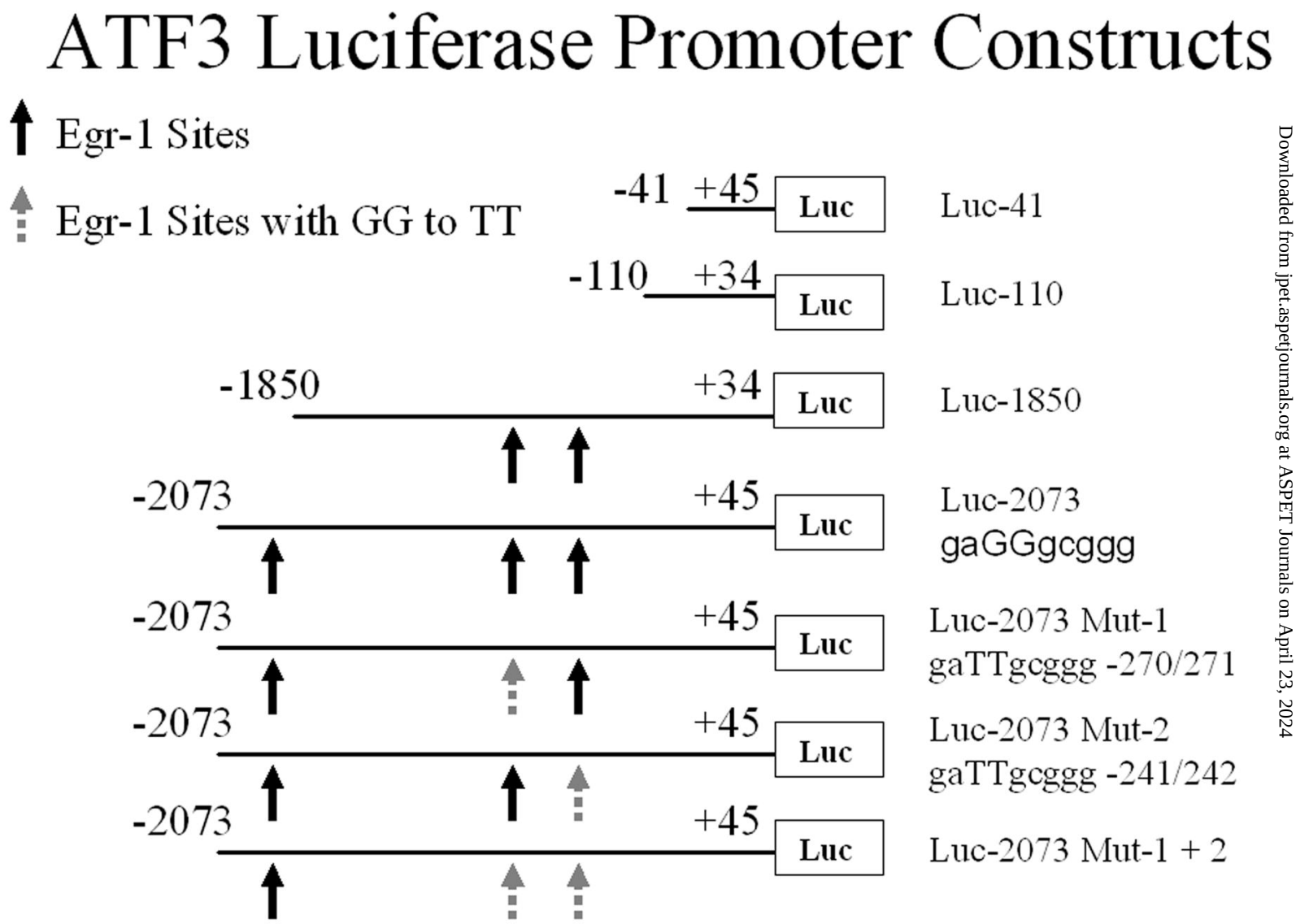

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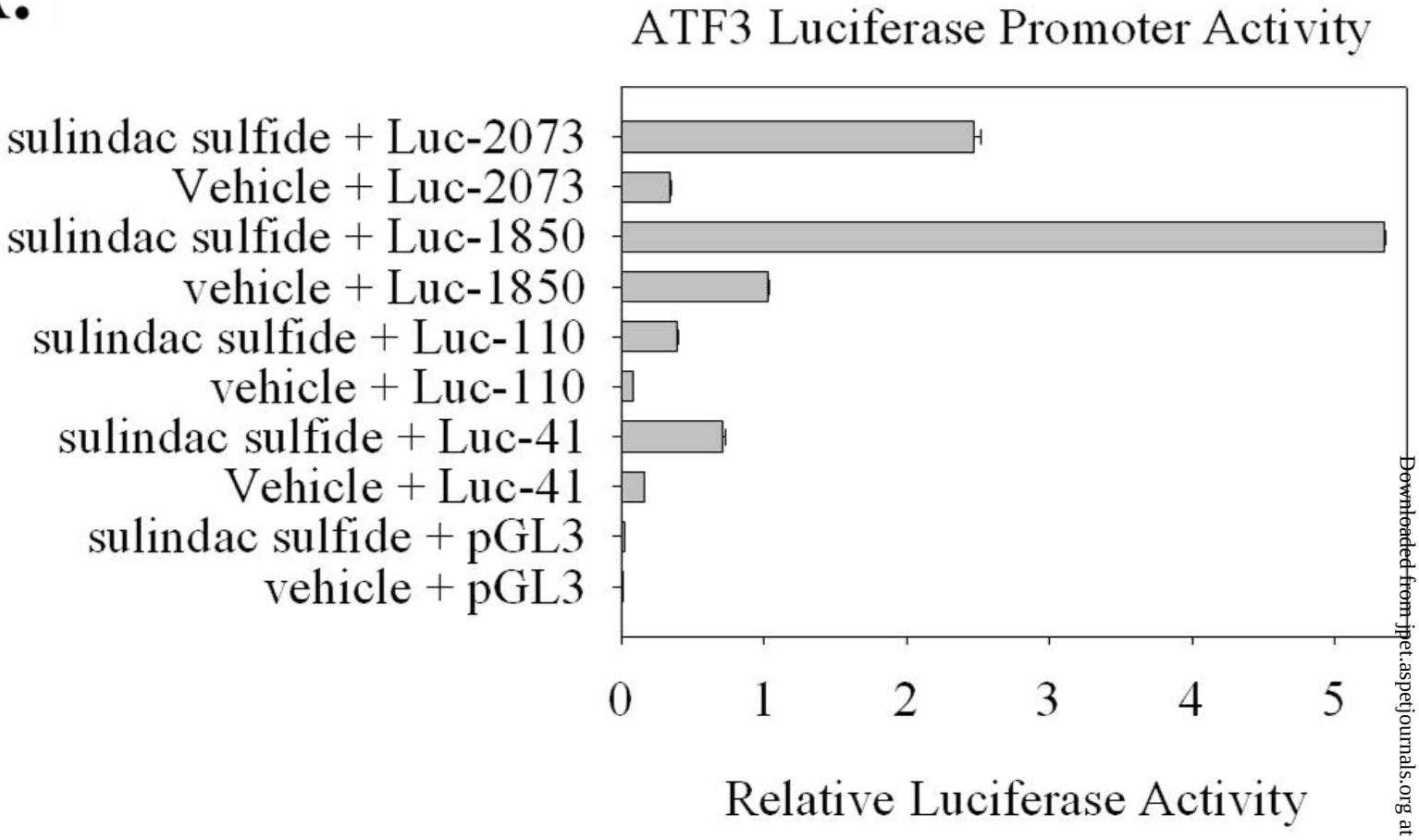
Fig. 4

A.



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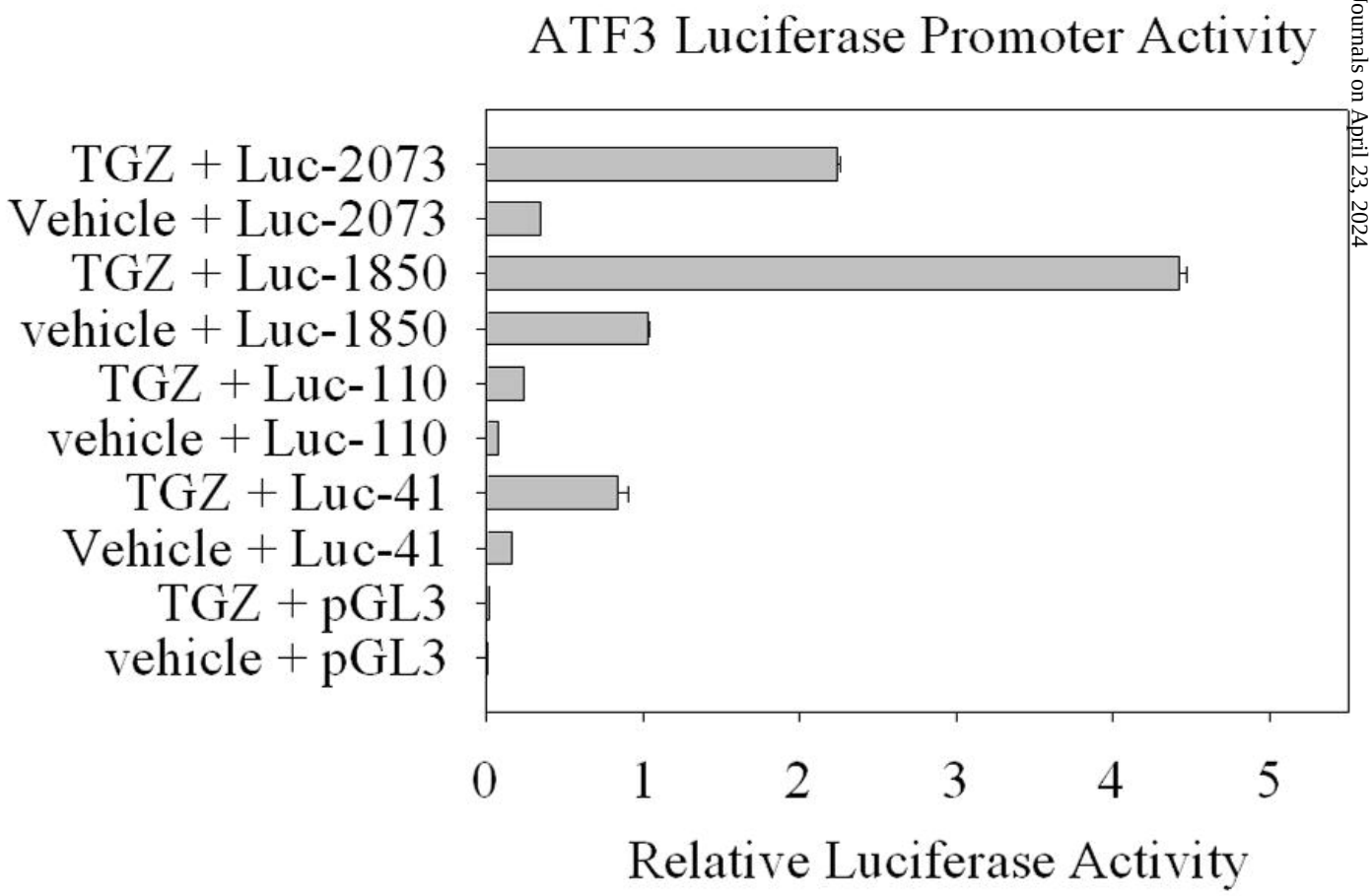

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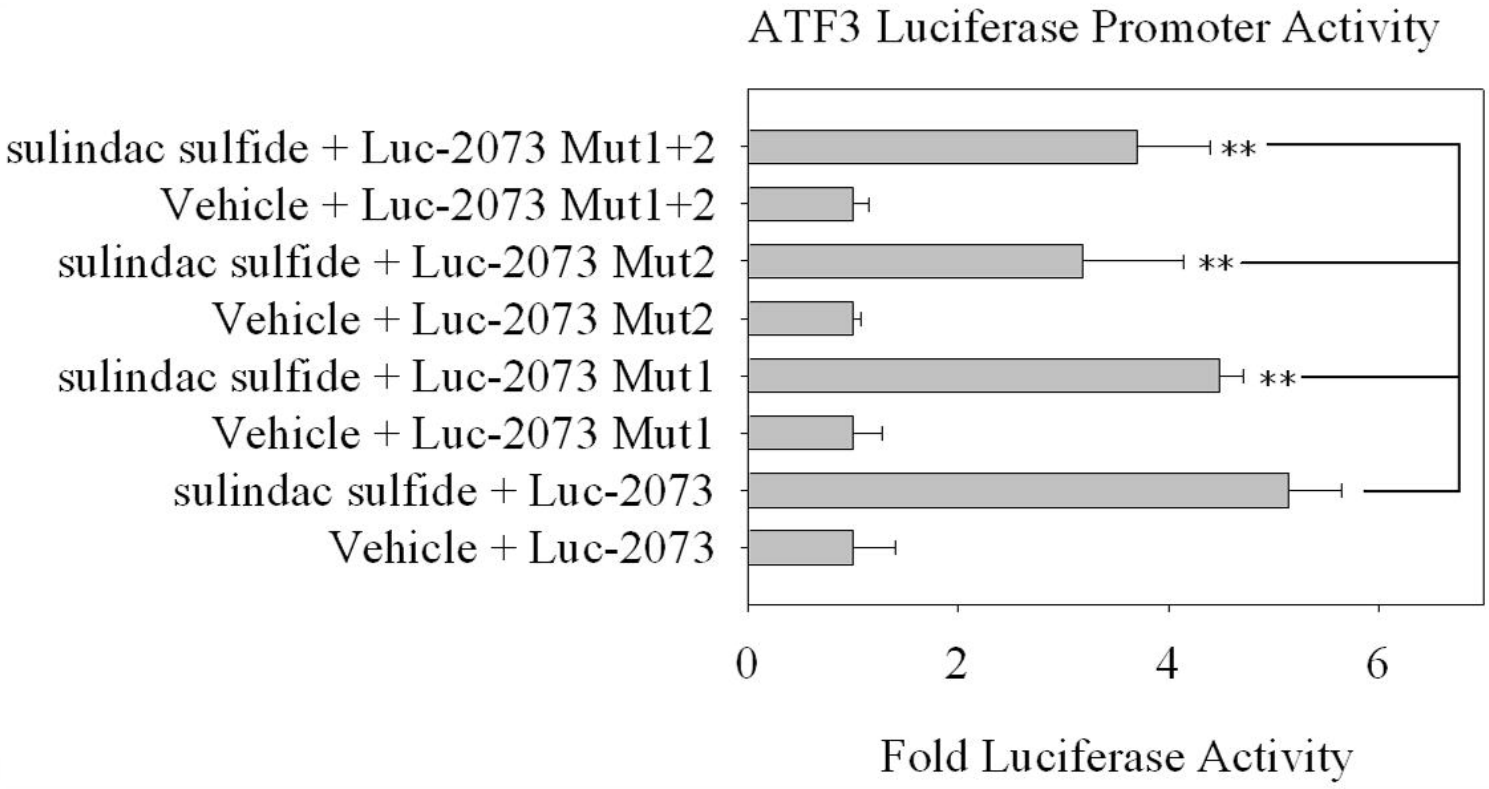
Fig. 4

C.



D.

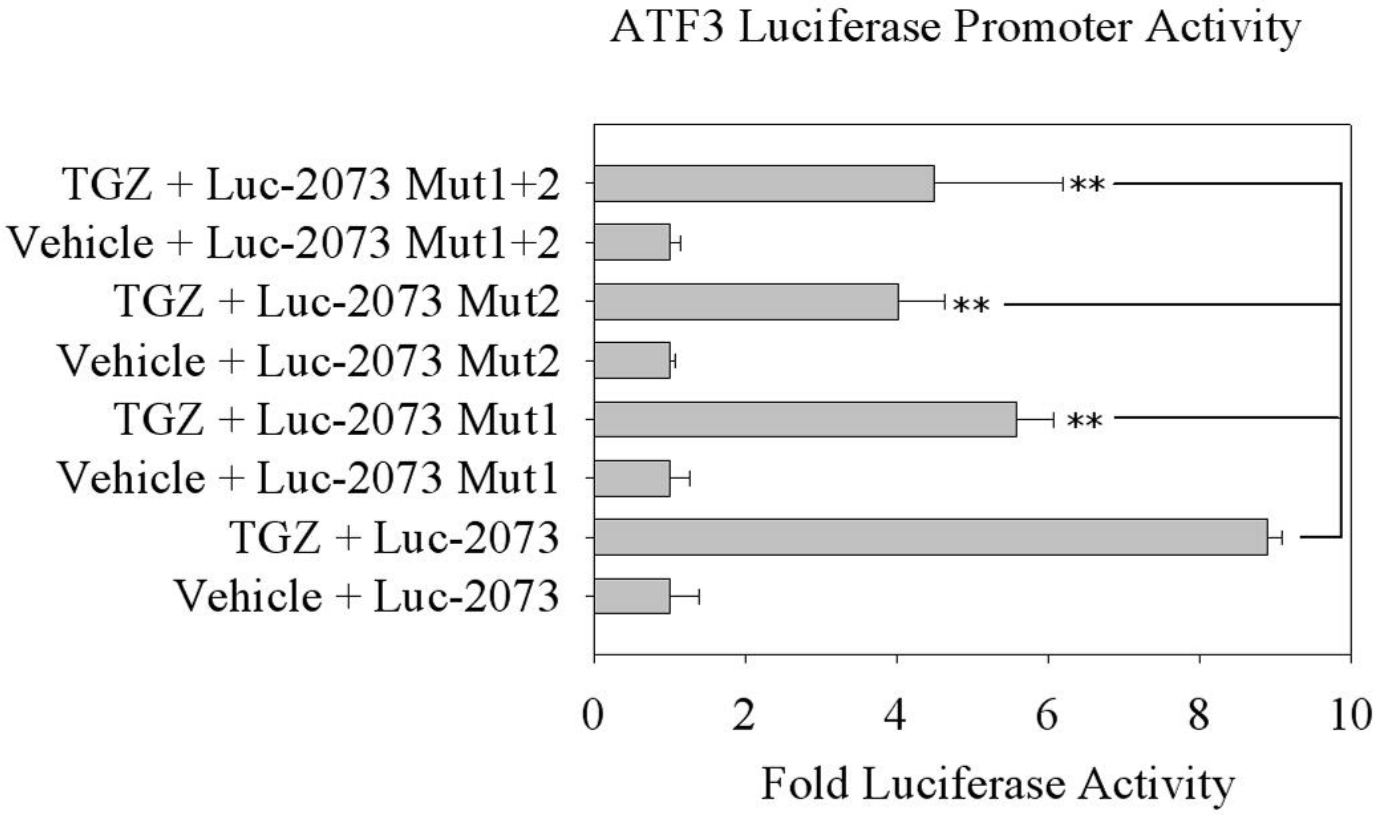
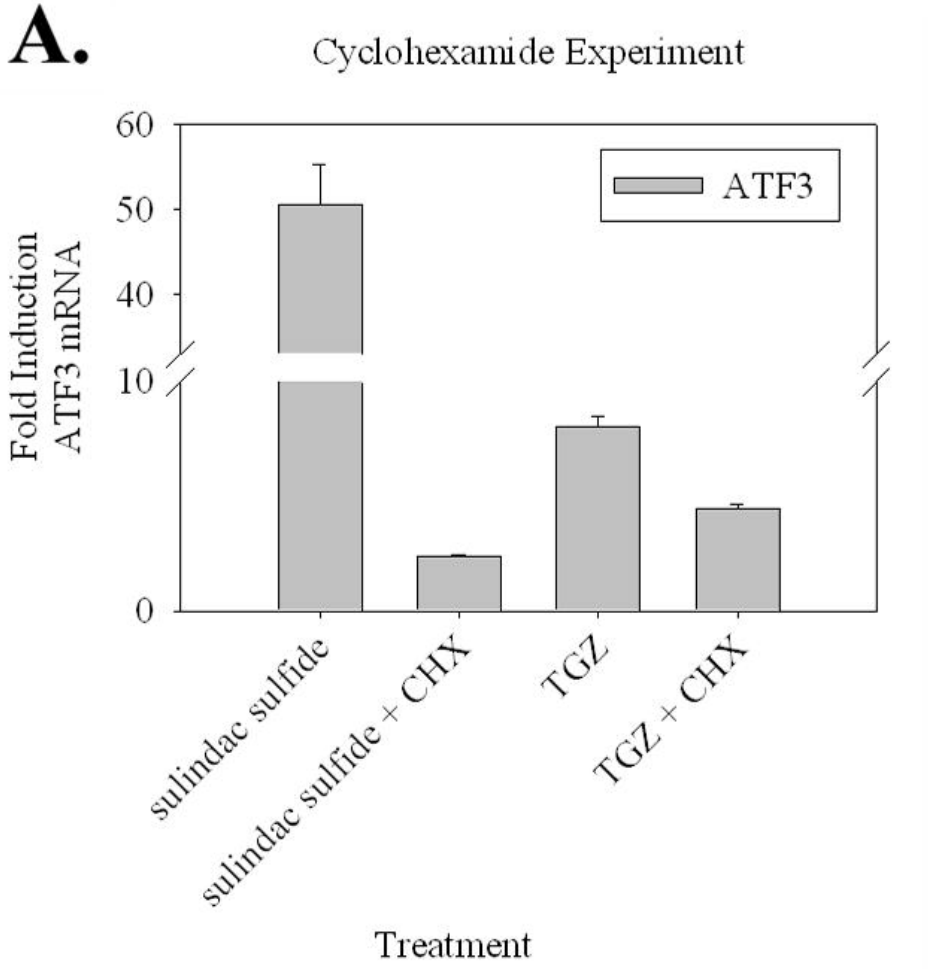


Fig. 5



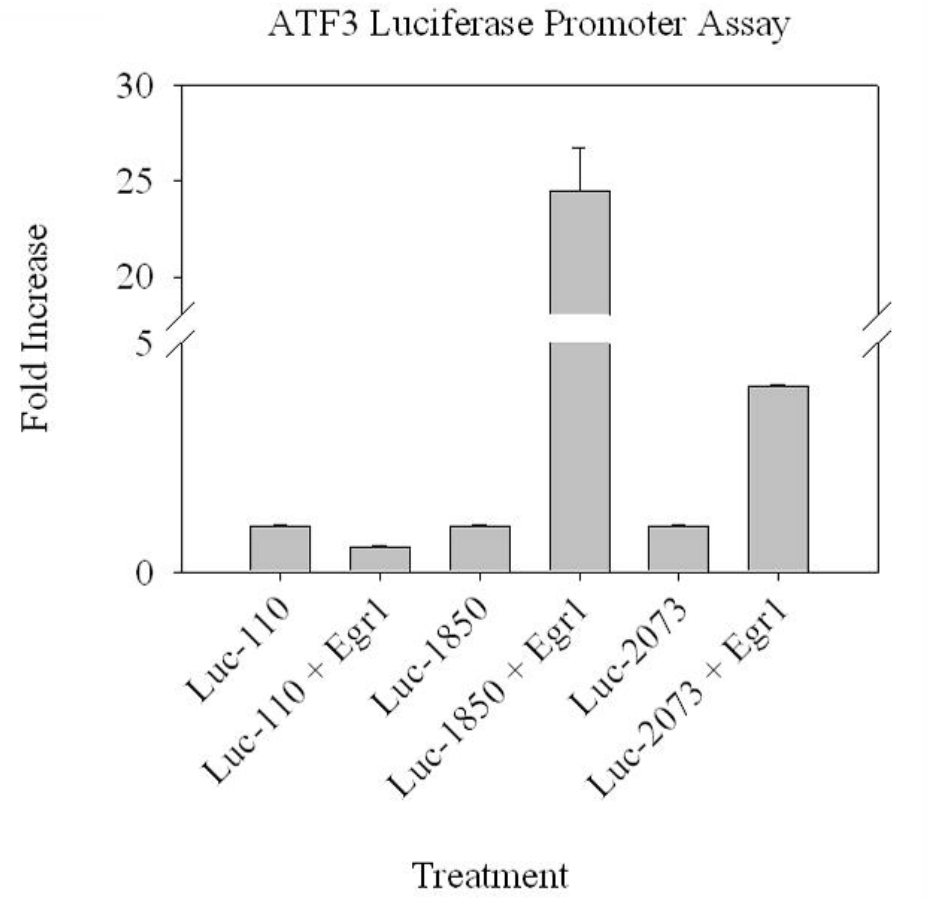
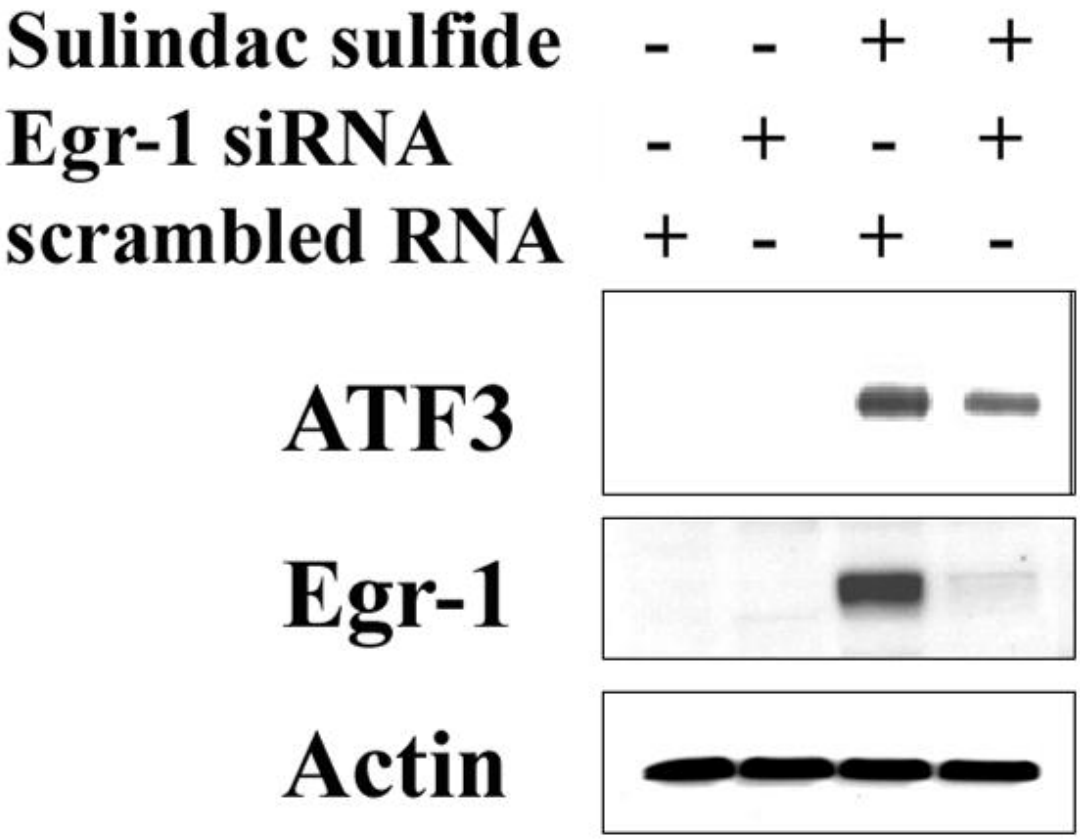


Fig. 6

C.



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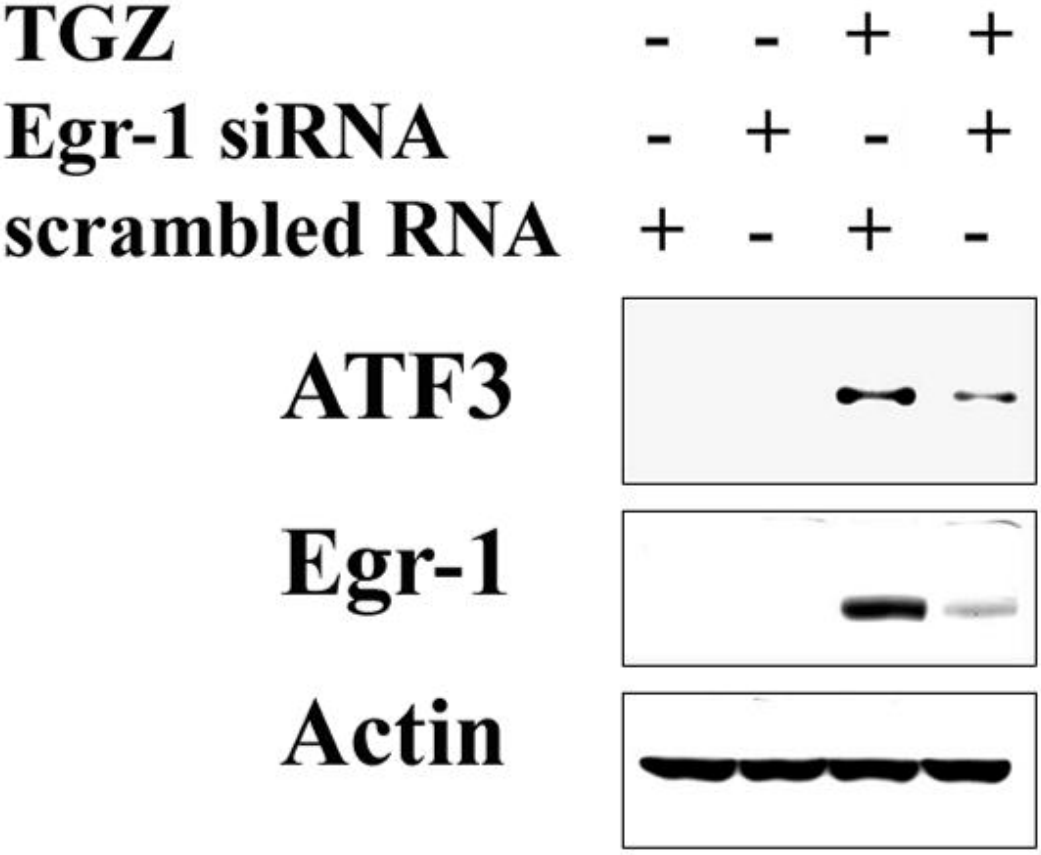

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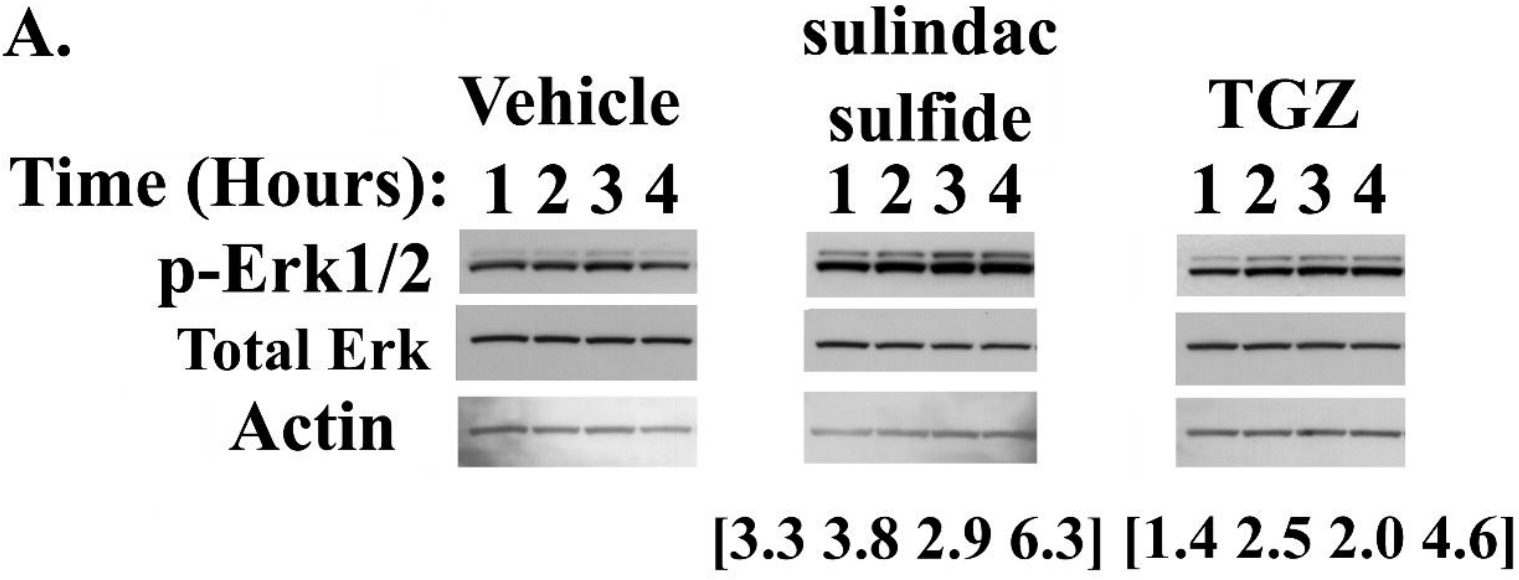
Fig. 6

A.



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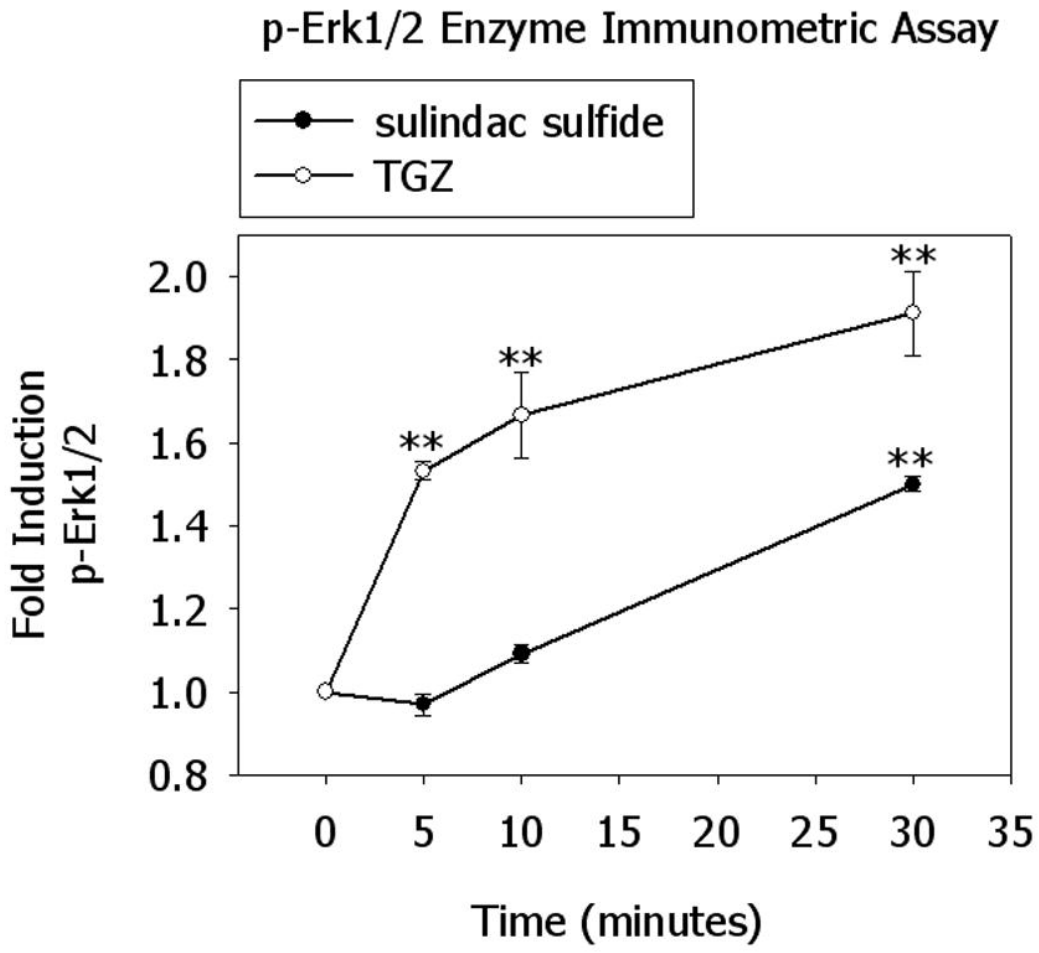

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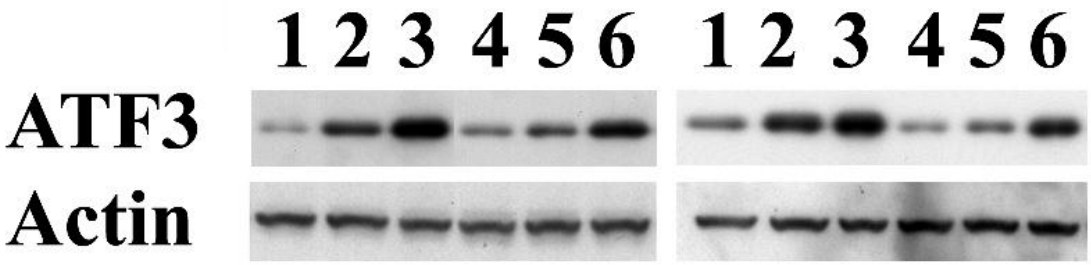
Fig. 6 C.



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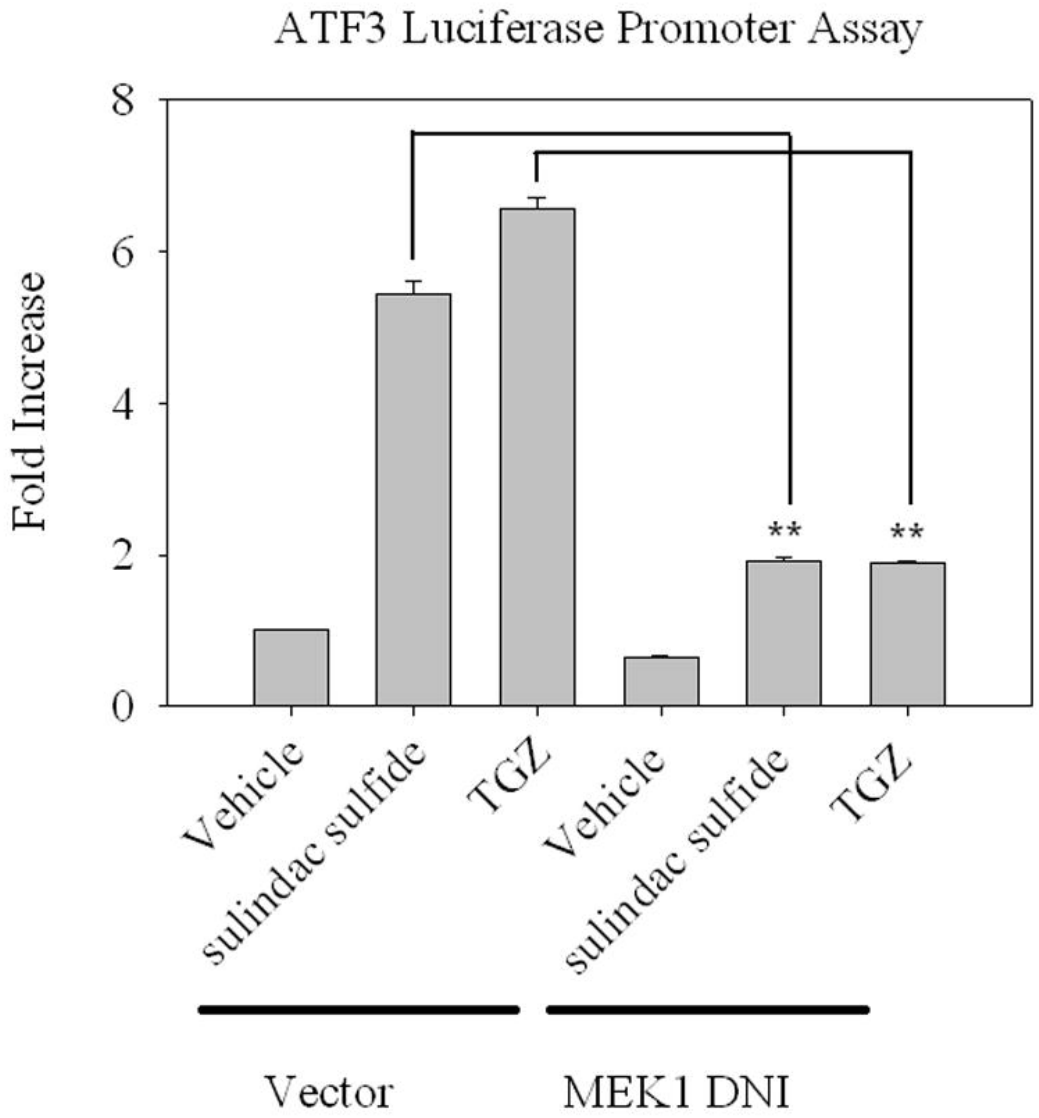

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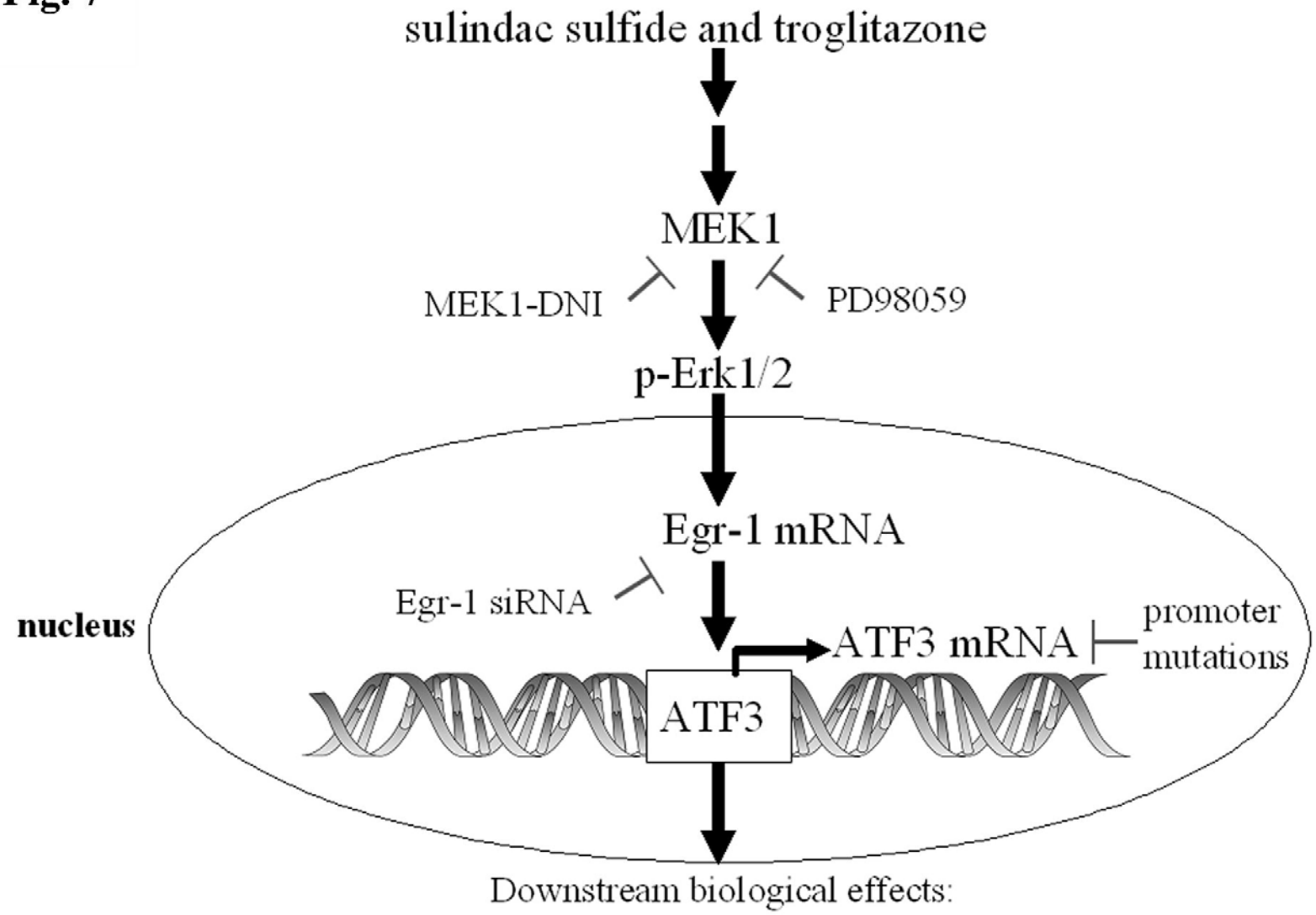
Fig. 7



alterations in gene expression, cell growth, apoptosis, and/or invasion