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Manipulation of the HIF-Vegf pathway rescues methyl tert-butyl ether (MTBE)-induced vascular lesions

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Abstract

Methyl tert-butyl ether (MTBE) has been shown to be specifically anti-angiogenic in piscine and mammalian model systems at concentrations that appear non-toxic in other organ systems. The mechanism by which MTBE targets developing vascular structures is unknown. A global transcriptome analysis of zebrafish embryos developmentally exposed to 0.00625–5 mM MTBE suggested hypoxia inducible factor (HIF)-regulated pathways were affected. HIF-driven angiogenesis via *vascular endothelial growth factor (vegf)* is essential to the developing vasculature of an embryo. Three rescue studies were designed to rescue MTBE-induced vascular lesions: pooled blood in the common cardinal vein (CCV), cranial hemorrhages (CH), and abnormal intersegmental vessels (ISV), and test the hypothesis that MTBE toxicity was HIF-Vegf dependent. First, zebrafish *vegfa* over-expression via plasmid injection, resulted in significantly fewer CH and ISV lesions, 46 and 35% respectively, in embryos exposed to 10mM MTBE. Then HIF degradation was inhibited in two ways. Chemical rescue by N-oxaloylglycine significantly reduced CCV and CH lesions by 30 and 32% in 10mM exposed embryos, and ISV lesions were reduced 24% in 5mM exposed zebrafish. Finally, a morpholino designed to knockdown ubiquitin associated von Hippel-Lindau protein, significantly reduced CCV lesions by 35% in 10mM exposed embryos. In addition, expression of some angiogenesis related genes altered by MTBE exposure were rescued. These studies demonstrated that MTBE vascular toxicity is mediated by a down regulation of HIF-Vegf driven angiogenesis. The selective toxicity of MTBE toward developing vasculature makes it a potentially useful chemical in the designing of new drugs or in elucidating roles for specific angiogenic proteins in future studies of vascular development.

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SUPPLEMENTARY DATA DESCRIPTION:

The supplemental data for this manuscript include array data files accessible at the NCBI GEO data bank via <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46371>, extra tables from the microarray analysis that were informational but contained some redundant information, the mRNA expression levels of the 2 hr NOG incubation used to chose a dose for Rescue Study 2, and pictures demonstrating that the VHL-MO designed for Rescue Study 3 induced the formation of additional angiogenic sprouts similar to those reported in the zebrafish VHL mutant.

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Keywords

Vascular Toxicity; Angiogenesis; Methyl tert-butyl ether; vascular endothelial growth factor; Hypoxia inducible factor; N-oxalylglycine; von Hippel-Lindau protein

INTRODUCTION

Angiogenesis occurs as a response to the hypoxic conditions that arise during tissue growth. Hypoxia stimulates the translocation of cytoplasmic hypoxia inducible factor 1- α (HIF1- α) into the nucleus, where it dimerizes with nuclear HIF1- β and binds to DNA at HIF response elements, driving the highly conserved process of angiogenesis primarily through upregulation of vascular endothelial growth factor (Vegf) (Wang and Semenza, 1993). Both exogenous and endogenous factors can disrupt angiogenesis by targeting the expression and function of Vegf and other important proteins. Unorganized, leaky, or stunted vascular structures are consequences of altered Vegf signaling (Carmeliet et al., 1996; Ferrara et al., 1996; Nasevicius *et al.*, 2000; Bahary et al., 2007). Previously, we have demonstrated that methyl tert-butyl ether (MTBE) specifically targets the developing vasculature in multiple model systems, both *in vivo* and *in vitro*, leading to abnormal vascular structures, increased hemorrhages, and disorganized endothelial cell arrangement (Bonventre et al., 2011; Kozlosky et al., 2012). During embryogenesis in the model vertebrate, zebrafish (*Danio rerio*), the vascular lesions induced by MTBE exposure are associated with decreased *vegfa* mRNA expression within the critical period established for the toxicant, between 6-somites to Prim-5 (Bonventre et al., 2011). We hypothesized that MTBE disrupts angiogenesis through a dysregulation of Vegf and associated pathways.

In the developing embryo, hypoxia is the primary inducer of angiogenesis (Ryan et al. 1998, Ramirez-Bergeron et al., 2004). Regulation of HIF1- α under normoxic conditions is crucial to maintaining appropriate vascularization. In the presence of abundant O₂, 2-oxoglutarate, and Fe²⁺, HIF1- α is hydroxylated on two conserved proline residues in the oxygen-dependent degradation domain by prolyl-4-hydroxylase domain (PHD) proteins. The proline hydroxylation allows for the von Hippel-Lindau (VHL) protein to bind and promote the ubiquitination and trafficking of HIF1- α to the proteasome (Ohh et al., 2000; Ivan et al., 2001; Jaakkola et al., 2001; Kim et al. 2006). PHD and VHL proteins indirectly regulate angiogenesis by controlling the quantity of HIF1- α in the cytoplasm. An excess of PHD co-substrate 2-oxoglutarate drives HIF1- α hydroxylation and its degradation, and therefore decreases Vegf expression and angiogenesis. Conversely, 2-oxoglutarate mimetics, e.g. N-oxaloylglycine or dimethyloxaloylglycine, competitively inhibit PHD activity and increase angiogenesis (Wirthner et al., 2007; van Rooijen et al., 2009). The von Hippel-Lindau syndrome, the disease from which the protein derives its name, is characterized by highly vascularized sporadic clear cell renal carcinomas associated with loss of function of VHL protein (Gnarra et al., 1994; Chew 2005). VHL inactivation in tumors leads to increased stabilization of HIF1- α , over-expression of HIF responsive genes, i.e. Vegf-A, and increased vascularization. As a result, PHD and VHL are valuable targets to use for studying HIF-Vegf driven angiogenesis.

Both transcription and mRNA stabilization of *vegfa* are increased by the hypoxia driven activation of HIF1 (Minchenko et al., 1994; Shima et al., 1995). Vegf-a belongs to a family of proteins that bind to extracellular portions of tyrosine kinase receptors, Vegf receptors (Vegfrs), to regulate the endothelial cell differentiation, proliferation, and migration, as well as the secretion of proteins required for tissue modification (Hollborn et al., 2007; Coultas et al., 2005; Matsumoto et al., 2005; Patel-Hett and D'Amore 2011). During vertebrate organogenesis, the relationship between HIF1 and Vegf is essential for normal development.

Our previous studies have implicated the Vegf pathway in MTBE vascular toxicity (Bonventre et al., 2011; Bonventre et al., 2012). Here, we used the Affymetrix Zebrafish Genome Array GeneChip[®] followed by Ingenuity Pathway Analysis[®] as an unbiased global transcriptome approach to discover possible modes of action of MTBE. In addition to predicting altered oxygen sensing and iron homeostasis, both closely associated with the cardiovascular system, the results suggested altered signaling of HIF-driven pathways associated with energy metabolism and cell survival. To test the hypothesis that MTBE induces vascular lesions by targeting the HIF1-Vegf pathway, three rescue studies were carried out to inhibit MTBE-induced vascular lesions (Figure 1A-F). First, zebrafish *vegfa* was over-expressed in the embryo by injecting a *vegfa* expression vector prior to MTBE treatment. Second, PHD hydroxylation of HIF1- α was chemically inhibited by co-exposure with a competitive inhibitor, N-oxalylglycine (NOG). Finally, a morpholino was designed to knockdown functional protein levels of VHL (VHL-MO) and block HIF1- α ubiquitination. By manipulating the components of the critical HIF-Vegf angiogenesis pathway, MTBE-induced vascular lesions were partially or fully rescued depending on the rescue scenario. The studies presented here provide an insight into the mechanism of MTBE toxicity, allude to the differential roles for HIF and Vegf in the developing vascular system, and suggest that MTBE could be a useful tool for future vascular toxicity studies.

2. METHODS

2.1 Animal Handling

Transgenic zebrafish *flil*-EGFPs (Fli1s), which express enhanced green fluorescent protein in all vascular endothelial cells (Lawson and Weinstein, 2002), were used for all experiments and maintained as previously described in Bonventre et al. (2011). Husbandry and embryonic exposure protocols (#08-025) were approved by the Rutgers University Animal Care and Facilities Committee. During exposures, embryos were incubated at 25 °C as previously described in Bonventre et al. (2011), and all embryos were selected to be at the same 512K developmental stage at the beginning of each experiment.

2.2 Chemicals

Methyl tert butyl ether (MTBE) was obtained from Sigma Aldrich [purity 99.0 %]. N-oxalylglycine was obtained from Enzo Laboratories (Farmingdale, NY). All exposure solutions were made the day of treatment with aerated embryo media (60 μ g/ml Instant Ocean in ddH₂O).

2.3 Global Gene Expression Analysis of MTBE Developmental Toxicity

Staged embryos (1k cell stage) were randomly selected and assigned to one of three time points (developmental stages): 6-somites, 21-somites, or Prim-5 (Kimmel et al., 1995). The time points were selected based on the critical period established for MTBE toxicity in the developing zebrafish, which also corresponds to development of the cardiovascular system (Bonventre et al., 2011; Isogai et al., 2001; Kimmel et al., 1995). To determine the effect of MTBE on global gene expression throughout early vertebrate development, three concentrations were chosen: 5.0 mM MTBE (induces vascular lesions and significantly decreases *vegfa* mRNA), 0.625 mM MTBE (NOAEL for MTBE vascular toxicity), and 0.00625 mM MTBE (100-fold lower than NOAEL) (Bonventre et al., 2011). Three biological replicates, each containing 15 group-housed embryos, were prepared in glass scintillation vials for each of the three developmental time points and MTBE treatment groups. Untreated embryos served as the control.

Total RNA extraction, hybridization to the Affymetrix Zebrafish Genome Array GeneChip[®], and data acquisition were performed by the Bionomics Research and

Technology Center in the Environmental Health & Occupational Sciences Institute in Piscataway, NJ. The array data were deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002). The accession number is GSE46371 and the data are accessible via <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46371>. All the data were analyzed using the GeneSpring GX[®] software package. A One-way Analysis of Variance (ANOVA) with a P = 0.05 cut off and a Student-Newman-Keuls post hoc test was used to determine significant changes in global gene expression. The Benjamini-Hochberg procedure was applied to the data to limit the false discovery rate. Normalized Log₂ data were transformed to fold changes, and a 1.5 fold cut off was applied to the data for further analysis in Ingenuity Pathway Analysis (IPA). The pathways and networks created in IPA are based on gene relationships published in peer-reviewed literature.

2.4 Rescue Study 1: Zebrafish *vegf-a* over-expression

The pCS2⁺ vector containing the zebrafish *vegf-a* sequence was obtained from Dr. Ruowen Ge at the National University of Singapore (Liang et al., 1998). The vector was transformed into DH5alpha competent cells (Protein Express) and amplified under ampicillin selection. Sequencing reactions using an SP6 forward primer and M13 reverse primer were performed to verify the *vegf-a* sequence. A pCS2⁺ vector control without the insert (empty vector) was also prepared by restriction digestion with EcoRI and re-ligation of the empty backbone. Based on previously reported Vegf over-expression studies in zebrafish (Habeck et al., 2002; Bahary et al., 2007), 80 pg of gel purified plasmid (QIAGEN Gel Extraction kit), with or without insert, was micro injected into each embryo at the 1-4 cell stages. The total injection volume was 8 nl.

2.5 Rescue Study 2: Prolyl-4-hydroxylase inhibitor N-oxalylglycine (NOG)

A range finding study was performed to determine an appropriate concentration of NOG to use for the rescue study since this is the first study using NOG in the zebrafish embryo model. The first concentrations tested were based on the published in vitro studies: 10-50µM for cell lystate studies and 500-1000µM for cell culture studies (Jaakkola et al., 2001; Wirthner et al., 2007). Embryos were first exposed to 20, 60, 100, 500, and 2500µM starting at the 512-cell stage in sealed glass scintillation vials with an N of 10 per concentration, and observed for 5 days for developmental defects as a result of exposure. Based on the range finding a study, a 2 hr incubation with 50, 200, 500µM NOG was performed at 21-somites to determine the ability of NOG to alter the expression of *vegf-a*, *vegf-c*, *vegfr-2*, *hif1-α*, *mmp-9*, and *elgn3* at those concentrations. The length of the incubation was arbitrarily chosen. Ultimately 200µM NOG was chosen for the chemical rescue studies.

2.6 Rescue Study 3: von Hippel-Lindau protein morpholino

An antisense morpholino to zebrafish von Hippel-Lindau protein (VHL-morpholino oligonucleotide [MO]), designed to target the intron 1-exon 2 splice site on the pre-mRNA sequence, was obtained from Gene Tools, LLC (Philomath, OR). The sequence of the VHL-MO was 5'-TGTCCTGACAGAACAGGAAAAACA-3', and was designed to delete exon 2, known to be associated with the VHL disease syndrome in humans (Gnarra et al., 1994). A standard control morpholino (CoMO) sequence from the Gene Tools was used as a control (Hillegass et al. 2007). A range finding study to observe embryonic survival and possible VHL-MO phenotypes was performed using 12.5, 25, 50, 100, 200, and 400µM of the VHL-MO. Embryos were injected at the 1-4 cells stage with a total injection volume of 8nl of the morpholino and observed daily. Based on the range finding study, 8nl of 25µM VHL-MO or 50µM of CoMO were used for the morpholino rescue experiments.

2.7 Morphological Examination of MTBE-induced vascular lesions

Following OECD 212 guidelines, embryos were exposed individually in sealed 4 ml glass vials to static, non-renewal concentrations of MTBE (nominal) at approximated the 512-cell stage. For Rescue Study 1, embryos were injected with either expression vector p-zfVEGF or an empty vector, and exposed to either 10mM MTBE or embryo media. Embryos injected with the empty vector but not treated with MTBE served as a control. For Rescue Study 2, embryos were exposed to either 5 or 10mM MTBE in the presence or absence of 200 μ M NOG. Untreated and NOG only treatments served as controls. Treatment of NOG began at the same time as the MTBE treatment. For Rescue Study 3, embryos were injected with either the VHL-MO or the control-MO (Co-MO), and exposed to 10mM MTBE or embryo media. Embryos injected with the Co-MO but not treated with MTBE served as a control. For both Rescue Study 1 and 3, uninjected embryos treated with 0 (embryo media - control) or 10mM MTBE were set up with each study to serve as quality control. All embryos were observed under light and fluorescence microscopy daily over the 5-day developmental period. The number of embryos exhibiting pooled blood in CCV (at the Prim-25 stage), cranial hemorrhages (at Pec Fin stage), and abnormal ISVs (at Protruding Mouth stage) were recorded (Figure 1A-F). The concentrations of MTBE used for the morphological rescue studies were chosen because they significantly induce vascular lesions in zebrafish embryo, while other malformations (i.e. toxicity to other tissues) are not apparent (Bonventre et al., 2011).

2.8 mRNA expression analysis of MTBE altered transcripts

The effect of *vegf-a* over-expression, PHD inhibition by NOG, or VHL-MO on rescuing the MTBE altered gene expression at 21-somites was determined with real time-quantitative polymerase chain reaction (RT-qPCR). Embryos were exposed to a static non-renewal treatment of 0 (control) or 5mM MTBE in glass scintillation vials from approximately the 512-cell stage and harvested at 21-somites. This concentration significantly decreases *vegf-a* mRNA expression in zebrafish embryos at 21-somites and was the lowest concentration of MTBE tested where 90% of the embryos exhibited vascular toxicity (Bonventre et al., 2011). Approximately 50 embryos were used per treatment and each treatment was performed in duplicate. Each experiment was repeated 2 times. RT-qPCR sample preparation was carried out as described in Bonventre et al. (2012). Primer sets were selected to work with the same RT-qPCR protocol: 35 cycles of 95°C for 15 sec and 60°C for 1 minute. All CTs were converted to copy numbers using standard curves for each gene and copy numbers were normalized to the housekeeping gene, 28s ribosomal RNA (Delaunay et al., 2000). Primers for *vegf-a*, *vegf-c*, *vegfr-2*, *hif1-a*, and *mmp-9* were previously published (Bonventre et al., 2011; Hillegass et al. 2008). The primer set for zebrafish *egln3* (BC066699) was developed using IDT PrimerQuest software: fwd 5'- GGA TTG CTG AGG ATC TTT CC- 3', rev 5'- GAT TCC TTC GAT CTG ACC AG-3'.

2.9. Statistical analyses of the Rescue Studies

Statistical analyses were performed using the SigmaPlot (version 11). Statistical differences in lesion occurrence in the individual morphology rescue studies (categorical data) were determined using Chi-square and Fisher Exact tests. Transcript levels of the mRNA analysis were analyzed using a two-way Analysis of variance (ANOVA) on average fold changes. The Holm-Sidak post hoc test was used where data were significant. The probability level for statistical significance was $p \leq 0.05$ for all studies.

3. RESULTS

3.1 The effect of MTBE on global expression during early vascular development

Embryos were exposed to 0.00625, 0.625, or 5mM MTBE until 6-somites, 21-somites, or Prim-5 stages for an unbiased global transcriptome analysis to discover possible modes of action of MTBE. Statistically significant changes in gene expression were not observed for any MTBE treatment, at any stage ($P > 0.05$). Therefore, the analysis of the effect of MTBE on global gene expression was performed on genes exhibiting at least a 1.5 fold change from control (Supp. Table 1). The greatest change in gene expression at any time point or treatment was less than 3 fold. A Venn diagram was created for each developmental stage to demonstrate the amount of overlap between each of the gene lists created for each MTBE treatment (Figure 2). The 6- and 21-somite stages had a similar number of unique genes differentially expressed, independent of treatment, but 21-somites had the greatest number of gene changes in the 3 different MTBE concentrations. The Prim-5 stage had fewer than 5 genes altered with any concentration of MTBE. The number of genes common to all 3 MTBE concentrations was greatest in 21-somites, suggesting similar pathways were altered by all 3 concentrations at that stage.

IPA was used to analyze differentially expressed genes in the 21-somite data set. For both 0.00625 and 5.0mM MTBE, “Cardiovascular System Development and Function” was the top physiological system predicted to be perturbed (p -values = $6.70E-05$ – $3.27E-02$ and $4.87E-05$ – $2.8E-02$, respectively), while “Tissue Morphology” was the top for 0.625mM MTBE (p -value = $6.75E-05$ – $2.37E-02$). “Hematological System Development and Function” was also ranked in the top 5 physiological systems for 5.0mM MTBE (p -value = $7.17E-04$ – $3.98E-02$). The top 10 differentially expressed genes predicted to be altered by each concentration of MTBE were identified and presented with summarized functions in (Supp. Tables 2A-C). With the exception of Cathepsin b (CTSB) in the 0.625 mM MTBE treatment group, all of the top 10 molecules had a greater than 1.5 fold change in all three MTBE treatment groups. The “top molecules” were then characterized as components of different cell responses or function (Table 2): cell stress (CTSB, CTSS, NPC2, OCM2, and SERPINA1), energy/metabolism (ACP5, APOA4, FBP1, MTHFD1), blood clotting (FGG), iron homeostasis (TF), oxygen sensing (EGLN3), innate immunity (CFI), and steroid synthesis (CYP11A1). ACP5, CFI, CTSS, and MTHFD1 were all up-regulated by each concentration of MTBE at 21-somites, while FBP1, NPC2, SERPINA1, and TF were down-regulated by each concentration. APOA4, CYP11A1, EGLN3, and OCM2 were all up-regulated by 0.00625mM MTBE, but down-regulated by 5mM MTBE. The top molecules were entered into IPA for a “custom list” data analysis in order to create custom networks based on the relationships between the molecules in the ‘Ingenuity® Knowledge Base (Genes and Endogenous Chemicals)’. The networks generated by IPA represent pathways predicted to be altered by MTBE exposure: “Cancer, Cardiovascular System Development and Function, Tumor Morphology” and “Lipid Metabolism, Small Molecule Biochemistry, Carbohydrate Metabolism” (Figure 3A,B). The scores for the two networks were 27 and 9 respectively, where scores of 2 or higher have at least a 99% confidence of not being generated at random. The top physiological system associated with the custom data set was the cardiovascular system (p -value = $6.487E-07$ – $4.98E-02$). VEGF, though not among the genes altered by 1.5 fold, was present in the first network for its relationship with 10 of the 14 top molecules. Many of the molecules differentially expressed by MTBE were associated with biological functions that can be related to HIF signaling, including oxygen sensing, blood clotting, energy/metabolism, and cell stress.

3.2 Rescue Study 1: Over expression of zebrafish *vegf-a*

Embryos were injected with an expression vector containing the zebrafish *vegf-a* coding sequence to determine if over-expression of the gene would rescue MTBE induced vascular lesions. Injections of the plasmid with or without the insert did not result in any significant developmental, vascular or other lesions, among untreated embryos. Exposure to 10mM MTBE induced a significant increase in the occurrence of vascular lesions in Empty Vector injected embryos as well as the non-injected positive controls, consistent with Bonventre et al. (2011). The percent of animals exhibiting pooled blood in the CCV was not significantly different between Empty Vector and p-zfVEGF injected embryos treated with MTBE (Figure 4A). Over-expression of *vegf-a* resulted in a 46% (± 5.5) fewer animals exhibiting MTBE-induced cranial hemorrhages, significantly less than Empty Vector treated with MTBE (Figure 4B). Similarly, there was a significant difference between Empty Vector and p-zfvegf injected embryos exhibiting abnormal ISVs following MTBE exposure (Figure 4C), where over expression resulted in 35% (± 5.8) fewer embryos exhibiting the lesion.

Exposure to 5mM MTBE resulted in a significant decrease in transcript levels of *vegf-a*, *vegf-c*, *vegfr-2*, *hif1-a*, and *elgn3* at 21-somites in the empty vector injected embryos (Empty Vector/MTBE) (Figure 5). *Mmp9* was not significantly reduced by exposure to 5 mM MTBE, consistent with previously reported studies (Bonventre et al., 2011; Bonventre et al., 2012). MTBE exposure did not significantly reduce the transcript levels of *vegf-a* or *-c* in embryos injected with p-zfvegf compared to control embryos (Empty Vector injected/no MTBE). However, the expression of these genes was also not significantly different from Empty Vector/MTBE embryos, suggesting that the rescue was only partial. The expression of *hif1-a* was not rescued by over-expression of *vegf-a* at 21-somites. For both *vegfr-2* and *elgn3*, p-zfvegf injection significantly decreased transcript levels and had no effect on the MTBE-induced suppression of the gene. Over-expression of *vegf-a* resulted in a statistically significant increase in *mmp-9* transcript levels compared to Empty Vector controls at 21-somites. This significant increase was not observed in p-zfvegf injected animals that were treated with MTBE.

3.3 Rescue Study 2: Chemical inhibition of PHDs by NOG

Embryos were first exposed to 20, 60, 100, 500, and 2500 μ M NOG at the 512-cell stage to establish a concentration that would not elicit toxicity under the conditions required for the morphology studies. Embryos exposed to 2500 μ M NOG were dead within minutes of treatment. At 24hr post treatment, greater than 20 % of the embryos exposed to 500 μ M NOG were dead, while the remainder appeared opaque and showed signs of morbidity. No morphological defects were observed in the embryos exposed to 20, 60 or 100 μ M NOG over the 5 day developmental period.

The 2hr incubation study at the 21-somite stage was performed to determine the ability of a non-lethal concentration of NOG to alter the expression of HIF1- α related genes in our *in vivo* embryo model system: *vegf-a*, *vegf-c*, *vegfr-2*, *hif1-a*, *mmp-9*, and *elgn3* (Supplemental Figure 1). Exposure to 50 μ M NOG for 2hr had no effect on the expression of any of the genes tested. The concentration of NOG that had the greatest effect on expression was 200 μ M, which significantly increased *vegf-c*, *hif1-a*, *mmp-9*, and *elgn3* levels. There was a dose dependent decreased in *vegf-a* transcript levels after a 2hr incubation with 200 and 500 μ M NOG. Finally, 500 μ M NOG exposure significantly decreased *elgn3*. Based on the results of these studies, 200 μ M NOG was chosen for Rescue Study 2.

Embryos were treated with 200 μ M of NOG, a competitive inhibitor of PHDs known to upregulate HIF responsive pathways, in an attempt to rescue MTBE induced vascular lesions. Treatment with NOG alone did not result in any significant lesions. There was a

significant increase in the incidence vascular lesions in 5 and 10 mM treated embryos was consistent with Bonventre et al. (2011). Co-exposure of embryos to 10mM MTBE and NOG resulted in a significant decrease in pooled blood in the CCV ($30\% \pm 11.6$) and cranial hemorrhage ($32\% \pm 3.9$) (Figure 6A, B). A complete rescue of the pooled blood in the CCV was observed in the 5mM MTBE plus NOG treatments which were not significantly different from NOG alone (background levels) (Figure 6A). Co-exposure with NOG resulted in $24\% (\pm 13.0)$ fewer exhibiting abnormal ISVs, a significant decrease from exposure to 5mM MTBE alone (Figure 6A,C). The percent of embryos exhibiting cranial hemorrhages was also decreased for 5mM MTBE plus NOG, but the decrease was not significantly different than 5mM MTBE alone (Figure 6 B, C). The occurrence of cranial hemorrhages in 5mM MTBE treatments is not as robust as it is with 10mM MTBE, which may explain why a significant difference was not observed.

The effectiveness of NOG at rescuing MTBE-altered gene expression was also tested. Exposure to 5mM MTBE significantly decreased mRNA transcripts for *vegfa*, *vegfc*, *vegfr-2*, *hif1- α* , and *egln3* at 21-somites consistent with Bonventre et al. (2011, 2012) (Figure 7). Exposure to 200 μ M NOG alone for 24hrs did not result in a significant difference in gene expression as compared to control embryos. In embryos treated with both MTBE and 200 μ M NOG, transcript levels of *vegfa* and *vegfc* were significantly greater than in embryos treated with MTBE alone. For both *vegfr-2* and *hif1- α* , expression was not significantly different from either the control or the MTBE alone embryos. There was no difference in *mmp-9* expression under any exposure condition. NOG did not rescue the significantly reduced transcript levels of *egln3* in embryos treated with MTBE.

3.3 Rescue Study 3: Antisense morpholino to zebrafish von Hippel-Lindau protein

Since VHL is critical for normal development, embryos were injected with a wide range of VHL-MO to determine a concentration that would not be lethal. Approximately 100 embryos were injected with 12.5, 25, 50, 100, 200, or 400 μ M of the VHL-MO and observed for 5 days. Mortality and lesion occurrence in the embryos were qualitatively assessed. Injection of both 200 and 400 μ M VHL-MO resulted in greater than 90% mortality in the embryos by day 5, with 100% of the remaining embryos exhibiting classic morpholino toxicity, a phenotype consequence of off target effects leading to general apoptosis and morbidity (Gerety and Wilkinson, 2011; Bedell et al., 2011). Morpholino toxicity was apparent in greater than 75% of the embryos injected with 100 μ M VHL-MO, and in approximately 50% of embryos injected with 50 μ M. However, observation of 50 μ M injected embryos with fluorescent microscopy revealed a number of embryos exhibiting additional aberrant vascular structures associated with the ISVs (Supp Figure 2 B, C). Embryos injected with 12.5 or 25 μ M VHL-MO appeared to develop normally. Therefore, 25 μ M VHL-MO was used for the rescue studies since it was the highest concentration tested that did not cause any morpholino toxicity in the embryos. The fluorescent labeling on the MO was observable in the embryos injected with 25 μ M VHL-MO 24hrs post injection, demonstrating that the MO was still present during the critical period for MTBE toxicity (data not shown).

Embryos were injected with the VHL-MO in an attempt to rescue MTBE induced vascular lesions. Treatment with 10 mM MTBE induced a significant increase of pooled blood in CCV, cranial hemorrhages, and abnormal ISVs in Co-MO injected embryos as well as non-injected positive controls, consistent with Bonventre et al. (2011). VHL morphants had a significant $35\% (\pm 4.5)$ decrease in embryos exhibiting pooled blood in CCV following exposure to MTBE when compared to exposed Co-MO injected embryos (Figure 8A). A similar trend was observed for MTBE induced cranial hemorrhages, however the results were not significant (Figure 8B). VHL-MO injection did not significantly change the percent

of embryos exhibiting MTBE induced abnormal ISVs when compared to Co-MO injected animals (Figure 8C).

The effect of VHL-MO on MTBE-altered gene expression was tested. Exposure to 5 mM MTBE significantly decreased mRNA transcripts for *vegfa*, *vegfc*, *vegfr2*, *hif1a*, and *egln3* at 21-somites consistent with Bonventre et al. (2011, 2012) (Figure 9). When embryos were treated with MTBE, VHL-MO injected embryos had significantly greater transcript levels of *vegfa*, *vegfc*, and *vegfr2*, than did the embryos injected with CoMO. The only gene tested that was significantly altered by the VHL-MO alone was *hif1a*, which was significantly upregulated at 21-somites. However, when VHL-MO injected embryos were treated with MTBE, the significant increase in *hif1a* transcript was inhibited. There was no difference in *mmp9* expression under any exposure condition. VHL-MO did not rescue the significantly reduced transcript levels of *egln3* in embryos treated with MTBE.

4. DISCUSSION

MTBE exhibits anti-angiogenic properties in piscine and mammalian model systems at concentrations ranging from 5-35mM. In Japanese medaka embryos (*Oryzias latipes*), MTBE completely inhibits vascular growth (Longo, 1995), while zebrafish embryos develop vascular lesions in the CCV, cranium, and dorsal vasculature (Bonventre et al., 2011). *In vitro* rodent and human endothelial cell cultures grow fewer, thinner, and shorter capillary-like tubes in the presence of MTBE, while Matrigel plugs containing the chemical displayed fewer vascular invasions in a mouse model (Kozlosky et al., 2012). The *in vivo* studies also demonstrate that MTBE does not appear to induce toxicity in other tissues directly at concentrations which elicit obvious vascular toxicity, suggesting that MTBE seems to target the vascular system by an unknown mechanism (Bonventre et al., 2011; Kozlosky et al., 2012). The gene-array analysis of MTBE-treated zebrafish embryos suggested altered HIF1 signaling, and these findings supported our previous work and were used to direct rescue studies targeting the HIF1-VEGF pathway. A loss of either Hif or Vegf causes aberrant vascularization in the embryo and lethality in murine knockout studies (Ryan et al., 1998; Carmeliet et al., 1996; Ferrara et al., 1996). Morpholino knockdown of Vegf-a or Vegfr2 in the zebrafish causes vascular lesions in the cranium and dorsal segmental vessels (Nasevicius et al., 2000; Bahary et al., 2007). Similar lesions observed in zebrafish exposed to MTBE are associated with a significant decrease in mRNA transcript levels of *vegfa*, *vegfc*, and *vegfr2* (Bonventre et al., 2011). By manipulating the Hif1-Vegf pathway, highlighted by the microarray and previous studies as a possible target of MTBE toxicity, we were able to significantly rescue multiple morphological and molecular endpoints affected by MTBE exposure, and propose a mode of action by which MTBE disrupts angiogenesis.

4.1 Global transcriptome analysis predicted cardiovascular system pathways were altered by MTBE and suggested altered HIF-driven pathways

The critical period of MTBE anti-angiogenesis in zebrafish occurs early in development, between 6-somites and Prim-5 stages of development, and precedes the appearance of vascular lesions by 1 day (Bonventre et al., 2011). The embryonic stage 21-somites was identified as an important stage for MTBE toxicity because the greatest number of genes were altered in the three concentrations of MTBE, and many were altered in more than one of the concentrations tested (Figure 1). The presence of “Cardiovascular System Development and Function” and “Hematological System Development and Function” pathways among the top physiological systems in the IPA analysis suggested that these systems were altered by MTBE exposure. The p-values represent the likelihood that the genes associated with the given biological system did not occur by random chance alone. The chance that any two genes from a small list of genes will be in the same pathway is very

small, so that fact that multiple genes from our array, altered by only 1.5 fold, are associated with the cardiovascular and other related systems provides further evidence that these pathways are a target of MTBE toxicity. Further, the top altered molecules (Table 1) share similar functional pathways, including energy metabolism/homeostasis and cell stress. While HIF is known to play a central role in the development and regulation of the cardiovascular system (via angiogenesis, hematopoiesis, vasodilation), it is also important to energy metabolism (via glycolysis, oxidative phosphorylation, citric acid cycle, lipid metabolism) and cell survival (via apoptosis, proliferation, migration, pH regulation) (Keith et al., 2012; Greer et al., 2012). Therefore, the similar functional pathways shared by the altered genes suggest that MTBE alters HIF-driven pathways.

The scores for both of the custom networks generated by IPA from the list of the top most altered genes were such that there was a greater than 99% confidence of the networks not being generated at random. The first custom network was associated with the cardiovascular system, indicating that the genes that changed the most were in some way related to this system (Figure 3A). The second network included the “small molecule biochemistry” pathway (Figure 3B), which was previously identified in another zebrafish Affymetrix gene-chip study that analyzed genes differentially regulated by a Vegf-Aa morpholino, one of two Vegf-As found in zebrafish due to a duplication event (Bahary et al., 2007). The similarity between the networks associated with the Vegf-Aa morphant and the networks identified with the MTBE treatments supports the hypothesis that MTBE toxicity occurs through Vegf-A dysregulation.

One notable gene of interest, *egl nine homolog 3 (egln3)*, was differentially regulated by 0.00625mM MTBE (+2.07 fold), a concentration that does not induce lesions, compared to 5mM MTBE (-1.78 fold) that does (Table 1). EGLN3, also known as PHD3, is part of the cellular oxygen sensing system, responsible for regulating HIF1- α levels in the cytoplasm. In addition to driving HIF- α degradation, EGLN3 is also upregulated by HIF activation (Berra et al., 2003; Marxsen et al. 2004). RT-qPCR validation confirmed that *egln3* transcript levels were significantly reduced by 5mM MTBE at 21-somites by approximately 2-fold (Figures 5, 7, 9). Vegf-a, vegf-c, and vegfr2 transcripts are significantly decreased at 21-somites in zebrafish embryos exposed to 5 mM MTBE (Bonventre et al., 2011); however, these genes were not among the list of genes altered in the array data set. One possible explanation for inconsistency between the RT-qPCR and the microarray is that mRNA transcripts of some genes up- or downregulated in cells at sites of localized hypoxia may not achieve concentrations in whole embryo lysate that are detectable by microarrays. For example, hypoxia did not alter the expression of either *erythropoietin* or *vegf* gene expression in a previous zebrafish microarray study (Ton et al., 2003). Identifying effects on specific genes may be confounded by the specificity of MTBE for a cell type or pathway at the concentrations tested. The reduced sensitivity of the microarray platform compared to RT-qPCR at a period of rapid development and transcriptional changes, using a toxicant that appears to preferentially target a cell type that composes a small portion of the total mass of an embryo, and the small sample size for the study, may have all contributed to the lack of significant gene expression changes in our array study. Despite this, the findings from our IPA analysis, in addition to the previous work on MTBE anti-angiogenesis (Bonventre et al., 2011; Kozlosky et al., 2012), directed our rescue studies to look upstream of Vegf-a expression toward HIF1 regulation of angiogenesis.

4.2 Over-expression of zebrafish *vegf-a* partially rescued cranial hemorrhages and abnormal ISVs

Both cranial hemorrhages and abnormal ISVs were rescued by over-expression of *vegf-a* (Figures 4B and C). These lesions are similar to those described in embryos injected with a

Vegf-a morpholino (Nasevicius et al., 2000; Bahary et al., 2007). Much like embryonic exposure to MTBE, the initial establishment of the axial vasculature was not affected in the Vegf-a morphant. In contrast, the development of the cranial vasculature and ISVs are Vegf-a dependent (Nasevicius et al., 2000; Bahary et al., 2007). Vegfr-2 is also important in ISV formation in zebrafish as both mutants and morpholinos have demonstrated (Habeck et al., 2002; Bahary et al., 2007). Over-expression of *vegfr-2* did not rescue the lack of ISV in Vegfr-2 mutant embryos indicating normal ISV development is dependent upon Vegfr-2 signaling (Habeck et al. 2002). Since over-expression of *vegfr-2* rescues MTBE-induced ISV abnormalities, it is likely that MTBE does not directly affect Vegfr-2, but rather it's ligand, Vegf-a.

Pooling of blood in the CCV is the first MTBE induced vascular lesion observed in developing zebrafish (Bonventre et al., 2011). While over-expression of zebrafish *vegfr-2* via, p-zfVEGF did not rescue the lesion, both co-exposure to NOG and a VHL morpholino significantly reduced the number of embryos exhibiting pooled blood in the CCV (Figures 4A, 6A, and 8A). MTBE is not the only toxicant known to disrupt normal CCV formation. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) inhibits CCV growth and regression in zebrafish embryos in an aryl hydrocarbon receptor-2 (AHR2)-dependent manner (Bello et al., 2004). Both HIF- α and AHR are known to bind to HIF- β . Multiple isoforms of HIF- α and HIF- β exist in both zebrafish and higher vertebrates, but the regulation of HIF- α by PHDs and VHL is similar (Keith et al., 2012; Greer et al., 2012). Therefore, PHD inhibition and VHL-MO have the potential to force the accumulation of all three HIF- α isoforms, and increase the availability of HIF- α s to bind to HIF- β s. In contrast, over expression of *vegfr-2* does not directly stimulate HIF- α /HIF- β activation. Together, the TCDD study and our MTBE-rescue studies, via different mechanisms, suggest future studies could examine a role for HIF- β activity in the formation of the CCV in zebrafish embryos.

4.3 Chemical inhibition of PHDs partially rescued all three MTBE-induced vascular lesions

One complication of using NOG to inhibit PHD hydroxylation of HIF- α is that NOG is a competitive inhibitor and therefore it does not completely prevent PHD activity unless the concentration of NOG exceeds that of the endogenous substrate, 2-oxoglutarate. The difference in survival between 200 and 500 μ M NOG in the preliminary studies could be explained by percent of PHD inhibition, indicating that in the 200 μ M NOG treatments, some level of HIF1- α hydroxylation still occurs. Therefore, the percent of MTBE rescued lesions in the NOG studies may be limited by concentration of the inhibitor, a parameter dictated by the inherent toxicity of the inhibitor, and not a reflection of the efficacy of the target. An alternative 2-oxoglutarate mimetic has been previously used in zebrafish embryos to simulate systemic hypoxia and drive angiogenesis pathways. Embryos exposed to dimethyloxalylglycine (DMOG) from 3 to 7 days post fertilization exhibited an increase in expression of *egln3*, *vegfr-2*, and *erythropoietin* mRNA compared with the controls (van Rooijen et al., 2009). However, embryonic exposure to DMOG prior to 3 days post fertilization was lethal. Due to the critical period of MTBE in zebrafish (6-somites to Prim 5 stages of development) and the experimental design (static, non-renewal in sealed glass vials), it was necessary to use a concentration of NOG that was not lethal under these conditions. The 2hr incubation to 200 μ M NOG altered the expression of angiogenesis related genes similar to DMOG (Supp Figure 1). In embryos exposed to MTBE and NOG, expression levels of both *vegfr-2* and *vegfr-3* were returned to normal control levels, and both *vegfr-2* and *hif1-a* levels were partially rescued (Figure 7). Alterations in gene expression were not apparent at the 21-somites in the NOG alone treatment, which may be attributed in part to metabolism of the 200 μ M NOG, resulting in concentrations of NOG that no longer altered mRNA at detectable levels.

4.4 Knockdown of VHL partially rescued the CCV lesion

The zebrafish VHL transheterozygote ($vhl^{hu2117}/vhl^{hu2081}$) mutant embryo model, described by van Rooijen et al., (2009, 2010), develops polycythemia, dilated blood vessels, aberrant intersegmental vessel sprouts, and increased Vegf-a/Vegfr2 signaling. Extra blood vessels were observed in the ISVs of embryos injected with 50 μ M VHL-MO, similar to those reported in the VHL mutant zebrafish (Supp Figure 2 A-C). The lower concentration of VHL-MO used for the rescue study, so as to not induce morpholino toxicity in the embryos, in addition to the transient nature of morpholino-based knock downs, may have limited the efficacy of the rescue. Like NOG, the VHL-MO should have resulted in an indirect increase of Vegf-a by forcing an accumulation of HIF1- α , and therefore driven hypoxia response pathways, including angiogenesis. However, the VHL-MO significantly rescued only pooled blood in the CCV ($35.2\% \pm 4.5$) (Figure 8A). The fact that *vegfr2*, *vegfr1*, and *vegfr3* transcripts were all returned to control levels in VHL-MO injected embryos treated with 5mM MTBE (Figure 9) suggests that if a higher concentration of VHL-MO could have been used, it is possible that a greater rescue of the MTBE-induced lesions might have occurred.

4.5 MTBE induces vascular lesions by targeting the HIF-VEGF driven angiogenesis

Manipulating components of the pathway rescued different endpoints of MTBE vascular toxicity, but for the most part did not result in 100% amelioration of the lesions. The differences may be a result of the inherent limitation of the treatment (i.e. the doses administered) or it may suggest distinct roles for Hif1 and Vegf in the development of different vascular structures. Because the appearance of a lesion is dependent upon exposure to MTBE between 6-somites and Prim-5 stages (Bonventre, et al., 2011), it is not the effect of a rescue parameter at the time of the lesion, but rather the effect on the HIF-Vegf pathway that occurs prior to 21-somites that presumably determines the rescue. A challenge with using the rescued-gene expression profile to explain differences in the rescues is that the knockdown of any given gene by MTBE is not very robust (usually not more than 2 fold). Therefore, observing significant rescues of the genes is difficult unless the expression of the gene is returned to control levels or higher. In addition, the rescued-gene profile may not accurately represent the protein expression within each rescue paradigm. Finally, exactly how each of our rescue parameters alters alternative pro-angiogenesis factors, such as the Angiopoietin/Tie system, is unknown, and could also contribute to the differences between the efficacy of the rescue studies. The fact that none of the rescue parameters were able to rescue the expression of *egln3*, may be the result of the incomplete rescues we observed in our studies. The decrease in *egln3* expression in MTBE exposed zebrafish, with both qPCR and the microarray, may indicate an effect on hypoxia-sensing mechanisms, the consequence of which may be decreased angiogenesis, by way of Vegf. A rescue of hypoxia-regulatory pathways may be more complex than simply increasing endogenous Vegf concentrations at 21-somites, and further studies would be required to assess whether or not there is a specific role for MTBE in altered hypoxia-sensing pathways.

Further evidence for the mode of action proposed here comes from the expression of *mmp-9* in the p-zfvegf injected embryos. When *vegfr2* was over-expressed in embryos via p-zfVEGF, *mmp-9* transcript levels were significantly increased (Figure 5), but when p-zfVEGF injected embryos were treated with MTBE, the upregulation was lost (Figure 5). Vegf-a is known to upregulate MMP-9 transcription and secretion via Vegfr signaling (Wang and Keiser, 1998; Pufe et al., 2002; Hiratsuka et al., 2002; Hollborn et al., 2007). Therefore, the lack of *mmp-9* upregulation in p-zfvegf injected embryos that were exposed to MTBE strongly suggests that MTBE reduces Vegf protein activity. Similarly, when embryos were injected with the VHL-MO, *hif1- α* was upregulated, but when the embryos were treated with MTBE, the upregulation was absent (Figure 9). Both are indirect examples of how MTBE is functionally knocking down different aspects the HIF-Vegf pathway. Both

examples indicate that MTBE, by direct or indirect mechanism, blocks the functions of key proteins involved in this pathway.

While the mechanism as to how MTBE interferes with angiogenesis is still unclear, there is enough evidence to support that the HIF-Vegf driven angiogenesis is at least one target pathway. The complexity of the pathways involved notwithstanding, the selectivity that MTBE has for developing vascular structures makes it an interesting chemical for the field of vascular toxicity and for future studies in the area of anti-angiogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Analysis of global gene changes in zebrafish embryos exposed to MTBE predicted altered HIF1 signaling
- Over expression of zebrafish *vegfa* rescues MTBE-induced vascular lesions
- Blocking HIF1- α degradation by inhibiting PHD or knocking down VHL rescues MTBE-induced vascular lesions
- HIF1-Vegf driven angiogenesis is a target for MTBE vascular toxicity

Figure 1. MTBE induced vascular lesions

(A) Control embryo exhibiting normal flow of red blood cells across the yolk sack in the broad common cardinal vein (CCV) of the Prim-25 stage zebrafish. (B) Arrow points to pooled blood in the CCV in an embryo exposed to 10mM MTBE. (C) Control embryo at the Pec Fin stage with normal circulation through the cranial vessels. (D) Embryo treated with 10 mM MTBE exhibiting a cranial hemorrhage. (E) Normal vascular structure of intersegmental vessels (ISVs) under GFP fluorescence in a control embryo; by the Protruding mouth stage all the vessels have circulating red blood cells through the vessels. (F) Arrows indicate vessels which were not fully formed and were not open for red blood cells to circulate through in an embryo exposed to 10mM MTBE. For more information on these lesions please refer to Bonventre et al., (2011).

Figure 2. Venn diagrams representing the genes differentially expressed by 1.50 at each developmental stage and concentration of MTBE

The 6-somite and 21-somite stages had similar total number of unique genes, while the Prim-5 stage had significantly few genes. The 21-somite stage had more genes in change within each treatment and more genes common to all three different concentrations of MTBE tested. (Refer to Supp. Table 1 for gene lists.)

Figure 3. Networks created by Ingenuity Pathway Analysis with a list of the top most differentially expressed genes by MTBE at 21-somites

Network (A) contains 10/14 molecules: ACP5, APOA1, CTSS, CTSB, CYP11A1, EGN3, OCM2, NPC2, SERPINA1, and TF. Network (B) contains 4/14 molecules: CFI, FBP1, FGG, and MTHFD1. The solid lines indicate a direct relationship between molecules, while dashed lines indicate an indirect relationship.

Figure 4. Rescue Study 1: Over-expression of zebrafish *vegf-a* rescued some MTBE-induced vascular lesions

A) Exposure to 10 mM MTBE induced a significant increased in pooled blood in the common cardinal vein (CCV) in non-injected, empty vector, and p-zfVEGF embryos compared to the respective controls. There was no significant difference between empty vector and p-zfVEGF injected embryos. B) Exposure to 10 mM MTBE induced cranial hemorrhages in non-injected and empty vector injected embryos, compared to controls, but not in embryos injected with p-zfVEGF. There was a significant difference between the percent of cranial hemorrhages in the empty vector and p-zfVEGF injected embryos exposed to MTBE (*). C) Exposure to 10 mM MTBE caused abnormal intersegmental vessels (ISVs) development in non-injected and empty vector injected embryos, but not in embryos injected with p-zfVEGF. There was a significant difference between the percent of

abnormal ISVs in the empty vector and p-zfVEGF injected embryos exposed to MTBE (*). Data represents the average of two independent studies where N=20-35 for each treatment group. Error bars represent the range of the studies. Asterisk denotes statistical significance between the treatment and its respective control, while the # denotes significant difference between the two marked bars ($P < 0.05$).

Figure 5. Rescue Study 1: The effect of *vegfa* over-expression on MTBE altered mRNA expression in angiogenesis related genes

The graph represents the average fold change in copy number quantification of mRNA transcripts for the genes of interest. Error bars are standard error. Two-way ANOVA was used to determine statistical differences between the treatment groups. Fold changes denoted by the same letter are not statistically different from one another. Statistical comparisons were made within genes only (similar lettering between genes does not imply similar statistical significance). Both *vegfa* and *vegfc* were partially rescued by over-expression of p-zfVEGF as they were not significantly different from either the empty vector control or the empty vector treated with MTBE. *Mmp-9* expression was significantly increased with p-zfVEGF injection, but not in the presence of MTBE.

Figure 6. Rescue Study 2: Co-exposure with N-oxalylglycine (NOG) rescued some MTBE-induced vascular lesions

A) Exposure to 5 or 10 mM MTBE induced a significant increased in pooled blood in the common cardinal vein (CCV). NOG significantly decreased the percent of embryos exhibiting this lesion in both co-treatments (*). B) Exposure to 10 mM MTBE significantly increased cranial hemorrhages. NOG significantly decreased the percent of embryos exhibiting this lesion in co-treatments with 10 mM MTBE (*). C) Exposure to 5 or 10 mM MTBE caused abnormal intersegmental vessel (ISV). Co-treatment with NOG rescued the occurrence of this lesion in the 5 mM co-treatments (*), but not with 10 mM MTBE. Data represents the average of three independent studies (N=22-25). Error bars represent the standard deviation of the three studies. Asterisk denotes statistical significance between the treatment and its respective control, while the # denotes significant difference between the two marked bars ($P < 0.05$).

Figure 7. Rescue Study 2: The effect of NOG co-treatment on MTBE altered mRNA expression in angiogenesis related genes

The graph represents the average fold change in copy number quantification of mRNA transcripts of the genes of interest. Error bars are standard error. Two-way ANOVA was used to determine statistical differences between the treatment groups. Fold changes denoted by the same letter are not statistically different from one another. Statistical comparisons were made within genes only (similar lettering between genes does not imply similar statistical significance). The expression of both *vegfr-a* and *vegfr-c* were significantly greater in the co-treatment than with MTBE alone. *Vegfr-2* and *hif1-α* were partially rescued.

Figure 8. Rescue Study 3: VHL-MO rescues an MTBE-induced vascular lesion

A) Exposure to 10 mM MTBE induced a significant increased in the prevalence of pooled blood in the common cardinal vein (CCV) in non-injected and control morpholino (CoMO) injected embryos, but not in VHL morphants. VHL-MO rescued the occurrence of pooled blood in the CCV following MTBE exposure (*). B) Exposure to 10 mM MTBE induced cranial hemorrhages in non-injected, CoMO, and VHL-MO injected embryos. VHL morphants were not protected against MTBE induced cranial hemorrhages. C) Exposure to 10 mM MTBE induced abnormal intersegmental vessel (ISV) development in non-injected, CoMO, and VHL-MO injected embryos. VHL morphants were not protected against MTBE induced abnormal ISV development. Data represents the average of three independent studies (N=17-38). Error bars represent the standard deviation of the three studies. Asterisk denotes statistical significance between the treatment and its respective control, while the # denotes significant difference between the two marked bars ($P < 0.05$).

Figure 9. Rescue Study 3: The effect of VHL-MO on MTBE altered mRNA expression in angiogenesis related genes

The graph represents the average fold change in copy number quantification of mRNA transcripts of the genes of interest. Error bars are standard error. Two-way ANOVA was used to determine statistical differences between the treatment groups. Fold changes denoted by the same letter are not statistically different from one another. Statistical comparisons were made within genes only (similar lettering between genes does not imply similar statistical significance). The expression of *vegfa*, *vegfc*, and *vegfr-2* were all significantly greater in the VHL-MO with MTBE than in the CoMo with MTBE, demonstrating a rescue. *Hif1-α* was significantly increase with VHL-MO, but was down to control levels in the presence of MTBE.

Table 1

Comparison of the top molecules altered by MTBE at 21-somites

Molecule	Name	Function	0.00625 mM	0.625 mM	5.0 mM
ACP5 *	Acid Phosphatase	Energy/Metabolism	+2.32	+2.19	+2.14
APOA4 *	Apolipoprotein	Energy/Metabolism	+2.13	+2.08	-1.87
CFI	Complement factor 1	Innate Immunity	+2.10	-1.69	+1.92
CTSB	Cathepsin B	Cell Stress	+2.90	<1.50	+1.61
CTSS *	Cathepsin S	Cell Stress	+2.28	+2.25	+2.24
CYP11A1 *	Cytochrome p450 11A1	Steroid Synthesis	+2.11	-1.82	-1.77
EGLN3	Egl Nine Homolog 3	Oxygen Sensing	+2.07	-1.63	-1.78
FBP1	Fructose-1,6-bisphosphatase	Energy/Metabolism	-1.87	-1.61	-1.74
FGG *	Fibrinogen Gamma Chain	Blood Clotting	-1.99	+2.03	-1.82
MTHFD1	Methylenetetrahydrofolate dehydrogenase	Energy/Metabolism	+1.84	-1.78	+1.57
NPC2	Niemann-Pick disease	Cell Stress	-1.93	-1.72	-1.60
OCM2 *	Oncomodulin 2	Cell Stress	+2.16	-1.95	-1.91
SERPINA1 *	Serine Proteinase inhibitor	Cell Stress	-1.92	-1.82	-1.86
TF	Transferrin	Iron Homeostasis	-1.79	-1.74	-1.66

* Indicates the molecules in the top 10 of all three MTBE treatment group.

+/- Indicates direction of fold change.