HHS Public Access

Phenotype anchoring in zebrafish reveals a potential role for matrix metalloproteinases (MMPs) in tamoxifen's effects on skin epithelium

Sean M. Bugel[a,*], **Leah C. Wehmas**[a], **Jane K. La Du**[a], and **Robert L. Tanguay**[a]

[a]Department of Environmental and Molecular Toxicology, Environmental Health Sciences Center, and the Sinnhuber Aquatic Research Laboratory, Oregon State University, Corvallis, Oregon, 97331, United States

Abstract

The zebrafish is a powerful alternative model used to link phenotypes with molecular effects to discover drug mode of action. Using a zebrafish embryo-larval toxicity bioassay, we evaluated the effects of tamoxifen - a widely used anti-estrogen chemotherapeutic. Zebrafish exposed to 10 μM tamoxifen exhibited a unique necrotic caudal fin phenotype that was rapidly induced regardless of developmental life-stage when treatment was applied. To define tamoxifen's bioactivity resulting in this phenotype, targeted gene expression was used to evaluate 100 transcripts involved in tissue remodeling, calcium signaling, cell cycle and cell death, growth factors, angiogenesis and hypoxia. The most robustly misregulated transcripts in the tail were matrix metalloproteinases *mmp9* and *mmp13a*, induced 127 and 1145 fold, respectively. Expression of *c-fos, c-jun*, and *ap1s1* were also moderately elevated (3–7 fold), consistent with AP-1 activity - a transcription factor that regulates MMP expression. Immunohistochemistry confirmed high levels of induction for MMP13a in affected caudal fin skin epithelial tissue. The necrotic caudal fin phenotype was significantly attenuated or prevented by three functionally unique MMP inhibitors: EDTA (metal chelator), GM 6001 (broad MMP inhibitor), and SR 11302 (AP-1 transcription factor inhibitor), suggesting MMP-dependence. SR 11302 also inhibited induction of *mmp9, mmp13a*, and a putative MMP target, *igfbp1a*. Overall, our studies suggest that tamoxifen's effect is the result of perturbation of the MMP system in skin leading to ectopic expression, cytotoxicity, and the necrotic caudal fin phenotype. These studies help advance our understanding of tamoxifen's non-classical mode of action and implicates a possible role for MMPs in tissues such as skin.

*Corresponding author: Oregon State University, Environmental Health Sciences Center, 1011 Agricultural and Life Sciences Building, Corvallis, OR 97331-7302, Phone: (541) 737-6500.

Graphical abstract

Keywords

matrix metalloproteinases; *mmp13a*; skin epithelium; tamoxifen; zebrafish

1.0. Introduction

The chemotherapeutic drug tamoxifen is a prodrug commonly used for its anti-estrogenic effects to treat various reproductive disorders and diseases (Jordan, 2003; 2005). Tamoxifen and the CYP3A4/CYP2D6 derived metabolite 4-hydroxytamoxifen act as potent estrogen receptor α antagonists through competitive inhibition in breast and other tissues (Jin *et al.*, 2005). These drugs also act as weak or partial agonists in endometrium and bone, and are thus classified as selective estrogen receptor modulators (SERMs). As a result of this activity, tamoxifen is primarily used for hormone therapy for prevention and treatment of ER-positive breast cancer for up to 10 years (Burstein *et al.*, 2014). Tamoxifen is also used secondarily to treat a number of other reproductive health issues, such as anovulatory disorders, gynecomastia, and McCune-Albright syndrome (Eugster *et al.*, 1998; Khan and Blamey, 2003; Steiner *et al.*, 2005). As a multi-purpose hormone therapeutic, tamoxifen use is likely to remain high and become increasingly common.

Aside from tamoxifen's clinical use for hormone therapy, its multifaceted bioactivity suggests potential for novel therapeutic applications. Many *in vitro* studies demonstrate tamoxifen to be highly cytotoxic and able to induce cellular transformation for a variety of cancerous cell types (Petinari *et al.*, 2004). At low micromolar concentrations that typically exceed SERM levels, tamoxifen is pro-apoptotic to cancer cells *in vitro*, including breast (Salami and Karami-Tehrani, 2003), glioma (Kim *et al.*, 2005), oral (Chu *et al.*, 2007), and prostate (El Etreby *et al.*, 2000). Tamoxifen modulates Ca^{2+} influx and triggers apoptosis in other cancer cell types such as bone (Lu *et al.*, 2002), bladder (Chang *et al.*, 2001), and liver (Kim *et al.*, 1999). In malignant melanoma cells, tamoxifen induces cell death through an IGF-dependent pathway (Kanter-Lewensohn, 2000). These diverse effects on cell viability are under investigation for potential alternative applications.

Despite proven therapeutic value, a number of adverse side effects complicate and limit tamoxifen use. Reported side effects include increased risks for endometrial and uterine cancer, menopausal symptoms, stroke, deep vein thrombosis, pulmonary embolism, heart disease, osteoporosis, uterine cancer, sleep problems, weight gain, anxiety and depression, irritability and mood swings (Ganz, 2001; Lorizio *et al.*, 2012). Gel based skin applications

(e.g. afimoxifene) can be used to reduce systemic levels of tamoxifen to ameliorate side effects and provide localized transdermal treatment (Goyal *et al.*, 2014). However, tamoxifen also induces skin-specific side effects including dermal fibrosis, epidermal atrophy, thinning of hair, thinning of skin, dryness, reduced vascularity, blistering, peeling, and loosening (Boström, 1999). For many drugs like tamoxifen and other emerging therapeutics, alternative *in vivo* approaches are needed to rapidly and comprehensively evaluate off-target effects.

The zebrafish model can be used to rapidly evaluate pharmaceuticals, such as tamoxifen, to identify novel effects and better understand drug side effects and toxicity (Barros *et al.*, 2008; Strahle and Grabher, 2010; MacRae and Peterson, 2015). This increasingly popular and rapid throughput model can link unique adverse phenotypes with molecular events to elucidate mechanisms of action for toxicants *in vivo* (Hill *et al.*, 2005; Bugel *et al.*, 2014; Truong *et al.*, 2014). Yet, many pharmaceuticals have not been thoroughly evaluated with this alternative model. Considering tamoxifen's widespread use and therapeutic value, we sought to evaluate tamoxifen using the zebrafish embryo-larval toxicity bioassay with a goal to elucidate previously unappreciated bioactivity. Using a phenotypic anchoring approach, we demonstrated that tamoxifen rapidly induces widespread cell death resulting in a necrotic caudal fin phenotype that is both p53 and estrogen receptor independent. Targeted gene expression analysis and immunohistochemistry identified high levels of induction for matrix metalloproteinases (MMPs) in the skin, and several inhibitors for MMPs ameliorate the phenotype. Collectively these studies suggest that tamoxifen rapidly activated a robust MMP response in skin.

2.0. Materials and methods

2.1. Chemicals

Tamoxifen (99%, ICI146,474, CAS: 10540-29-1) was obtained from MP Biomedicals (Santa Ana, CA). Ethylenediaminetetraacetic acid (99%, EDTA, CAS: 60-00-4) and 17β-estradiol (98%, E2, CAS: 50-28-1) were obtained from Sigma-Aldrich (St. Louis, MO). Pifithrin α hydrobromide (98%, CAS: 63208-82-2), SR 11302 (98%, CAS: 160162-42-5), and G-15 (GPER antagonist, >99%, CAS: 1161002-05-6) were obtained from Tocris Bioscience (Minneapolis, MN). GM 6001 (98%, CAS: 142880-36-2) was obtained from Enzo Life Sciences (Farmingdale, NY). Dimethyl sulfoxide (99.9%, DMSO, CAS: 67-68-5) was obtained from Avantor Performance Materials (Center Valley, PA). All chemicals stocks were prepared in DMSO.

2.2. Zebrafish husbandry

Adult zebrafish (*Danio rerio*) were maintained on a 14:10 light:dark cycle at the Sinnhuber Aquatic Research Laboratory at Oregon State University (Corvallis, OR), in accordance with protocols approved by the Oregon State University Institutional Animal Care and Use Committee and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Wild type tropical 5D were used for all studies except the apoptosis study, which required a secA5-YFP transgenic line to visualize apoptosis *in vivo* (van Ham *et al.*, 2010). Prior to studies, chorions were enzymatically removed from embryos at 4-5 hours post

fertilization (hpf) with pronase (Roche, Indianapolis, IN) following procedures by Usenko *et al.* (2007). Dechorionated embryos individually transferred into clear BD 353075 Falcon 96-well plates (Corning, Corning, NY) contained 100 μL embryo medium (15 mM NaCl, 0.5 mM KCl, 1 mM CaCl$_2$, 0.15 mM KH$_2$PO$_4$, 0.05 mM Na$_2$HPO$_4$, 1 mM MgSO$_4$, 0.05 mM NaHCO$_3$).

2.3. Exposure protocols

Exposures were either static throughout development, or staggered to determine developmental windows of sensitivity (Fig. 1). At the onset of exposure, treatments prepared in embryo medium were added to achieve the desired final concentration in 0.1% DMSO. Exposure plates were covered with parafilm and wrapped in aluminum foil to minimize chemical photo degradation, and incubated at 28.5 °C. Embryos were developmentally exposed from 6–120 hpf in an initial study to determine developmental toxicity, and observed daily for morphological defects (e.g. pericardial and yolk sac edemas, axial and craniofacial defects, circulatory effects, etc.). Concentrations tested were 1, 2.5, 5, 10, and 15 μM. Subsequent exposures to characterize the caudal fin phenotype were conducted at 48–72 hpf. For those studies, the necrotic caudal fin phenotype was the primary observation and scored as either present/absent or by severity for inhibitor rescue studies depending on how far the phenotype progressed (Fig. 1). For inhibitor rescue studies (EDTA, G-15, GM 6001, pifithrin α, SR 11302), inhibitors were co-added at previously determined maximum tolerable no observable adverse effect level (NOAEL) concentrations simultaneously with tamoxifen.

2.4. Microinjection of morpholinos

Morpholino antisense oligonucleotides were used to investigate the potential involvement of *tp53* and nuclear estrogen receptors (*esr1, esr2a, esr2b*) in the onset of the necrotic fin lesion caused by tamoxifen, using techniques adapted from Nasevicius and Ekker (2000). Morpholinos were designed and synthesized by Gene Tools, LLC (Philomath, OR) and were used to knock-down expression in an attempt to ameliorate the phenotype. Morpholinos were microinjected into 1–2 cell stage embryos (2–4 nL) directly into the yolk-stream and viable animals were selected and manually dechorionated at 24 hpf. The standard control morpholino (C-MO) used was 5′ -CCT CTT ACC TCA GTT ACA ATT TAT A-3′ and was injected at matching concentrations. The standard translation blocking *tp53*-MO: 5′ -GCG CCA TTG CTT TGC AAG AAT TG-3′ was injected at 1 mM, which has been widely used in zebrafish literature and our laboratory (Bill *et al.*, 2009; Miller *et al.*, 2012). The *esr1*-MO: 5′ -CAT GTA AAA CAG GCT GGT CAC CTT G-3′ is an E3I3 splice blocking morpholino injected at 1.5 mM, and was designed and validated by Griffin *et al.* (2013). Both *esr2a*-MO and *esr2b*-MO were newly validated. The *esr2a*-MO: 5′ -GTA CTT TCA GAG AGT CTT ACC TTG T-3′ is an E5I5 splice blocking morpholino and was injected at 1.5 mM. The *esr2b*-MO 5′ -CAC ACC TGT CGA AAC ACA CAA GAA C-3′ is an I4E5 splice blocking morpholino and was injected at 0.5 mM.

2.5. Analysis of mRNA expression by quantitative real-time polymerase chain reaction (qRT-PCR)

Messenger RNA expression for 100 transcripts of interest was evaluated using qRT-PCR methods adapted from Bugel *et al.* (2013). Transcripts selected for analysis were for genes involved in pathways identified in literature implicated or related to tamoxifen's effects in various tissues, and were primarily involved in tissue remodeling, calcium signaling, cell cycle and cell death, growth factors, angiogenesis and hypoxia (Suppl. Data Table 1). Briefly, total RNA was isolated from whole animal larvae and isolated tail tissue using RNAzol® RT (Molecular Research Center, Inc., Cincinnati, OH). For whole animal expression analysis, four pools of 12 larval animals were sampled at 3, 6, and 12 hours post-exposure (hpe). For tail specific expression analysis, four pools of 80 tails were sampled 6 hpe. Tails were cut using a glass microdissection blade at the ventral pigment gap (Fig. 1). Isolated total RNA was converted to complementary DNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA). qRT-PCR was performed using a StepOnePlus™ Real-Time PCR System with Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA). Primers used are listed in Suppl. Data Table 1, and were purchased from Integrated DNA Technologies (Coralville, IA). Primers were designed to amplify 75–400 bp fragments of each transcript and were verified for specificity using the NCBI BLAST. OligoAnalyzer (IDT) was used to evaluate primer sets for hetero/homo-dimers ($G > -9$), formation of hairpins (T_m of hairpin $\ll T_m$ of oligo) and T_m of primers (60 ± 2 °C). Melt curves were generated for each run to ensure a single product, and product size was verified by gel electrophoresis. *β-actin* was used as a housekeeping transcript for normalization, and relative expression was quantified using the Ct method (Pfaffl, 2001).

2.6. Whole mount immunohistochemistry (IHC) for MMP13a

For *in situ* whole mount IHC visualization of MMP13a protein expression in the caudal fin in response to tamoxifen, an IgG rabbit polyclonal anti-MMP-13a (hinge) antibody for detecting both proenzyme and cleaved active MMP was used (Anaspec, Inc., Fremont, CA, Cat. No. 55114). The manufacturer's protocol was adapted with minor changes. Briefly, animals were fixed in 4% paraformaldehyde in PBS at 4 °C overnight, then rinsed 3 times in PBSTx for 10 minutes each, then blocked in blocking buffer 1 (PBSTx with 5% BSA, 5% NGS, 0.1% DMSO) for 6 hours at 4 °C. Primary antibody was applied overnight in blocking buffer 2 (PBSTx with 0.5% BSA, 0.1% DMSO) with a 1:500 dilution at 4 °C. Primary was rinsed off with PBSTx 2×5 minutes, then 4×30 minutes. AlexaFluor 555 goat anti-rabbit IgG secondary antibody (1:5000 dilution, Molecular Probes, Eugene, OR) was applied in blocking buffer 2 at room temperature for 2 hours. Secondary was washed off with PBSTx 2×5 minutes, then 4×30 minutes. Stained embryos were then visualized on an inverted Zeiss Axiovert 200M epifluorescence microscope using a Zeiss Axiocam HR camera (Carl Zeiss, Oberkochen, Germany).

2.7. Data and statistical analyses

Statistical tests were performed using SigmaPlot™ (v. 11.0) or R (v. 3.2.2), and a p-value 0.05 was regarded as significantly different for all studies. For discrete presence/absence

data, a Fisher's exact test was used to compare control versus treatment. Gene expression data was evaluated using either one-way ANOVA (Student-Newman-Keuls), or Student's t-test (Wilcoxon when normality/variance failed). For gene expression, GENE-E (v. 3.0.204) was used to generate heat maps only for significantly altered transcripts with a ≥ 1.5 fold change for whole animal samples and ≥ 2 fold change for isolated tail samples.

3.0. Results

3.1. Phenotypic characterization of tamoxifen toxicity in zebrafish

A series of developmental exposure studies were conducted to evaluate the effects of tamoxifen using the zebrafish embryo-larval toxicity bioassay (Fig. 1). Initially, embryos were exposed to tamoxifen (1–15 μM, static non-renewal) throughout development, from 6–120 hpf, and observed daily for morphological defects and mortality (N = 24 per treatment). There was no significant developmental toxicity (morphology or mortality) observed for 1, 2.5, and 5 μM tamoxifen at any time point (Fig. 2A). However, 10 μM and 15 μM significantly induced a highly specific necrosis-like caudal fin defect in 100% of animals by 24 hpf, with some mortality at 15 μM. With continued exposure, >75% of animals died in the 10 μM group, while all animals perished with 15 μM by 120 hpf (Fig. 2A).

Acute exposures within discrete developmental windows were conducted to determine whether the fin defect was due to perturbation of tail formation during embryogenesis (Fig. 1). Embryonic and larval zebrafish were exposed to 10 μM tamoxifen for 24 hours starting at 6, 24, 48, 72, and 96 hpf (N = 24 per group). Within all exposure windows, tamoxifen significantly induced the necrotic caudal fin phenotype in 100% of treated animals, indicating developmental stage-independence (Fig. 2B). The necrotic phenotype was also recapitulated in adult animals acutely exposed to 10 μM tamoxifen (Fig. 2C). Fin necrosis and skin lesions were more widespread in the adult and observed in pectoral fins and skin universally.

To characterize the onset and progression of the caudal fin phenotype, a study was conducted using 48 hpf larvae exposed to 10 μM tamoxifen. Larvae at 48 hpf were chosen for subsequent studies because tail tissue is differentiated, and enabled the use of morpholinos for later functional studies. Transgenic secA5-YFP zebrafish allowed for non-invasive *in vivo* visualization of apoptosis within the necrotic fin phenotype (van Ham *et al.*, 2010). Tamoxifen rapidly induced robust changes in cell morphology at the apex of the caudal fin by 3 hpe, which progressively worsened by 6, 12 and 24 hpe (Fig. 3). Apoptosis was observed as early as 3 hpe, and co-localized to the lesion as it advanced. Apoptotic cells were typically adjacent proximally to the necrotic tissue, in normal appearing tissue that later became necrotic-like. A time-lapse video of the development of the phenotype demonstrated that tamoxifen rapidly induced the phenotype starting with a necrotic-like lesion and propagating into adjacent phenotypically normal tissue (Suppl. Data Video 1). Initially, changes in cell morphology rapidly occurred by 3 hpe followed by swelling and lysis of apical cells by 6 and 12 hpe. Leukocytes were also observed migrating towards the distal part of the damaged tail tissue. Proximal to the caudal fin, skin was observed to behave increasingly fluid-like as the phenotype progressed.

3.2. Evaluation of gene expression

Targeted gene expression was evaluated for 100 transcripts in 48 hpf larval animals acutely exposed to 10 μM tamoxifen. Whole animal expression was evaluated at 3, 6, and 12 hpe, whereas tail specific expression was evaluated only at 6 hpe. Tamoxifen treatment rapidly induced significant and robust changes in expression for a number of transcripts in both the whole animal and isolated tails (Fig. 4 and Suppl. Data Table 2). Generally, whole animal gene expression was concordant between time points and with the 6 hpe tail dataset. In the whole animal, the number of transcripts and the magnitude fold change generally increased with exposure time. At 3 hpe, expression of five transcripts were significantly elevated in the whole animal (*mmp9 > mmp13a > tnfb > c-fos > igfbp1a*), all of which were also significantly elevated in the 6 hpe tail dataset. At 6 hpe, expression of eleven transcripts were significantly elevated in whole animal samples, ten of which were similarly elevated in the tail dataset (top 6: *mmp9 > mmp13a > tnfb > timb2b > igfbp1a > c-fos*), and one that was not (*epcam*). At 12 hpe, 26 genes were elevated in whole animal samples, 17 of which were also significantly increased in the tail specific dataset (top 10: *mmp13a, mmp9, igfbp1a, timp2b, tnfb, igfbp1b, c-fos, cdkn1a, tnfa, c-jun*).

Overall, the highest number of transcripts with significantly altered expression levels was observed in the isolated tail at 6 hpe (41 out of 100 tested transcripts). Focusing on the 6 hpe dataset, transcripts for tissue remodeling and growth factor pathway genes were the most robustly altered. These involved several matrix metalloproteinases and other genes involved in MMP regulation or function, such as tissue specific inhibitors, and insulin-like growth factors. The top 10 transcripts most robustly altered (by fold change) in the 6 hpe tail specific dataset were: *mmp13a* (1146 fold) > *igfbp1a* (150 fold) > *mmp9* (127 fold) > *timp2b* (40 fold), *mmp11a* (-19 fold) > *igfbp1b* (16 fold) > *tnfb* (14 fold) > *timp4* (-11 fold) > *hif1aa* (8 fold) > *nfkb2* (7 fold). Similarly, the top six transcripts most altered in the 6 hpe whole animal dataset were: *mmp9* (54 fold) > *mmp13a* (42 fold) > *tnfb* (7 fold) > *timp2b* (4 fold) > *igfbp1a* (3 fold) = *c-fos* (3 fold). In the 6 hpe tail dataset, a number of transcripts for genes involved in cell cycle/cell death were significantly altered with modest fold changes (± 3–6 fold), notably *c-fos, c-jun, tp63, tnfa, tnfb*. A number of transcripts for genes involved in calcium signaling were also significantly different with low fold-changes (± 2–3 fold), specifically the calmodulins, calpains, and cadherins.

3.3. Investigation of the potential role for p53 in the necrotic caudal fin phenotype

To explore the possibility for a *tp53* role in mediating the tamoxifen induced caudal fin lesion, anti-sense morpholinos and chemical inhibition was used to suppress p53 signaling. For these studies, 48 hpf larval animals were exposed to 10 μM tamoxifen with and without morpholino or inhibitor, and observed at 3, 6, 12, and 24 hpe (N = 24). The *tp53* translation blocking morpholino is widely used in zebrafish literature to knock-down mRNA expression and concentrations used were similar to those effectively used previously (Bill *et al.*, 2009; Miller *et al.*, 2012). The inhibitor, pifithrin α, directly binds the p53 DNA-binding domain, though there may be off-target effects on related tp63 and tp73 because of sequence similarity (Levrero *et al.*, 2000). The concentration of pifithrin α used (5 μM) was similar to effective concentrations previously used and was a maximum tolerable NOAEL concentration (Davidson *et al.*, 2014; Duffy and Wickstrom, 2014). In the present study,

neither method of p53 suppression, morpholino antisense knockdown nor pifithrin α inhibition provided any beneficial effect against the tamoxifen induced caudal fin lesion at any observed time point (Fig. 5). The tamoxifen treatments, with or without morpholino/ inhibitor, produced caudal fin necrosis in 100% of animals. Severity between all treatment groups treated or co-treated with tamoxifen were qualitatively similar at all evaluated time points.

3.4. Demonstration of an MMP-dependent role for tamoxifen toxicity in the necrotic caudal fin epithelium

The role of matrix metalloproteinases as key mediators of the necrotic caudal fin phenotype was investigated using the 48 hpf larval exposure paradigm. First, whole mount *in situ* immunohistochemistry was used to visualize MMP13a protein expression in the caudal fin in response to tamoxifen. Similar to the mRNA study, MMP13a protein expression was robustly induced by 10 μM tamoxifen at 6 hpe in the caudal fin lesion of all treated animals (Fig. 6). Low level, MMP13a expression was widely observed in a ring like pattern resembling a secreted protein specifically localized to large skin cells across the entire body of control animals. The antibody used detects both pro-enzyme and active cleaved MMP13a.

Next, the MMP-dependence of the tamoxifen-induced caudal fin phenotype was evaluated using three functionally unique MMP inhibitors: EDTA, GM 6001 and SR 113302. EDTA is a divalent metal cation chelator commonly used to broadly inhibit MMP activity, and the NOAEL concentration (1 mM) used in our studies was consistent with those used previously (Hillegass *et al.*, 2007). GM 6001 is a broad competitive inhibitor that complexes with the zinc binding site of MMPs to inhibit enzymatic activity. The maximum tolerable NOAEL for GM 6001 (100 μM) has been previously used (Hillegass *et al.*, 2007). SR 11302 is an inhibitor to AP-1, a transcription factor responsible for regulating MMP gene expression. SR 11302 was used at 10 μM, the maximum tolerable NOAEL. For these studies, severity of the lesion was evaluated on a scale from 1–5 (Fig. 1). Only EDTA completely prevented the development of the tamoxifen-induced fin lesion at 3, 6, 12 and 24 hpe. Co-treatment of tamoxifen with EDTA in embryo medium (EM) resulted in 0% of animals exhibiting the lesion, compared to 100% of animals when treated with tamoxifen in EM without EDTA (Fig. 7A). GM 6001 and SR 113302 only partially ameliorated the severity of the lesion, but did not block the prevalence of the lesion (Fig. 7B). Co-treatment of tamoxifen with both GM 6001 and SR 113302 moderately reduced the severity of the necrotic caudal fin phenotype at all evaluated time points, though only slightly better than each inhibitor tested alone when co-treated with tamoxifen (Fig. 7B, only 6 and 12 hpe are shown). A gene expression study confirmed the ability of SR 11302 to inhibit the robust induction of the two primary MMP transcripts of interest, *mmp9* and *mmp13a*, and a putative downstream MMP target, *igfbp1a*. For this study, tail tissue was isolated at 3 hpe, and co-treatment of tamoxifen with SR 11302 significantly inhibited induction of *mmp9, mmp13a and igfbp1a* by 28.9%, 64.4% and 44.4%, respectively (Fig. 7C).

3.5. Evaluation of tamoxifen's anti-estrogenic potency and potential role of estrogen receptors

To determine the anti-estrogenic potency of tamoxifen in larval zebrafish, a co-exposure study was conducted from 6–120 hpf with either 0.1% DMSO (control), E2 (100 nM 17β-estradiol), tamoxifen, or a combination of E2 + tamoxifen (Fig. 8A–B). NOAEL concentrations of tamoxifen tested were 0.1, 1, and 5 μM. Gene expression was measured in whole animal samples for estrogen responsive transcripts (A) *vtg1*, a highly specific hepatic biomarker, and (B) *cyp19a1b*, the highly specific brain aromatase. Robust inductions of both estrogen biomarkers were significantly inhibited by 1 and 5 μM, but not 0.1 μM tamoxifen co-treatment. Hepatic *vtg1* expression was inhibited by greater than 98% at 1 μM tamoxifen and above (Fig. 8A). Induction of brain aromatase was inhibited by 82% at 1 μM tamoxifen, and 96% at 5 μM tamoxifen (Fig. 8B). Together, this demonstrated that the necrotic caudal fin phenotype, which developed at 10 μM tamoxifen and higher, exceeded concentrations that elicited highly potent antiestrogenic effects on gene expression.

To explore the potential role of estrogen receptors in the tamoxifen induced caudal fin lesion, either the chemical inhibitor G-15 was used to antagonize the g-protein estrogen receptor (GPER), or splice blocking morpholinos were used to knock-down expression of nuclear estrogen receptors (Fig. 8C). For these studies, 48 hpf larval animals were exposed to 10 μM tamoxifen with and without inhibitor or individual morpholinos, and observed at 3, 6, 12, and 24 hpe (N = 24). The concentration of G-15 (25 μM) was previously determined to effectively inhibit 5 dpf developmental toxicity of the GPER agonist G-1 in zebrafish in our lab (data not shown), and others (Jayasinghe and Volz, 2012). The *esr1* morpholino was developed and validated by Griffin *et al.* (2013) and used at similar concentrations, and those for *esr2a* and *esr2b* were newly developed and validated prior to this study. In the present study, inhibition of the membrane g-protein estrogen receptor with G-15 or knockdown of nuclear estrogen receptors using morpholinos did not ameliorate nor provide a beneficial effect against the tamoxifen induced caudal fin lesion at any observed time point. The tamoxifen treatments, with or without morpholino or inhibitor, produced the caudal fin defect in 100% of animals and were qualitatively the same at all time points.

4.0. Discussion

In the present studies, we used the zebrafish embryo-larval developmental toxicity bioassay as an alternative model to define the effects of tamoxifen, a highly prescribed pharmaceutical with diverse bioactivity in many tissue and cell types. A comprehensive evaluation of tamoxifen's toxicity *in vivo* may provide a better understanding of secondary effects and alternative modes of action (Ganz, 2001; Lorizio *et al.*, 2012). To our knowledge, tamoxifen has not been fully evaluated in zebrafish, with the exception of its specific effects on the cardiovascular, hepatic, and reproductive systems (Burns *et al.*, 2005; van der Ven *et al.*, 2007; Bopp and Lettieri, 2008). Our studies demonstrated that acute exposure to tamoxifen rapidly induced a unique tail lesion regardless of developmental life-stage when tamoxifen was applied (Fig. 2). In a high-throughput developmental toxicity screen of 1060 compounds, relatively few chemicals (79) induced any type of adverse effects on the caudal fin in zebrafish, suggesting similar effects to be uncommon (Truong *et*

al., 2014). It was therefore the focus of these studies to investigate the causative mode of action for this unique phenotype to further elucidate tamoxifen's bioactivity.

Tamoxifen induced the caudal fin phenotype as rapidly as 3 hpe, as observed with both secA5-YFP transgenic zebrafish and time-lapse video (Fig. 3, Suppl. Data Video 1). This finding paralleled previous studies, which found tamoxifen to be cytotoxic and pro-apoptotic *in vitro* to multiple cell types at low micromolar concentrations (Petinari *et al.*, 2004). Apoptotic cells were typically adjacent to the necrotic fin tissue, suggesting apoptosis to be a secondary effect. The misregulation of transcripts involved in cell cycle and cell death pathways reflected the widespread tissue damage (Fig. 4 and Suppl. Data Table 2). Altered expression of regulatory molecules associated with cell death suggested potential for a *tp53* pathway mediated effect, including *c-fos, c-jun, cdkn1a (p21), nfkb2, tnfa, tnfb*, and *tp53*. Tumor protein p53 is a pleiotropic transcription factor that drives a variety of cellular responses and programmed cell death, including both apoptosis and necrosis (Chipuk and Green, 2006; Baumann, 2012). The aforementioned transcripts identified in our study are similarly involved in cellular responses and interact with *tp53* through a variety of mechanisms (Elkeles *et al.*, 1999; Ryan *et al.*, 2000; Lohr *et al.*, 2003; Pastor *et al.*, 2010). We therefore attempted to mitigate the necrotic caudal fin phenotype using two methods of *p53* suppression. However, neither morpholino knockdown of *tp53* nor chemical inhibition by pifithrin α ameliorated the phenotype, suggesting the effect to be *tp53*-independent (Fig. 5). In addition, significantly altered transcript levels were observed for several calmodulins, caspases and calpains, which are involved with programmed cell death signaling cascades (Schuler *et al.*, 2000; Harwood *et al.*, 2005). Calmodulins and calpains are a family of calcium-dependent genes involved in cell cycle progression, gene expression, and cell death (Goll *et al.*, 2003). For many cell types, tamoxifen's *in vitro* cytotoxicity involved calcium signaling (Kim *et al.*, 1999; Chang *et al.*, 2001; Lu *et al.*, 2002). However, changes in expression for *calm, casp* and *capn* genes were generally not robust or compelling (Fig. 4). Taken together, our data suggested the apoptosis to be secondary to the widespread tissue damage, and not likely directly involved with initiation of the phenotype.

Overall, a number of genes in other related pathways were significantly misregulated in the tail, providing valuable molecular insight into the onset and progression of the phenotype. The most robustly misregulated transcripts were the MMPs (Fig. 4 and Suppl. Data Table 2). This family of calcium-dependent zinc-containing endopeptidases is responsible for extracellular matrix degradation and tissue remodeling critical for regulation of skin physiology, function, and diseases (Page-McCaw *et al.*, 2007). They play important roles in tumor progression for various cancers (e.g. melanoma), through alteration of tumor microenvironment thereby affecting proliferation, migration, invasion and metastasis (Hofmann *et al.*, 2000; Kessenbrock *et al.*, 2010; Lu *et al.*, 2012). The zebrafish has great potential as a versatile tool for studying chemical effects on MMP function *in vivo* (Crawford and Pilgrim, 2005). Our studies identified *mmp9* and *mmp13a* as the primary MMPs involved in the necrotic caudal fin phenotype, based on robust induction and inhibition studies (Figs. 6–7). Like many MMPs, these two respond to toxicants and drugs and are potential therapeutic targets for treating various diseases (Overall and Kleifeld, 2006; Vartak and Gemeinhart, 2007). In the present studies, robust induction of MMPs

offered an explanation for cytotoxicity in the necrotic caudal fin phenotype considering their function as endopeptidases and their roles in extracellular gelatin and collagen matrix degradation. Further support for a role of MMPs in the tamoxifen caudal fin phenotype came from the complete or partial amelioration of the phenotype by MMP inhibitors (Fig. 7). Three MMP inhibitors chosen for their differential mechanism of action ameliorated the necrotic caudal fin phenotype and were: EDTA, a divalent metal cation chelator; GM 6001, a broad-spectrum MMP competitive inhibitor, and SR 11302, an inhibitor of AP-1 transcription factor signaling. EDTA and GM 6001 provided direct evidence that the MMPs were involved in the development of the necrotic caudal fin phenotype, and SR 11302 suggested that the ectopic induction of *mmp9* and *mmp13a* in skin by tamoxifen was mediated through an AP-1 regulated pathway. AP-1 is an inducible transcriptional complex composed of *c-fos* and *c-jun* proteins that broadly modulate MMP gene expression, and as a family, nearly all have AP-1 binding sites in their promoters (Benbow and Brinckerhoff, 1997). In our studies, expression of *c-fos, c-jun*, and the *ap1s1* subunit were all moderately elevated (3–7 fold), consistent with elevated AP-1 activity (Fig. 4 and Suppl. Data Table 2). Numerous estrogen receptor ligands, including tamoxifen, transactivate AP-1 through ERβ binding (Paech, 1997). Our dataset found that tamoxifen modestly decreased isolated tail tissue expression of *esr2a* (–2.7 fold), the zebrafish ERβ ortholog (Fig. 4 and Suppl. Data Table 2). Additionally, tamoxifen is a ligand for the membrane g-protein estrogen receptor (GPER), which can subsequently activate *c-jun/c-fos* (Maggiolini *et al.*, 2004; Prossnitz and Barton, 2011). However, the failure of *esr1, esr2a* and *esr2b* morpholinos and the GPER specific antagonist (G-15) to provide a beneficial effect suggested the caudal fin phenotype to be ER-independent (Fig. 8C). Furthermore, robust changes in expression were observed for multiple IGF transcripts (*igf1a, igfbp1a* and *igfbp1b*), which are known to be cleaved and activated by MMPs and play roles in physiological and pathological conditions (McCawley and Matrisian, 2001; Samani *et al.*, 2007). These results relate to findings by Kanter-Lewensohn (2000) where tamoxifen induced apoptosis in malignant melanoma cells *in vitro* by an IGF-dependent pathway. Taken together, these studies suggest a critical role for MMPs, though they were not able to determine whether the caudal fin phenotype was due to direct MMP action (i.e. extracellular matrix breakdown), or a secondary MMP-mediated downstream response (e.g. IGF signaling). Future studies may address this and use tamoxifen to further understand how MMPs and IGFs are ectopically regulated in skin *in vivo*.

The skin epithelium is a relatively simple structure in the zebrafish, particularly in the larval animal (Le Guellec *et al.*, 2004; Chang and Hwang, 2011). Embryonic and larval zebrafish have only two epidermal layers: the surface enveloping layer and epidermal basal layer. Cell types include keratinocytes, ionocytes and mucous cells. Skin in adult zebrafish has an additional intermediate layer and club cells. These differences provided an advantage in using the simpler larval model to characterize the necrotic caudal fin phenotype and elucidate the mode of action. The simple larval caudal fin is a non-muscular limb limited to actinotrichia (bony ray precursor), neurons, and skin, while lacking vasculature in the region where the necrotic phenotype originated. Immunohistochemistry for MMP13a demonstrated a specific and widespread pattern of expression across the entire body of the larval animal in very large epithelial cells (Fig. 6). The distribution, morphology, and size of the stained

epithelial cells were consistent with ionocytes based on studies by Kwong *et al.* (2013). Ionocytes are widespread in zebrafish skin and have several sub-types, generally defined by their dominantly expressed ion pump (Janicke *et al.*, 2007; Chang and Hwang, 2011). Specific ionocytes sub-types can be regiospecific in larvae, possibly offering an explanation for the caudal fin specificity at this life-stage. Unlike the larval animal, the effect was not restricted to a particular region of the adult skin and was observed broadly. Furthermore, the response in the ionocytes may be a secondary effect, due to signals from adjacent cells, such as keratinocytes, which are known to have a highly reactive MMP responsive to certain chemicals (Murphy *et al.*, 2004). Further studies using cell specific stains would be necessary to positively identify other skin cell types involved.

The aqueous concentrations used in our study to produce the necrotic caudal fin phenotype are relatable to blood plasma levels reported in patients using low- and high-dose tamoxifen therapy, and also *in vitro* studies demonstrating cytotoxic effects of tamoxifen. Patients using low-dose tamoxifen hormone therapy typically have sub-micromolar blood plasma levels, ranging from 0.1 to 0.3 μM (MacCallum *et al.*, 2000; Gallicchio *et al.*, 2004; Langenegger *et al.*, 2006), whereas low-micromolar levels of 1–10 μM and greater can be achieved with high dose therapy (Ducharme *et al.*, 2003). A relatively high concentration of tamoxifen (10 μM) was required to elicit the caudal fin phenotype. This concentration exceeded SERM concentrations that are typically in the low to sub-micromolar range. In our studies, the anti-estrogenic effects of tamoxifen on the induction of E2 responsive genes was only observed at 1 μM (Fig. 8A–B). In contrast, van der Ven *et al.* (2007) showed in a partial life-cycle study with zebrafish that exposure to tamoxifen at ~0.1 μM resulted in reproductive toxicity in adults, suggesting larvae to be less sensitive to tamoxifen than later stages. Furthermore, the concentrations used to produce cytotoxicity *in vitro* with a broad range of cell types typically exceeded 10 μM (Kanter-Lewensohn, 2000; Kim *et al.*, 2005; Chu *et al.*, 2007). Therefore, concentrations used to elicit effects on the MMP system and the resulting necrotic caudal fin phenotype in our studies may be pharmacologically relevant.

Taken together, our studies use a phenotype anchoring approach to provide evidence to suggest a role for MMPs in mediating tamoxifen's effects in skin epithelium. In summary, we discovered a unique necrotic caudal fin phenotype that was rapidly induced after exposure to tamoxifen. The onset of this phenotype correlated with robust induction of several MMP (*mmp9* and *mmp13b*) genes as well as genes involved with the AP-1 transcriptional factor complex (*ap1s1, c-fos, c-jun*) that regulates MMP expression. The necrotic caudal fin phenotype was attenuated by three unique MMP inhibitors, one of which was an AP-1 inhibitor that also inhibited the robust induction of MMP genes. We demonstrated that perturbation of the MMP system in skin results in ectopic MMP expression, cytotoxicity and ultimately a unique necrotic caudal fin phenotype, implicating MMP involvement as the mode of action. This action is likely due to either direct MMP action through extracellular matrix breakdown, or an MMP-mediated downstream secondary effect. This may help to understand tamoxifen's effects for other cell types and tissues, and perhaps implicates a role for MMPs in skin-specific side effects (Boström, 1999). Overall,

our study highlights the potential for zebrafish to be used to evaluate unique effects for new, old, and emerging pharmaceuticals for mode of action discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

E2	17β-estradiol
hpe	hours post exposure
hpf	hours post fertilization
MMP	matrix metalloproteinase

References

Barros TP, Alderton WK, Reynolds HM, Roach AG, Berghmans S. Zebrafish: an emerging technology for in vivo pharmacological assessment to identify potential safety liabilities in early drug discovery. Br. J. Pharmacol. 2008; 154:1400–1413. [PubMed: 18552866]

Baumann K. Cell death: multitasking p53 promotes necrosis. Nat. Rev. Mol. Cell Biol. 2012; 13:480–481. [PubMed: 22781904]

Benbow U, Brinckerhoff CE. The AP-1 site and MMP gene regulation: What is all the fuss about? Matrix Biol. 1997; 15:519–526. [PubMed: 9138284]

Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC. A primer for morpholino use in zebrafish. Zebrafish. 2009; 6:69–77. [PubMed: 19374550]

Bopp SK, Lettieri T. Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line. BMC Pharmacol. 2008; 8:8. [PubMed: 18513395]

Boström Å. Radiation Recall: Another Call With Tamoxifen. Acta Oncologica. 1999; 38:955–960. [PubMed: 10606426]

Bugel SM, Tanguay RL, Planchart A. Zebrafish: A marvel of high-throughput biology for 21 century toxicology. Current environmental health reports. 2014; 1:341–352. [PubMed: 25678986]

Bugel SM, White LA, Cooper KR. Inhibition of vitellogenin gene induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin is mediated by aryl hydrocarbon receptor 2 (AHR2) in zebrafish (Danio rerio). Aquat. Toxicol. 2013; 126:1–8. [PubMed: 23142599]

Burns CG, Milan DJ, Grande EJ, Rottbauer W, MacRae CA, Fishman MC. High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. Nat. Chem. Biol. 2005; 1:263–264. [PubMed: 16408054]

Burstein HJ, Temin S, Anderson H, Buchholz TA, Davidson NE, Gelmon KE, Giordano SH, Hudis CA, Rowden D, Solky AJ, Stearns V, Winer EP, Griggs JJ. Adjuvant endocrine therapy for women with hormone receptor-positive breast cancer: american society of clinical oncology clinical practice guideline focused update. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2014; 32:2255–2269. [PubMed: 24868023]

Chang H-T, Huang J-K, Wang J-L, Cheng J-S, Lee K-C, Lo Y-K, Lin M-C, Tang K-Y, Jan C-R. Tamoxifen-induced Ca 2+ mobilization in bladder female transitional carcinoma cells. Arch. Toxicol. 2001; 75:184–188. [PubMed: 11409540]

Chang WJ, Hwang PP. Development of zebrafish epidermis. Birth defects research. Part C, Embryo today : reviews. 2011; 93:205–214.

Chipuk JE, Green DR. Dissecting p53-dependent apoptosis. Cell Death Differ. 2006; 13:994–1002. [PubMed: 16543937]

Chu ST, Huang CC, Huang CJ, Cheng JS, Chai KL, Cheng HH, Fang YC, Chi CC, Su HH, Chou CT, Jan CR. Tamoxifen-induced [Ca2+]i rises and Ca2+-independent cell death in human oral cancer cells. Journal of receptor and signal transduction research. 2007; 27:353–367. [PubMed: 18097937]

Crawford BD, Pilgrim DB. Ontogeny and regulation of matrix metalloproteinase activity in the zebrafish embryo by in vitro and in vivo zymography. Dev. Biol. 2005; 286:405–414. [PubMed: 16112664]

Davidson W, Ren Q, Kari G, Kashi O, Dicker AP, Rodeck U. Inhibition of p73 function by Pifithrin-α as revealed by studies in zebrafish embryos. Cell Cycle. 2014; 7:1224–1230. [PubMed: 18418056]

Ducharme J, Fried K, Shenouda G, Leyland-Jones B, Wainer IW. Tamoxifen metabolic patterns within a glioma patient population treated with high-dose tamoxifen. Br. J. Clin. Pharmacol. 2003; 43:189–193. [PubMed: 9131952]

Duffy KT, Wickstrom E. Zebrafish tp53 knockdown extends the survival of irradiated zebrafish embryos more effectively than the p53 inhibitor pifithrin. Cancer Biology & Therapy. 2014; 6:675–678. [PubMed: 17426443]

EBCTCG. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. The Lancet. 2005; 365:1687–1717.

El Etreby MF, Liang Y, Lewis RW. Induction of apoptosis by mifepristone and tamoxifen in human LNCaP prostate cancer cells in culture. The Prostate. 2000; 43:31–42. [PubMed: 10725863]

Elkeles A, Juven-Gershon T, Israeli D, Wilder S, Zalcenstein A, Oren M. The c-fos Proto-Oncogene Is a Target for Transactivation by the p53 Tumor Suppressor. Mol. Cell. Biol. 1999; 19:2594–2600. [PubMed: 10082525]

Eugster EA, Shankar R, Feezle LK, Pescovitz OH. Tamoxifen Treatment of Progressive Precocious Puberty in a Patient With McCune-Albright Syndrome † 420. Pediatric Research. 1998; 43:74–74.

Gallicchio L, Lord G, Tkaczuk K, Danton M, Lewis LM, Lim CK, Flaws JA. Association of tamoxifen (TAM) and TAM metabolite concentrations with self-reported side effects of TAM in women with breast cancer. Breast Cancer Res Treat. 2004; 85:89–97. [PubMed: 15039600]

Ganz PA. Impact of Tamoxifen Adjuvant Therapy on Symptoms, Functioning, and Quality of Life. JNCI Monographs. 2001; 2001:130–134.

Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. Physiol. Rev. 2003; 83:731–801. [PubMed: 12843408]

Goyal A, Mansel R, Le Nestour E, Masini-Etévé V. Topical tamoxifen gel (afimoxifene) is associated with low plasma levels of 4-OHT while achieving therapeutic local antiestrogenic effect in premenopausal women with cyclical mastalgia. Cancer Res. 2014; 69:2154.

Griffin LB, January KE, Ho KW, Cotter KA, Callard GV. Morpholino-mediated knockdown of ERalpha, ERbetaa, and ERbetab mRNAs in zebrafish (Danio rerio) embryos reveals differential regulation of estrogen-inducible genes. Endocrinology. 2013; 154:4158–4169. [PubMed: 23928376]

Harwood SM, Yaqoob MM, Allen DA. Caspase and calpain function in cell death: bridging the gap between apoptosis and necrosis. Ann. Clin. Biochem. 2005; 42:415–431. [PubMed: 16259792]

Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol. Sci. 2005; 86:6–19. [PubMed: 15703261]

Hillegass JM, Villano CM, Cooper KR, White LA. Matrix metalloproteinase-13 is required for zebra fish (Danio rerio) development and is a target for glucocorticoids. Toxicol. Sci. 2007; 100:168–179. [PubMed: 17728286]

Hofmann UB, Westphal JR, Van Muijen GN, Ruiter DJ. Matrix metalloproteinases in human melanoma. The Journal of investigative dermatology. 2000; 115:337–344. [PubMed: 10951266]

Janicke M, Carney TJ, Hammerschmidt M. Foxi3 transcription factors and Notch signaling control the formation of skin ionocytes from epidermal precursors of the zebrafish embryo. Dev. Biol. 2007; 307:258–271. [PubMed: 17555741]

Jayasinghe BS, Volz DC. Aberrant ligand-induced activation of G protein-coupled estrogen receptor 1 (GPER) results in developmental malformations during vertebrate embryogenesis. Toxicol. Sci. 2012; 125:262–273. [PubMed: 21984484]

Jin Y, Desta Z, Stearns V, Ward B, Ho H, Lee KH, Skaar T, Storniolo AM, Li L, Araba A, Blanchard R, Nguyen A, Ullmer L, Hayden J, Lemler S, Weinshilboum RM, Rae JM, Hayes DF, Flockhart DA. CYP2D6 genotype, antidepressant use, and tamoxifen metabolism during adjuvant breast cancer treatment. J. Natl. Cancer Inst. 2005; 97:30–39. [PubMed: 15632378]

Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat. Rev. Drug Discov. 2003; 2:205–213. [PubMed: 12612646]

Kanter-Lewensohn L. Tamoxifen-induced cell death in malignant melanoma cells: possible involvement of the insulin-like growth factor-1 (IGF-1) pathway. Mol. Cell. Endocrinol. 2000; 165:131–137. [PubMed: 10940491]

Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010; 141:52–67. [PubMed: 20371345]

Khan HN, Blamey RW. Endocrine treatment of physiological gynaecomastia. BMJ. 2003; 327:301–302. [PubMed: 12907471]

Kim J-A, Kang YS, Jung M-W, Lee SH, Lee YS. Involvement of Ca2+ influx in the mechanism of tamoxifen-induced apoptosis in HepG2 human hepatoblastoma cells. Cancer Lett. 1999; 147:115–123. [PubMed: 10660097]

Kim YJ, Lee CJ, Lee U, Yoo YM. Tamoxifen-induced cell death and expression of neurotrophic factors in cultured C6 glioma cells. Journal of neuro-oncology. 2005; 71:121–125. [PubMed: 15690126]

Kwong RW, Kumai Y, Perry SF. The role of aquaporin and tight junction proteins in the regulation of water movement in larval zebrafish (Danio rerio). PloS one. 2013; 8:e70764. [PubMed: 23967101]

Langenegger T, Wahl P, Schiesser D, Thurlimann B. Plasma levels of tamoxifen, N-desmethyl tamoxifen and anastrozole in a patient with metastatic breast cancer and chronic hemodialysis. Breast Cancer Res Treat. 2006; 100:177–181. [PubMed: 16688477]

Le Guellec D, Morvan-Dubois G, Sire JY. Skin development in bony fish with particular emphasis on collagen deposition in the dermis of the zebrafish (Danio rerio). Int. J. Dev. Biol. 2004; 48:217–231. [PubMed: 15272388]

Levrero M, De Laurenzi V, Costanzo A, Gong J, Wang JY, Melino G. The p53/p63/p73 family of transcription factors: overlapping and distinct functions. J. Cell Sci. 2000; 113(Pt 10):1661–1670. [PubMed: 10769197]

Lohr K, Moritz C, Contente A, Dobbelstein M. p21/CDKN1A mediates negative regulation of transcription by p53. J. Biol. Chem. 2003; 278:32507–32516. [PubMed: 12748190]

Lorizio W, Wu AH, Beattie MS, Rugo H, Tchu S, Kerlikowske K, Ziv E. Clinical and biomarker predictors of side effects from tamoxifen. Breast Cancer Res Treat. 2012; 132:1107–1118. [PubMed: 22207277]

Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. J. Cell Biol. 2012; 196:395–406. [PubMed: 22351925]

Lu Y-C, Jiann B-P, Chang H-T, Huang J-K, Chen W-C, Su W, Jan C-R. Effect of the Anti-Breast Cancer Drug Tamoxifen on Ca2+ Movement in Human Osteosarcoma Cells. Pharmacology and Toxicology. 2002; 91:34–39. [PubMed: 12193259]

MacCallum J, Cummings J, Dixon JM, Miller WR. Concentrations of tamoxifen and its major metabolites in hormone responsive and resistant breast tumours. Br. J. Cancer. 2000; 82:1629–1635. [PubMed: 10817496]

MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. Nat. Rev. Drug Discov. 2015; 14:721–731. [PubMed: 26361349]

Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, Montanaro D, Musti AM, Picard D, Ando S. The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-

estradiol and phytoestrogens in breast cancer cells. J. Biol. Chem. 2004; 279:27008–27016. [PubMed: 15090535]

McCawley LJ, Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore! Curr. Opin. Cell Biol. 2001; 13:534–540. [PubMed: 11544020]

Miller GW, Labut EM, Lebold KM, Floeter A, Tanguay RL, Traber MG. Zebrafish (Danio rerio) fed vitamin E-deficient diets produce embryos with increased morphologic abnormalities and mortality. The Journal of nutritional biochemistry. 2012; 23:478–486. [PubMed: 21684137]

Murphy KA, Villano CM, Dorn R, White LA. Interaction between the aryl hydrocarbon receptor and retinoic acid pathways increases matrix metalloproteinase-1 expression in keratinocytes. J. Biol. Chem. 2004; 279:25284–25293. [PubMed: 15075337]

Nasevicius A, Ekker SC. Effective targeted gene 'knockdown' in zebrafish. Nat. Genet. 2000; 26:216–220. [PubMed: 11017081]

Overall CM, Kleifeld O. Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. Nat. Rev. Cancer. 2006; 6:227–239. [PubMed: 16498445]

Paech K. Differential Ligand Activation of Estrogen Receptors ER and ER at AP1 Sites. Science. 1997; 277:1508–1510. [PubMed: 9278514]

Page-McCaw A, Ewald AJ, Werb Z. Matrix metalloproteinases and the regulation of tissue remodelling. Nat. Rev. Mol. Cell Biol. 2007; 8:221–233. [PubMed: 17318226]

Pastor DM, Irby RB, Poritz LS. Tumor necrosis factor alpha induces p53 up-regulated modulator of apoptosis expression in colorectal cancer cell lines. Diseases of the colon and rectum. 2010; 53:257–263. [PubMed: 20173470]

Petinari L, Kohn LK, de Carvalho JE, Genari SC. Cytotoxicity of tamoxifen in normal and tumoral cell lines and its ability to induce cellular transformation in vitro. Cell Biol. Int. 2004; 28:531–539. [PubMed: 15261161]

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:e45. [PubMed: 11328886]

Prossnitz ER, Barton M. The G-protein-coupled estrogen receptor GPER in health and disease. Nature reviews. Endocrinology. 2011; 7:715–726.

Ryan KM, Ernst MK, Rice NR, Vousden KH. Role of NF-kappaB in p53-mediated programmed cell death. Nature. 2000; 404:892–897. [PubMed: 10786798]

Salami S, Karami-Tehrani F. Biochemical studies of apoptosis induced by tamoxifen in estrogen receptor positive and negative breast cancer cell lines. Clin. Biochem. 2003; 36:247–253. [PubMed: 12810152]

Samani AA, Yakar S, LeRoith D, Brodt P. The role of the IGF system in cancer growth and metastasis: overview and recent insights. Endocr. Rev. 2007; 28:20–47. [PubMed: 16931767]

Schuler M, Bossy-Wetzel E, Goldstein JC, Fitzgerald P, Green DR. p53 Induces Apoptosis by Caspase Activation through Mitochondrial Cytochrome c Release. J. Biol. Chem. 2000; 275:7337–7342. [PubMed: 10702305]

Steiner AZ, Terplan M, Paulson RJ. Comparison of tamoxifen and clomiphene citrate for ovulation induction: a meta-analysis. Human reproduction. 2005; 20:1511–1515. [PubMed: 15845599]

Strahle U, Grabher C. The zebrafish embryo as a model for assessing off-target drug effects. Disease models & mechanisms. 2010; 3:689–692. [PubMed: 20876356]

Truong L, Reif DM, St Mary L, Geier MC, Truong HD, Tanguay RL. Multidimensional in vivo hazard assessment using zebrafish. Toxicol. Sci. 2014; 137:212–233. [PubMed: 24136191]

Usenko CY, Harper SL, Tanguay RL. In vivo evaluation of carbon fullerene toxicity using embryonic zebrafish. Carbon. 2007; 45:1891–1898. [PubMed: 18670586]

van der Ven LTM, van den Brandhof E-J, Vos JH, Wester PW. Effects of the Estrogen Agonist 17β-Estradiol and Antagonist Tamoxifen in a Partial Life-Cycle Assay with Zebrafish (Danio Rerio). Environ. Toxicol. Chem. 2007; 26:92. [PubMed: 17269465]

van Ham TJ, Mapes J, Kokel D, Peterson RT. Live imaging of apoptotic cells in zebrafish. FASEB J. 2010; 24:4336–4342. [PubMed: 20601526]

Vartak DG, Gemeinhart RA. Matrix metalloproteases: underutilized targets for drug delivery. J Drug Target. 2007; 15:1–20. [PubMed: 17365270]

Highlights

- Tamoxifen rapidly induced a unique necrotic caudal fin phenotype in zebrafish

- Apoptosis co-localized temporally and spatially in the necrotic tail

- The necrotic fin phenotype was p53, GPER and ER independent

- The necrotic fin phenotype was dependent on ectopic MMP induction and activity in skin

- The necrotic fin phenotype occurred at concentrations exceeding anti-estrogenic effects
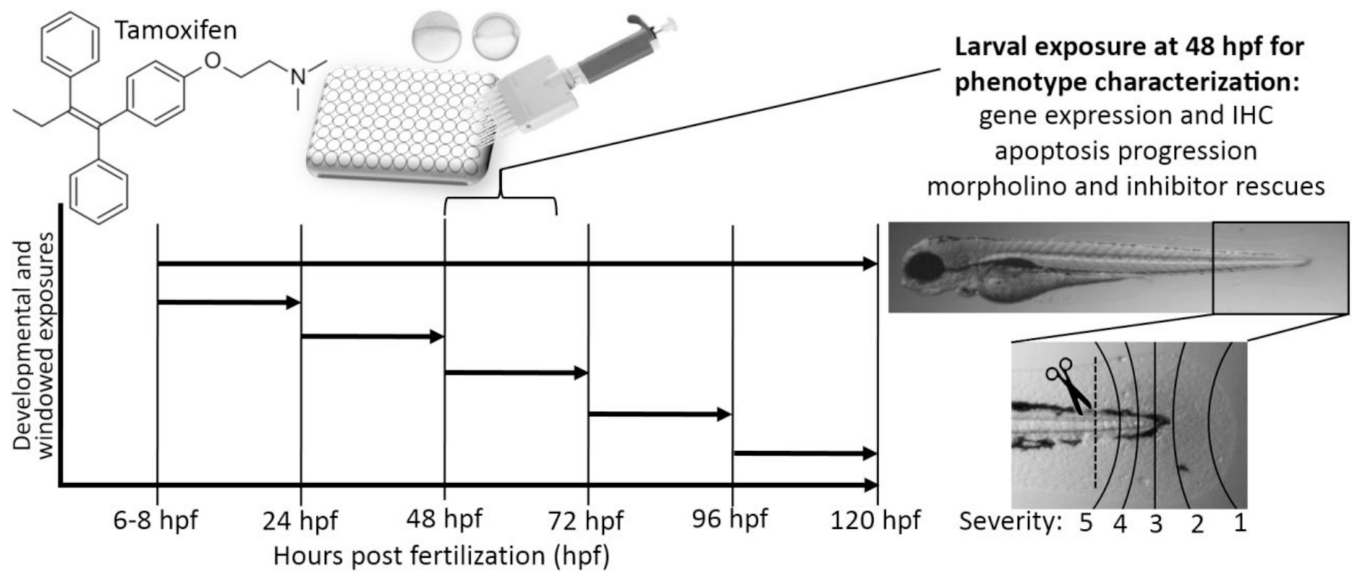
Figure 1.
Schematic of the exposure and study paradigm used to evaluate tamoxifen's effects in the larval zebrafish model. Initially, embryos were developmentally exposed 6–120 hours post fertilization (hpf) and observed daily for toxicity. Embryo and larval animals were then exposed in 24 hour periods to determine the window of sensitivity. For most studies, larval 48 hpf animals were exposed to tamoxifen to characterize the phenotype and elucidate the mode of action using transgenic strains, gene expression, IHC and inhibitor rescue studies. Gene expression was evaluated in whole animal and isolated tails cut at the pigment gap (dashed line). For inhibitor rescue studies, phenotype progression was scored from 1 (least) to 5 (most severe).
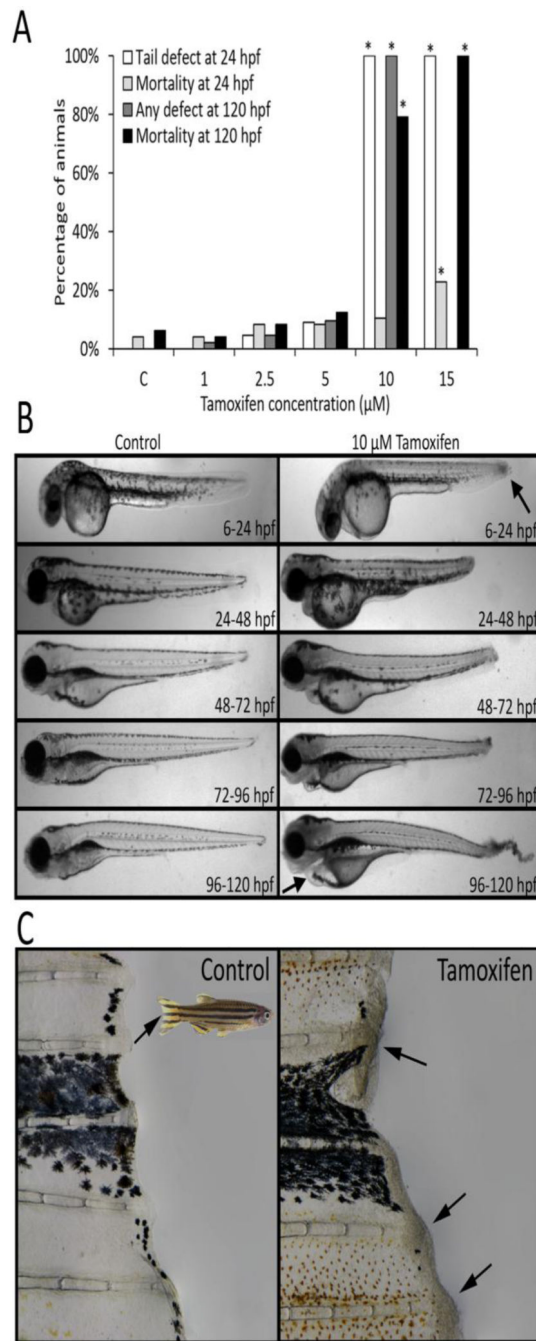
Figure 2.
Exposure to tamoxifen induced a necrotic tail lesion in zebrafish that was not dependent on life-stage or development. (A) Zebrafish developmentally exposed to 10 μM tamoxifen or greater from 6–120 hours post fertilization (hpf) showed the presence of a necrotic tail phenotype at 24 hpf, and high levels of mortality at 120 hpf with continued exposure. *Indicates significantly elevated percentage relative to controls (Fisher's exact test, p ≤ 0.05, N = 48 per treatment). (B) Exposure to 10 μM tamoxifen for 24 hours beginning at any time point throughout embryonic development significantly induced the tail lesion in 100% of

animals (Fisher's exact test, p 0.05, N = 24 per group). (C) Adult zebrafish exposed to 10 μM tamoxifen for 1.5 hours exhibited similar necrotic lesions widespread on the caudal fin (shown), pectoral fins, and skin of the body. All control and treatment groups contained 0.1% DMSO.
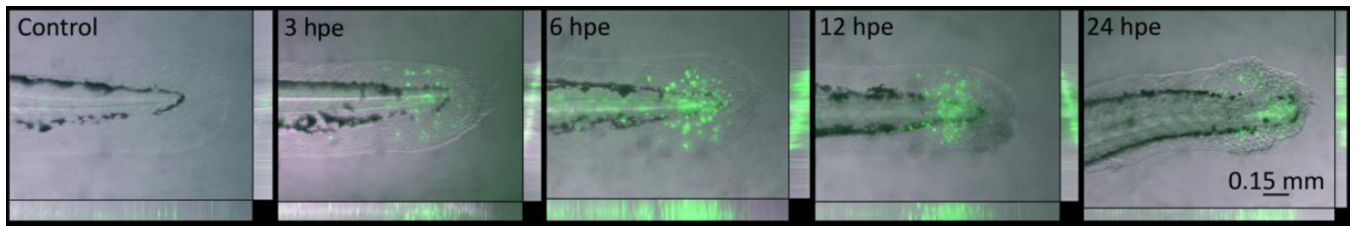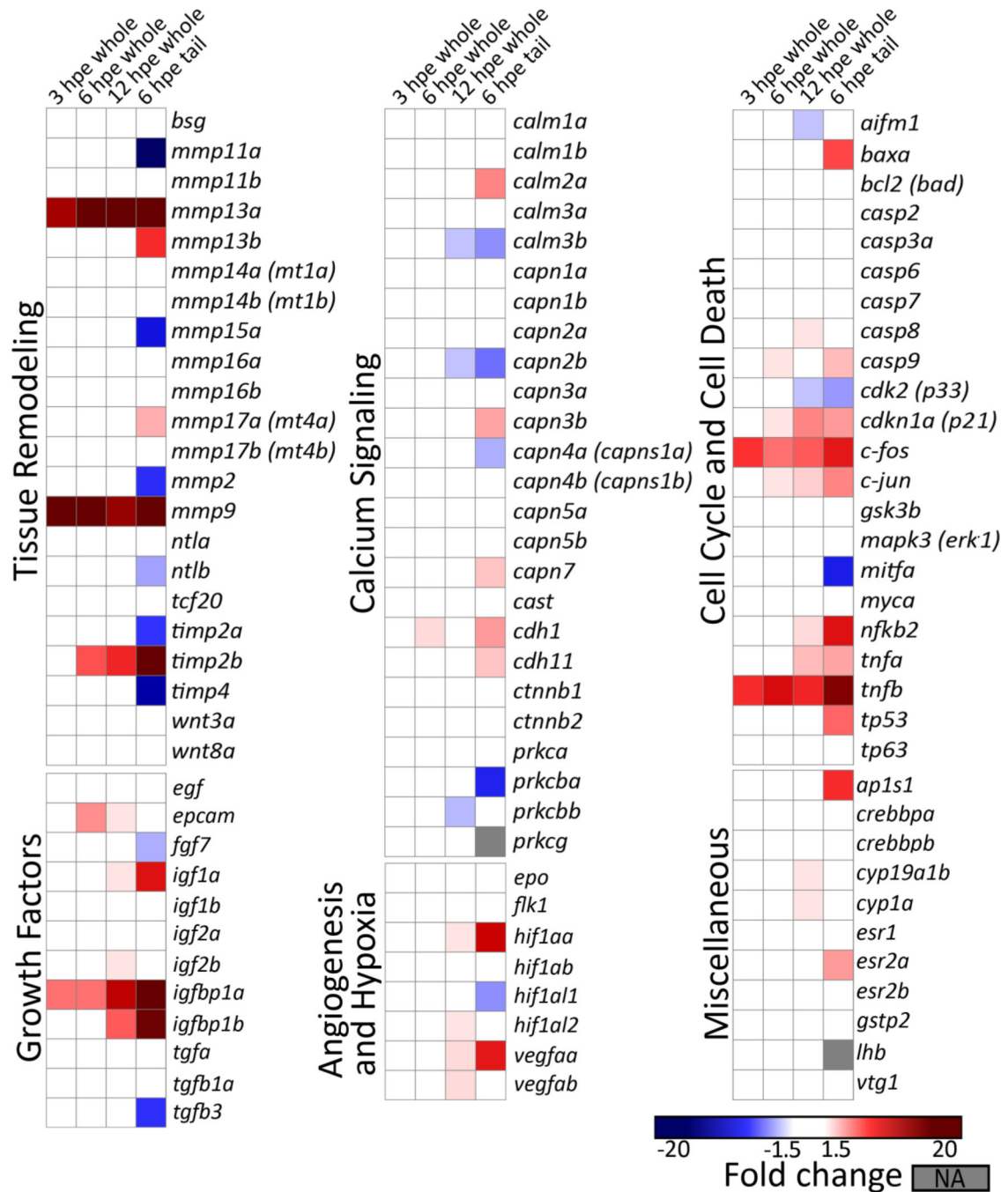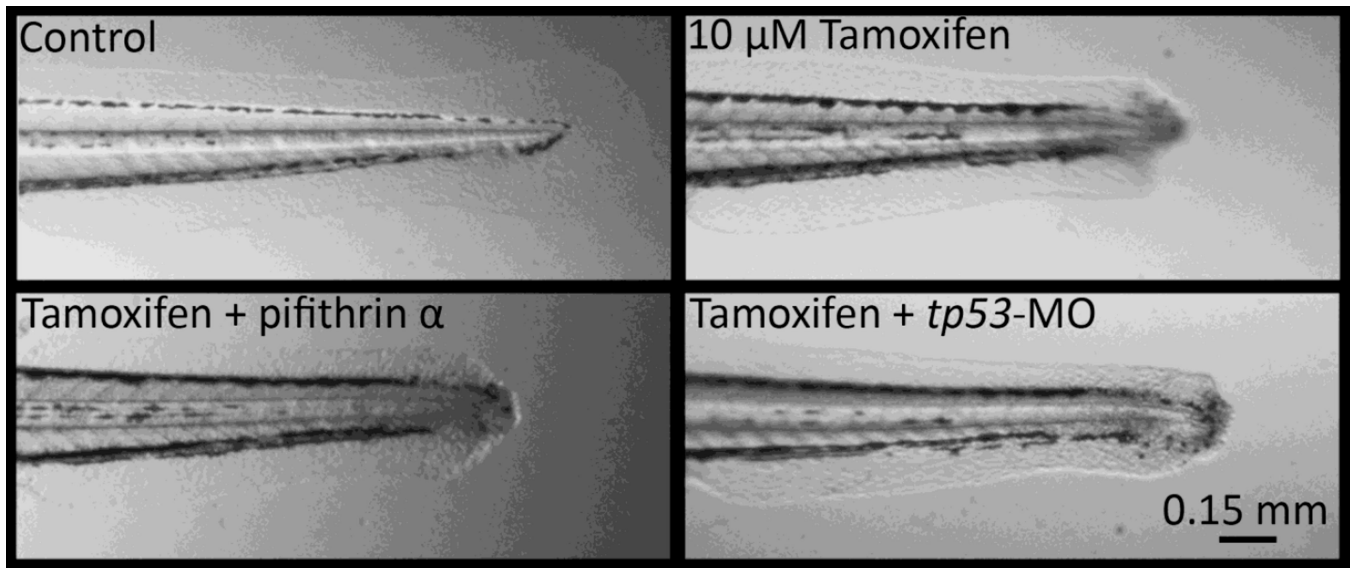
Figure 3.
Tamoxifen rapidly induced the necrotic caudal fin phenotype in 48 hour post fertilization (hpf) larvae exposed to 10 μM tamoxifen, and apoptosis co-localized temporally and spatially with the lesion. The secA5-YFP transgenic strain was used to visualize apoptosis *in vivo*. Representative z-stack photomicrographs at 3, 6, 12 and 24 hours post exposure (hpe) are shown.

Figure 4.

Heat map for targeted mRNA gene expression analysis revealed significant effects on transcripts and pathways involved in tissue remodeling, growth factors, and cell cycle and death. Gene expression was evaluated in larval animals treated with 10 μM tamoxifen at 48 hours post fertilization (hpf). Whole animal expression was evaluated at 3, 6 and 12 hours post exposure (hpe), and tail specific expression only at 6 hpe in isolated tails (cut at pigment gap). Data represents fold changes (treatment to control ratio) calculated using the Ct method. Only significantly altered transcripts with a fold change greater than the

cutoff are shown (Student's t-test or Wilcoxon when normality/variance failed, p   0.05, N = 4 replicates with either 12 whole animals each or 75 tails each). The fold change cutoff for whole animal and isolated tail samples was   1.5 and   2, respectively. Gray shaded cells (NA) indicate expression below detection.
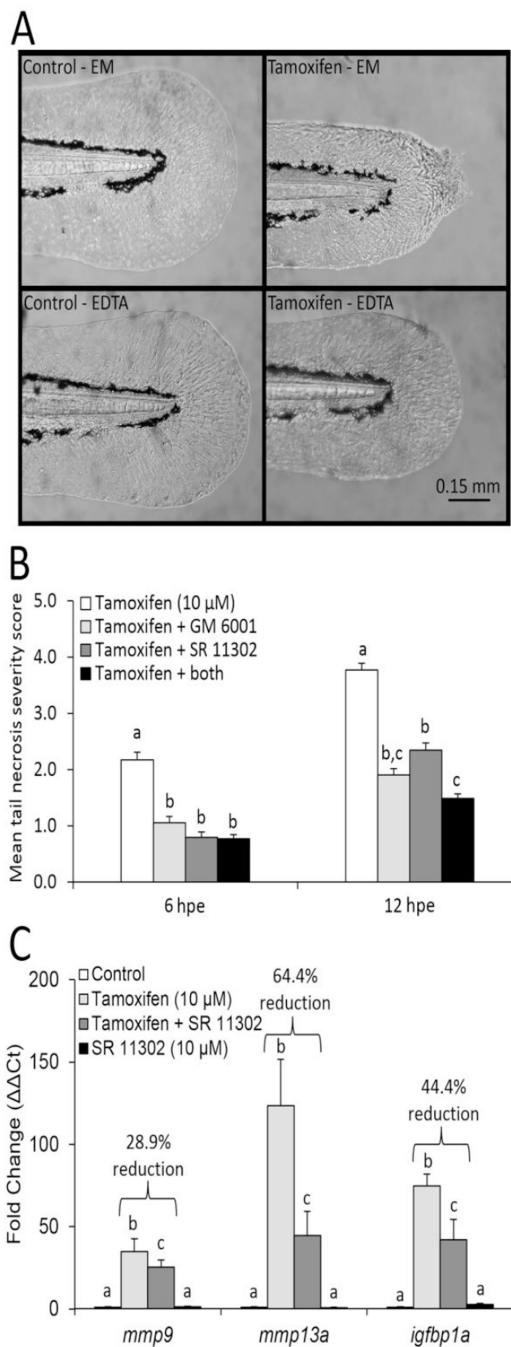
Figure 5.
Suppression of tumor protein 53 with either a specific inhibitor (5 μM pifithrin α) or morpholino gene knockdown (*tp53*-MO) did not prevent nor ameliorate the necrotic caudal fin phenotype induced by 10 μM tamoxifen in 48 hours post fertilization (hpf) larval animals. Photomicrographs are 12 hours post exposure (hpe), and similarly, no amelioration of severity was observed at 3, 6, or 24 hpe. A control morpholino group was included. 100% of animals exposed to tamoxifen exhibited the phenotype with or without inhibitor/ morpholino (N = 24 per group).
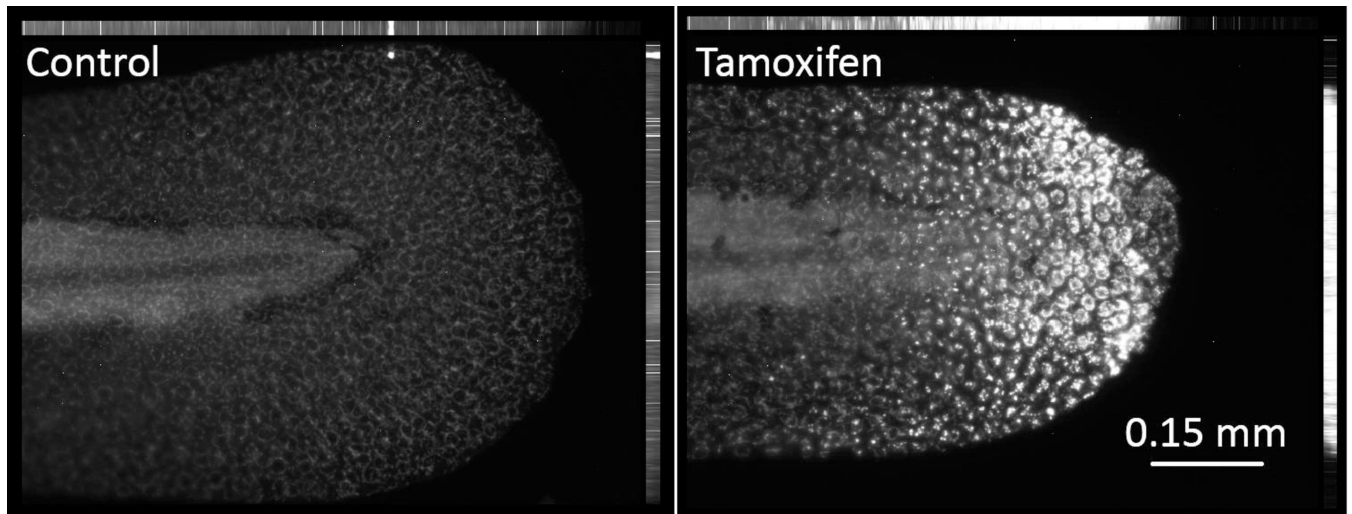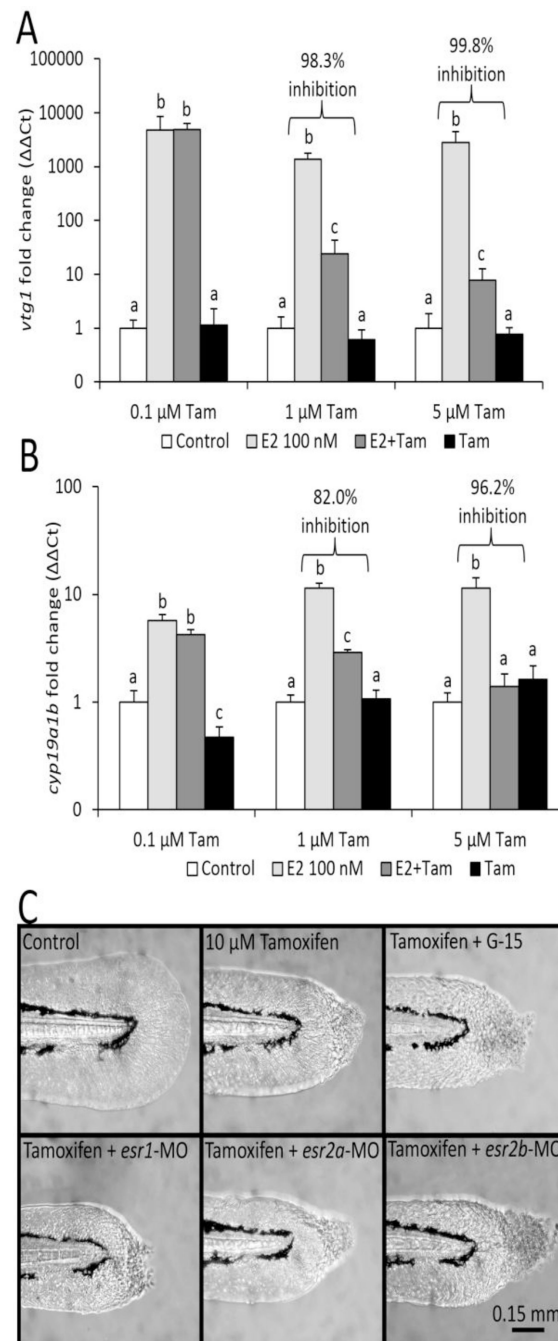
Figure 6.
Whole mount *in situ* immunohistochemistry analysis revealed robust and widespread induction of MMP13a protein expression in skin epithelial ionocytes in the area surrounding the necrotic caudal fin lesion. Photomicrographs are shown for 48 hours post fertilization (hpf) larval animals treated with 10 μM tamoxifen at 6 hours post exposure (hpe). MMP13a expression was localized primarily to skin epithelial ionocytes in controls at low level, and high levels in treated animals. Both proenzyme and cleaved active MMP13a are co-measured.

Figure 7.
MMP inhibitors broadly prevented or ameliorated the severity of the necrotic caudal fin phenotype induced by 10 μM tamoxifen in 48 hour post fertilization (hpf) larval animals. (A) 1 mM EDTA completely blocked the phenotype at 3, 6, 12 and 24 hours post exposure (hpe). Example photomicrographs show 6 hpe. Tamoxifen treatment in EDTA did not appear qualitatively different than control groups in embryo medium (EM) or EDTA, while tamoxifen treatments in embryo medium induced the phenotype in 100% of animals (Fisher's exact test, p ⩽ 0.05, N = 36 animals per group). (B) Co-treatment of 10 μM

tamoxifen with 100 µM GM 6001 or 10 µM SR 11302, reduced the severity of the necrotic caudal fin phenotype. Data are reported as mean ± stdev. Bars not labelled with the same letter are significantly different (ANOVA on ranks, Student-Newman-Keuls, p 0.05, N = 36 per group). (C) SR 11302, an AP-1 transcription factor inhibitor, significantly reduced the robust tail specific induction of *mmp9, mmp13a* and *igfbp1a* by 10 µM tamoxifen at 3 hpe. Fold changes (CT) are relative to the 0.1% DMSO control group. Data are reported as mean ± stdev. Bars not labelled with the same letter are significantly different (ANOVA on ranks, Student-Newman-Keuls, p 0.05, N = 4 replicates with 75 tails each). Percent inhibition was calculated using fold change of the co-treatment group relative to the tamoxifen group.

Figure 8.
Tamoxifen was highly anti-estrogenic at 1 μM or greater, though the necrotic caudal fin lesion was not regulated by estrogen receptors. Developmental exposure to 100 nM 17β-estradiol (E2) from 6–120 hours post fertilization (hpf) robustly induced estrogen responsive genes (A) *vtg1* and (B) *cyp19a1b* at 120 hpf, which were significantly inhibited by co-treatment with 1 and 5 μM, but not 0.1 μM tamoxifen. Gene expression was evaluated in whole animals and fold changes (CT) are relative to the 0.1% DMSO control group. Data are reported as mean ± stdev. Bars not labelled with the same letter were significantly

different (ANOVA on ranks, Student-Newman-Keuls, p   0.05, N = 4 replicates with 12 animals each). Percent inhibition was calculated using the fold inductions for the co-treatment group relative to the E2 group. (C) Co-treatment with G-protein estrogen receptor antagonist G-15 (25 μM) or morpholino gene knockdown of *esr1, esr2a*, or *esr2b* did not prevent nor ameliorate the necrotic caudal fin phenotype induced by 10 μM tamoxifen in 48 hpf larval animals. Photomicrographs are 6 hours post exposure (hpe), and similarly, no amelioration of severity was observed at 3, 12, or 24 hpe. Control groups included morpholino and inhibitor alone. 100% of animals exposed to tamoxifen exhibited the phenotype with or without morpholino or inhibitor (N = 24 per group).