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Antiangiogenic, antimigratory and antiinflammatory effects of 2-methoxyestradiol in zebrafish larvae

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ABSTRACT

2-Methoxyestradiol (2ME), an endogenous metabolite of 17β-estradiol, has been previously reported to possess antiangiogenic and antitumor properties. Herein, we demonstrate that the effects of this antiangiogenic steroid can be readily assayed in live zebrafish, introducing a convenient and robust new model system as a screening tool for both single cell and collective cell migration assays. Using the *in vitro* mammalian endothelial cell line EA.hy926, we first show that cell migration and angiogenesis, as estimated by wound assay and tube formation respectively, are antagonized by 2ME. In zebrafish (*Danio rerio*) larvae, dose-dependent exposure to 2ME diminishes (1) larval angiogenesis, (2) leukocyte recruitment to damaged lateral line neuromasts and (3) retards the lateral line primordium in its migration along the body. Our results indicate that 2ME has an effect on collective cell migration *in vivo* as well as previously reported anti-tumorigenic activity and suggests that the molecular mechanisms governing cell migration in a variety of contexts are conserved between fish and mammals. Moreover, we exemplify the versatility of the zebrafish larvae for testing diverse physiological processes and screening for antiangiogenic and antimigratory drugs *in vivo*.

1. Introduction

Nearly two decades ago, the estrogen metabolite 2-methoxyestradiol (2ME) was recognized as a potent inhibitor of angiogenesis and thus, of cancer (Fotsis et al., 1994). 2ME was later shown to also restrict tumor growth itself by affecting numerous cellular processes such as microtubule dynamics, up-regulation of p53, down-regulation of c-myc, cell cycle arrest, stimulation of apoptosis and production of reactive oxygen species. Collectively, these studies suggest a pleiotropic mode of action for this molecule (D'Amato et al., 1994; Kato et al., 2008; Zhang et al., 2009; Mueck and Seeger, 2010; Salama et al., 2012; Choi and Zhu, 2012). 2ME also inhibits the hypoxia-inducible factor 1α and progesterone receptor pathways, which have been implicated in tumor metastasis (Mabjeesh et al., 2003; Dubey and Jackson, 2009; Salama et al., 2009; Quezada et al., 2010). Additional reports indicate 2ME has anti-inflammatory properties, possibly through the inhibition of neutrophil recruitment mediated by pro-inflammatory cytokines including TNF-α (Issekutz and Sapru, 2008; Shand et al., 2011; Stubelius et al., 2011). Additional evidence has shown that 2ME inhibits the ability of circulating inflammatory cells to adhere to and infiltrate vascular lesions (Kurokawa et al., 2007).

The vast majority of functional studies assigning biological roles for 2ME have been carried out *in vitro* and a lesser number of studies have relied on *in vivo* systems, mostly tumor models (reviewed in Pribluda et al., 2000; Schumacher and Neuhaus, 2001; Chourasia and Joy, 2008; Shand et al., 2011). While serum concentrations of 2ME in the picomolar range are present in cycling women, these levels could reach nanomolar or possibly low micromolar values by late pregnancy (Berg et al., 1983). At pharmacological doses (1–10 μM), 2ME manifests antitumor and antiangiogenic effects in preclinical cancer models (Mooberry, 2003; Choi and Zhu, 2012). Currently, 2ME is being analyzed clinically to treat a variety of cancers and other pathologies (Matei et al., 2009; Tevaarwerk et al., 2009; Dubey and Jackson, 2009; Harrison et al., 2011; Guo et al., 2012; Machado-Linde et al., 2012).

Genomic conservation with other vertebrates and the availability of genetic tools (transgenics and mutants) has made the zebrafish an excellent model for understanding development, physiology and disease (Hassel et al., 2012; Konantz et al., 2012). Given the optical transparency of embryos and larvae, most studies have been carried out during the early life stages in this animal. Among other applications,

Abbreviations: GFP, green fluorescent protein; 2ME, 2-methoxyestradiol; ISVs, intersegmental vessels; DLAV, dorsal anastomotic vessels; PAV, parachordal anastomotic vessels.

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the zebrafish has recently been presented as a powerful model system in which to monitor angiogenesis in vivo (Seng et al., 2004; Staton et al., 2009; Letamendia et al., 2012). In the zebrafish, the formation of blood vessels begins during the first day after fertilization and, by the second day, the heart commences beating and a simple vascular system carries the circulation. Parts of the early vasculature, such as the intersegmental vessels (ISVs), are formed by the process of neoangiogenesis (Blum et al., 2008). Visualization of this vasculature has been achieved by either microangiography, *in situ* hybridization, antibody stains or by using transgenic fish lines that express fluorescent proteins in endothelial cells (reviewed in Mckinney and Weinstein, 2008). This latter method has the advantage that intervention is not required to follow development of the vascular system in live fish. Furthermore, small molecules or any water-soluble compound can be added to the incubation medium and the development of the blood vessels monitored over time. In this fashion, several compounds have been shown to be anti-angiogenic (Isenberg et al., 2007; Tran et al., 2007; Kalén et al., 2009; Wang et al., 2010). As hundreds or thousands of zebrafish embryos can be obtained daily, high-throughput screens, which incorporate automated procedures, have been developed and may deliver ample opportunities for drug and compound analysis (Kalén et al., 2009; Tong et al., 2009; Vogt et al., 2009).

Cell migration has also been assayed in the zebrafish, although reliable high-throughput methods to screen for this cellular behavior have yet to be developed. During zebrafish embryogenesis numerous cell migration events occur, which may offer the potential for effective monitoring of cell behaviors in diverse contexts thus presenting opportunities for testing candidate compounds on migratory cells. One such tissue is the primordium of the posterior lateral line. This is formed by a cohesive group of about 120 cells that, between the first and second day of life, traverse the entire length of the embryo's body to lay down the mechanosensory lateral line system (Ghysen and Dambly-Chaudière, 2007; Ma and Raible, 2009). We have previously shown that this group of cells is an ideal system in which to score for migration defects after exposure of embryos to small molecules or compounds (Villablanca et al., 2008; Gallardo et al., 2010). A second useful system for cell migration analysis consists of following the behavior of early leukocytes. These can be induced to migrate to the site of a wound as an inflammatory response is quickly mounted in embryos and larvae (Redd et al., 2006; Renshaw et al., 2006; Hall et al., 2007; d'Alençon et al., 2010). As before, both in the case of the migrating posterior lateral line primordium and of early leukocytes, the availability of transgenic lines that specifically label the moving cells against an unlabeled background immensely facilitates scoring for effects and quantification.

Herein, we examine the antiangiogenic and antimigratory effects of the anti-cancer drug 2ME using live zebrafish embryos and we compare these effects with those seen in mammalian cell *in vitro* assays. Our results demonstrate the utility of zebrafish *in vivo* assays for testing the effects of 2ME on diverse physiological processes and for further screening of antiangiogenic and antimigratory drugs.

2. Materials and methods

2.1. Cell culture and reagents

EA.hy926 human endothelial cells (Emeis and Edgell, 1988) were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, NY, USA/Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) (Invitrogen/Sigma Aldrich), 100-U/mL penicillin G, and 100-mg/mL streptomycin sulfate (Invitrogen) at 37 °C with 5% CO_2. 2-methoxyestradiol (2ME; STERALOIDS, Newport, RI, USA) was dissolved as a stock at 1.5×10^{-2} M in ethanol and applied at final concentrations stated in the figure legends.

2.2. In vitro cell migration assay

EA.hy926 cells (1×10^5 cells/well) were seeded in 24-well plates and grown to reach 100% confluence. The cell monolayer was disrupted by scraping the surface with a 200-μL pipette tip and, after washing with PBS, cells were incubated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM F12) medium in the absence of serum (a condition that allows survival and migration of cells, but not proliferation) and were incubated with 2-methoxyestradiol (2ME 0.5 and 1.0 μM) or with vehicle (EtOH) as a control. After 24 h of incubation, migration of EA.hy926 cells was recorded using an inverted phase contrast photomicroscope (Olympus CKX41) with 20× objective. The area of wound sealing was calculated using the NIH ImageJ software. Results are given as the average ± SE and statistical comparisons were performed by Kruskal–Wallis nonparametric ANOVA with Dunn's post test.

2.3. In vitro angiogenic assay

In vitro endothelial tube formation was carried out as previously described (Aranda and Owen, 2009). Matrigel (200 μL, BD Biosciences, CA, USA) was added to each well of a 24-well plate and allowed to polymerize for 1 h at 37 °C. EA.hy926 cells were suspended in culture medium with a pro-angiogenic cocktail (PAC) that allows increase basal endothelial cell reorganization, as used previously for screening anti-angiogenic activity (Aranda and Owen, 2009). One milliliter of medium containing 4×10^4 cells was added to each well coated with matrigel. Cells were incubated for 6 h at 37 °C and photographed with a 20× objective. Ten representative images per well were obtained and analyzed. The angiogenic index was quantified as previously described (Aranda and Owen, 2009). Results are showed as average ± SE and statistical comparisons were done by using a Kruskal–Wallis nonparametric ANOVA with Dunn's post test.

2.4. Zebrafish husbandry and experimental conditions

Zebrafish (*Danio rerio*) embryos and larvae were obtained from our breeding colony and were wild type (AB strain) or of the following transgenic strains: *Tg(fli1a:EGFP)y1* or *fli1:GFP* (Lawson and Weinstein, 2002), *Tg(−8.0cldnb:lynEGFP)zf106* or *cldnB:GFP* (Haas and Gilmour, 2006), *Tg(pou4f3:GAP-GFP)s356t* or *brn3c:GFP* (Xiao et al., 2005) and *Tg(mpx:GFP)i114* or *mpx:GFP* (Renshaw et al., 2006). Embryos were raised in Petri dishes at 28 °C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and 0.1% methylene blue), as described (Haffter et al., 1996). Embryonic and larval ages are expressed in hours post-fertilization (hpf). Incubations were carried out for the required time and embryos were scored for effects on the structures/cell types of interest. Representative photographs show the effects observed in over 90% of the fish in each treatment condition. Images were obtained using an Olympus MD10 fluorescent dissecting stereoscope and recorded with a Leica DLI30 digital camera. Images were adjusted for size and orientation with Photoshop 7.0 (Adobe) in unmodified form and organized with Pages 09 (Apple); statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

2.5. In vivo angiogenesis assay

For examining effects of 2ME on angiogenesis, 4 to 5 *fli1:GFP* transgenic embryos were distributed into single wells (1–2 mL final volume) of 12 well plates. 2ME was added when embryos reached 10 hpf to avoid effects on early development. Control fish were treated in parallel with vehicle (EtOH) at the highest concentration used in 2ME treated fish (as is the case for all experiments involving zebrafish larvae). Each concentration was tested in triplicate and four independent experiments were carried out and averages were calculated. Statistical

comparisons were made by using Kruskal–Wallis nonparametric ANOVA with Dunn's post-test adjustment. For measuring effects of 2ME on angiogenesis, we monitored formation of the ISV vessels in the trunk/tail region of 32 hpf *fli1:GFP* larvae using a fluorescence dissecting scope. Correct formation of an ISV was evaluated depending on whether it spanned the distance between the ventral and dorsal longitudinal vessels; the five ISVs immediately anterior to the end of the yolk extension were scored in this manner and an average of correctly formed vessels was calculated for at least 15 fish per condition in each experiment (see Fig. 2).

2.6. Collective cell migration assay

Larvae of the *cldnB:GFP* transgenic strain were raised for the first 10 h after fertilization as before and 2ME or ethanol was added to the medium at the indicated concentration. For determining the effects of 2ME on collective cell migration, we measured the position of the migrating lateral line primordium at 30 hpf in the *cldnB:GFP* transgenic larvae. The larva was arbitrarily divided into five different regions (1 to 5) and the position of primordia for at least 30 fish per condition was recorded (see Lecaudey et al., 2008; Fig. 3). The number of deposited neuromasts was scored for at least 30 fish per condition and three independent experiments were carried out. Results for both primordium migration and neuromast deposition are shown as averages ± SE and statistical comparisons were performed by Kruskal–Wallis nonparametric ANOVA with Dunn's post test.

2.7. Inflammation/leukocyte migration assay

Finally, for quantification of leukocyte migration, we used the ChIN assay (d'Alençon et al., 2010) using *mpx:GFP* transgenic fish (Renshaw et al., 2006). Briefly, 56 hpf larvae were incubated with the indicated doses of 2ME for 1`h and were then exposed to 10 μM $CuSO_4$ for 40 min to induce damage to superficial sensory organs and inflammation of these sites with neutrophils. The number of neutrophils infiltrating the area where the sensory organs are located provides a measure of the degree of inflammation produced. Neutrophils are counted in at least 15 larvae per condition and three independent experiments were carried out. Results are shown as average ± SE and statistical comparisons were done by using a Kruskal–Wallis nonparametric ANOVA with Dunn's post test.

3. Results and discussion

3.1. 2ME inhibits endothelial cell migration in response to a scratch wound

Angiogenesis is a complex cellular process that is mediated through changes in cell adhesion, cell migration and endothelial cell realignment to form tubules. We therefore initially sought to determine if 2ME could inhibit migratory behavior in cultured endothelial cells. We employed a well-established wound-healing assay with EA.hy926 cells (Edgell et al., 1983, 1990), which were cultured in DMEM F12 in absence of serum. A wound was introduced by creating a scratch in a confluent monolayer of EA.hy926 cells and, after 24 h of incubation, the extent to which the wound was sealed was photographed and quantified. Control EA.hy926 cells were able to achieve an average of 68% sealing of the wound (Fig. 1a). In contrast, EA.hy926 cells incubated with 0.5 and 1 μM 2ME beginning immediately after the scratch wound showed approximately 6% and 16% sealing, respectively, suggesting that 2ME inhibits wound-induced migration in this cell line (Fig. 1a). We confirmed that the 2ME antimigratory effect was not caused by a decrease on cell viability by MTS (not shown), suggesting that 2ME specifically inhibits migration in this human endothelial cell line. Microtubule disruption altered levels of ROS through inhibition of SOD and down-regulation

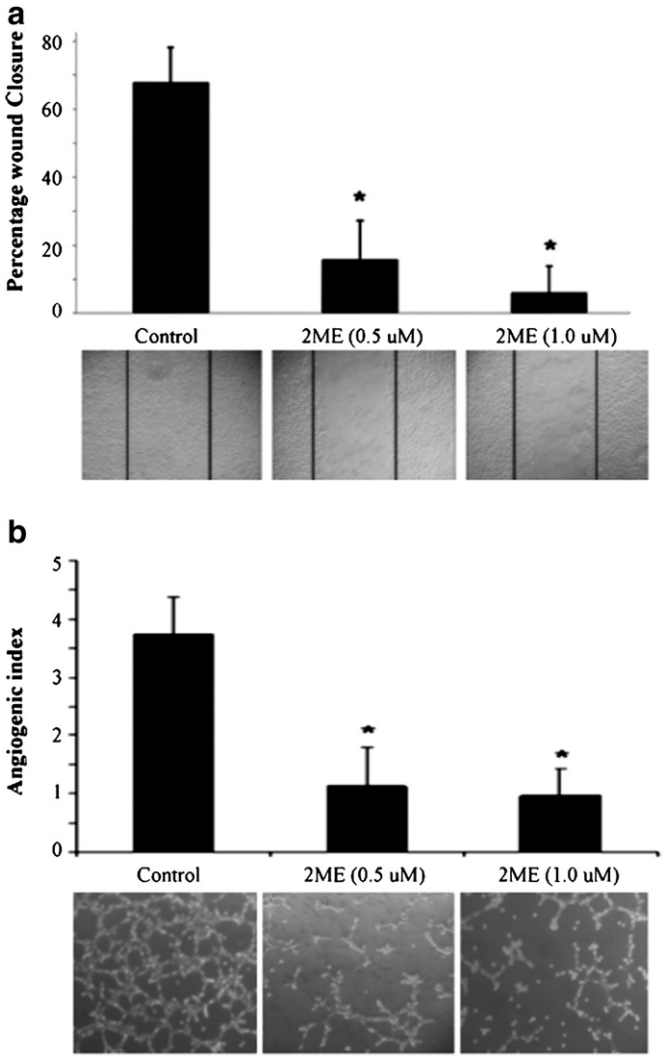

Fig. 1. 2ME inhibits endothelial cell line migration and angiogenesis in vitro. (a) EA.hy926 wound healing assay. EA.hy926 cells were grown to confluence in a 24-well plate and incubated in IMDM medium 5% serum overnight. EA.hy926 cells monolayers were scratched with a pipette tip and cultured with IMDM medium in absence of serum to stimulate migration. Cells were treated with IMDM in absence serum alone (Control), 0.5 μM 2ME, or 1.0 μM 2ME. Cell migration was photographed by phase contrast microscopy over an 8 h time course after wound scratch. Representative images of wound sealing at 24 h after wound scratch are shown in the lower panel. The level of cell migration into the wound scratch was quantified as the percentage of wound sealing at 24 h using NIH ImageJ software. $^*p<0.05$ Kruskal–Wallis post test Dunn. Values represent averages SE of four independent measurements along the wound scratch. Note the percentages of wound sealing under the different conditions at 24 h after the wound scratch were reproducibly observed in at least five independent experiments. (b) Tube forming *in vitro* assay. EA.hy926 cells were suspended in culture medium containing a pro-angiogenic cocktail (PAC) with or without 2ME (0.5 μM and 1.0 μM) and 4×10^4 cells were added to individual wells (12 well plates) coated with matrigel. Cells were incubated at 37 °C and photographed using inverted phase contrast photomicroscope, with a 20× objective. Ten representative images per well are taken and transferred to the computer for image analysis. The angiogenic index was quantified as we described previously (Aranda and Owen, 2009). $^*p<0.05$ Kruskal–Wallis post test Dunn. Values represent averages SE of four independent measurements. Representative images are shown in the lower panel. As we previously observed, 2ME inhibits tube formation compared to control conditions (EA.hy926 incubated with PAC).

of phosphorylated-ERK1/2 (MAPK) and phosphorylated-Akt are effects of 2ME that potentially contribute to the reduced migration (Kamath et al., 2006; Kato et al., 2008; Barchiesi et al., 2010; Quezada et al., 2010). 2ME is known to inhibit endothelial cell proliferation (Yue et al., 1997), however herein we demonstrate that the endothelial cell migratory ability is impaired. This observation is in accordance with previous reports that 2ME can inhibit smooth

muscle cell migration in both a wound healing and Boyden chamber assay (Yin et al., 2010) and with the antimigratory effects reported in cultured human and rat aortic vascular smooth muscle cells (Dubey and Jackson, 2009). In addition, c-myc and HIF1 alpha are inhibited by 2ME, two proteins involved with the angiogenic process (Mabjeesh et al., 2003; Seng et al., 2004; Chow et al., 2008; Zhang et al., 2009). Furthermore, in the modified murine pre-B-cell line Ba/F3, 2ME altered motility, adhesion and migration was coupled with a reduction in the number of filopodia and lamellipodia (Sattler et al., 2003). As migration is essential for invasion, this observation may suggest another mechanism as to how 2ME reduces cancer cell invasiveness (Garcia et al., 2006; Seeger et al., 2006; Quezada et al., 2010; Hung et al., 2012).

3.2. 2ME inhibits endothelial tube formation in vitro

To evaluate whether inhibition of EAhy.926 migration by 2ME also leads to inhibition of angiogenesis, we used a previously reported *in vitro* assay (Bauer et al., 1992; Aranda and Owen, 2009). As we and many other groups have previously described, EA.hy926 cells form capillary-like structures in matrigel in the presence of a pro-angiogenic cocktail (PAC) and this remodeling of cell shapes and organization can be quantified. EA.hy926 cells were suspended in culture medium with a pro-angiogenic cocktail (PAC) with or without 2ME (0.5 μM and 1.0 μM). 4×10^4 cells were then added to matrigel-coated wells. Cells were incubated for 6 h at 37 °C and cultures were photographed for quantification of the angiogeneic index. As we previously observed (Aranda and Owen, 2009), albeit at different assay times and concentrations, 2ME abrogated the formation of endothelial tubular structures (Fig. 1b). This inhibition observed in EA.hy926 tube formation suggests that 2ME is blocking angiogenesis at an early stage, as migration is fundamental to endothelial sprouting and the growth and invasion process.

3.3. Inhibition of angiogenesis in zebrafish by 2ME

We wished to validate the properties of 2ME detected in cultured mammalian cells using an *in vivo* system. The transparent zebrafish embryo offers a powerful live whole-animal model for analysis of antiangiogenic activity (Kalén et al., 2009; Staton et al., 2009). These assays are made more straightforward with the use of *fli1:GFP* transgenic fish, which express GFP in endothelial cells thus labeling all of the vasculature (Lawson and Weinstein, 2002). During zebrafish development, vessels begin to form one day after fertilization and circulation commences during the second day of life. The embryonic vascular system grows both by vasculogenesis and angiogenesis. Specifically, intersegmental, parachordal and dorsal anastomotic vessels (ISV, PAV and DLAV, respectively) grow by sprouting angiogenesis (Blum et al., 2008). Furthermore, zebrafish embryos and larvae have been used extensively for testing of drugs and bioactive compounds by incubation of the fish in water containing the dissolved molecule of interest (Wang et al., 2010). As 2ME is a lipid-soluble compound dissolved in EtOH, we did not add DMSO as a solubilizing agent. 10 h post fertilization (hpf) fish embryos were placed in multi-well culture dishes and 2ME was added to the medium at different dilutions. Embryos were observed and photographed at 24 and 48 hpf under fluorescent illumination to evaluate the growth of blood vessels and angiogenesis in particular. While control larvae at this stage show complete formation of ISVs, their development was strongly inhibited by incubation of fish with 2ME and the strength of the effect was proportional to the concentration of 2ME used (Fig. 2a–h). In order to quantify the effect, we considered the ISVs that normally span from dorsal to ventral in the five somites anterior to the anus (brackets in Fig. 2) and scored them for completeness at 48 hpf; ISVs that did not reach the DLAV were scored as incomplete (Fig. 2f, black arrowhead). From our quantitative analysis (Fig. 2i) we established that no statistically significant effect was elicited by 0.5 μM or 1.0 μM 2ME (the dotted lines in Fig. 2i indicate the 95% confidence interval). However, treatment with 2.0 μM 2ME caused significant defects in angiogenesis while 4.0 μM 2ME completely abolished vessel growth. Beginning at 5.0 μM 2ME and higher concentrations, developmental defects were evident in the larvae, indicating toxicity of the compound at these concentrations (Fig. 2h and not shown). We conclude that, as *in vitro*, angiogenesis *in vivo* is strongly inhibited by 2ME. Although we cannot compare these results to those obtained using *in vitro* mammalian systems, where the steroid is added directly to the cell culture, it is interesting to note that the majority of the effects observed by 2ME occur in the low micromolar range. Although in human, micromolar concentrations of 2ME may be theoretically reached in the ovary during pregnancy, the generally accepted concentration of 2ME in circulation is in the femtomolar to nanomolar range (Berg et al., 1983). Previous studies have reported that in protein inhibition and microtubule binding, the effective doses are in the 0.5–2.0 μM range (reviewed in Dubey and Jackson, 2009; Quezada et al., 2010). As the initial endogenous concentrations of this metabolite are unknown, as are the final concentrations reaching the interior of zebrafish embryos in our experiments, we cannot conclude if the effects observed herein are due pharmaceutical concentrations of 2ME nor if they reflect a potential physiological action of this metabolite.

3.4. 2ME inhibits collective cell migration

As we and others have shown that 2ME exposure reduces the migratory potential of mammalian cells in culture (Sattler et al., 2003), we used two zebrafish assays for determining whether we could replicate this effect *in vivo*, both in the context of collective and of single cell migration. The migrating primordium of the posterior lateral line (PLL) consists of a coherent group of cells that traverses the body from the head to the tail of the developing fish embryo in order to lay down the mechanosensory organs of the lateral line system (Ghysen and Dambly-Chaudière, 2007). This developmental feature has been proposed as a convenient assay for cell migration, since the molecules governing directionality and movement of these cells are conserved with other cell migration events (Villablanca et al., 2008; Friedl and Gilmour, 2009; Gallardo et al., 2010). Transgenic *cldnB:GFP* fish, in which migrating primordium PLL cells are labeled with GFP, were incubated in 2ME as before and were examined at 30 hpf (Fig. 3). We divided the embryo's longitudinal axis into five sections (Fig. 3a) and scored for the position of the primordium in control and experimental animals, as reported previously (Lecaudey et al., 2008). At 30 hpf in control fish, the primordium has migrated down the length of the body and has reached a position past the yolk extension (Fig. 3b). In a manner directly proportional to the concentration of 2ME used, the primordium was retarded in its migration along the body, indicating that this metabolite has an effect on collective cell migration *in vivo* (Fig. 3c–i). Quantification of the position of the PLL primordium in treated fish showed significant effects at concentrations of 2.0 μM 2ME and higher (Fig. 3j). Again, strong developmental effects were detected in larvae that were exposed to 4.0 μM 2ME or higher. As another measure of the effects of 2ME on PLL formation, we also counted the number of neuromasts (clusters of sensory organs deposited by the primordium along its trajectory) formed in each treatment. Again, concentration dependent effects were observed, though significant effects using this criterion were observed starting at 4.0 μM 2ME. These results suggest that at concentrations that are effective *in vitro*, 2ME is also a strong inhibitor of collective cell migration *in vivo*.

3.5. Antiinflammatory effects of 2ME in zebrafish

A useful assay for evaluating individual cell migration in zebrafish larvae takes advantage of the potent innate immune response induced

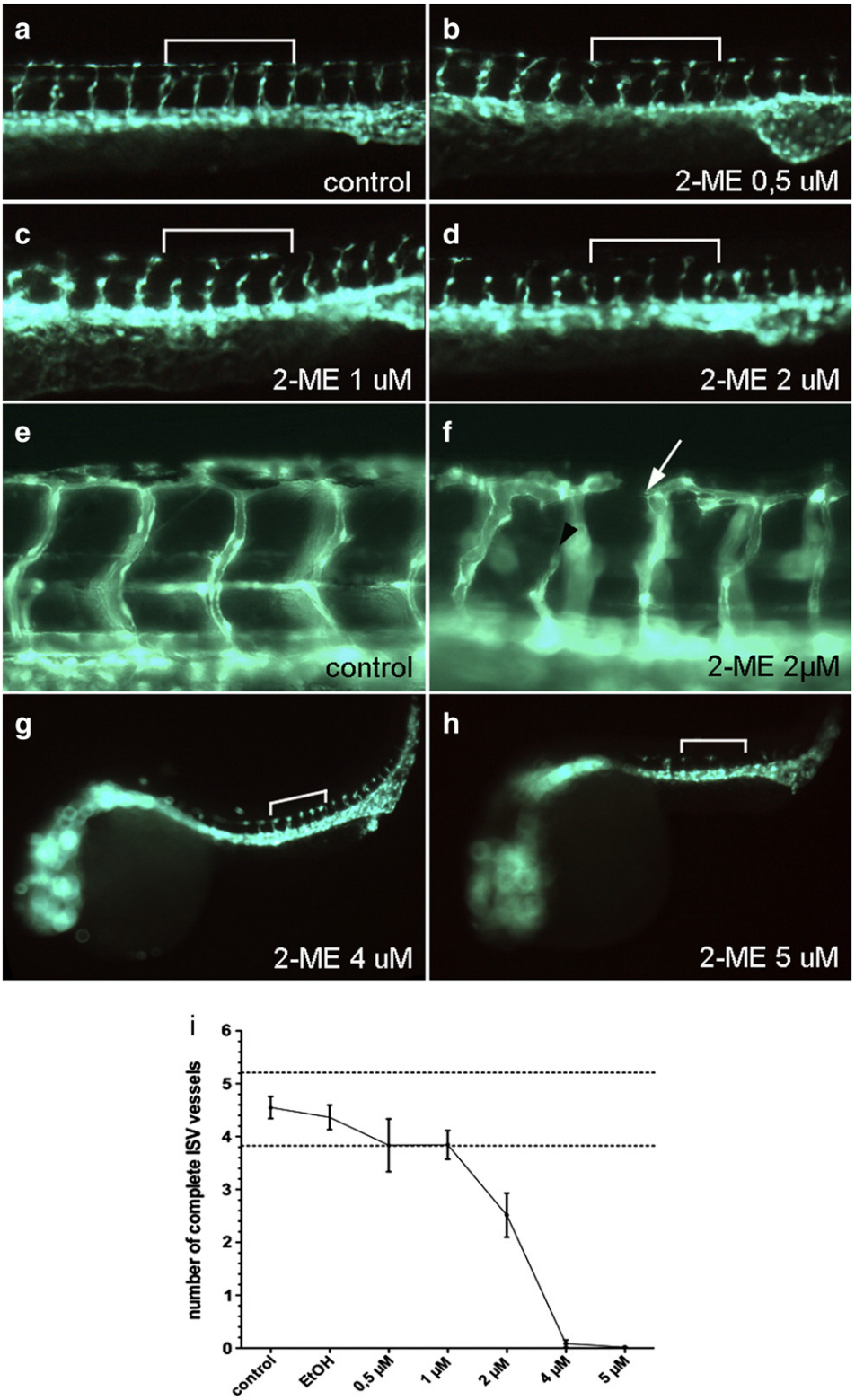

Fig. 2. Antiangiogenic effects of 2ME. Zebrafish embryos of the *fli1:GFP* transgenic line were incubated with different concentrations of 2ME and formation of the intersegmental vessels (ISVs) was evaluated. The five ISVs immediately anterior to the anus (a-d, brackets) were considered. While control larvae show normal development of ISVs (a), 2ME exposed larvae display inhibition of ISV formation in a concentration dependent manner (b-h). Quantification of those ISVs that reached the DLAV (i) shows significant inhibition of angiogenesis at 2.0 μM 2ME and higher ($p > 0.001$). 2.0 μM 2ME exposure inhibited ISV development (f, black arrowhead) and caused DLAV defects (f, white arrow). Data are presented as average ± SE from at least 30 larvae per condition and three independent experiments were carried out. Comparisons were performed by using a Kruskal–Wallis non-parametric ANOVA with Dunn's post test. Dashed lines represent 95% confidence interval for number of complete ISV vessels (i).

after damage to lateral line neuromasts (d'Alençon et al., 2010). In this assay, larvae are incubated in 10 μM $CuSO_4$ for 30 min to 2 h and infiltration of leukocytes (neutrophils and macrophages) to the horizontal myoseptum, where neuromasts are located, is monitored. Neutrophils are specifically identified by GFP expression in *mpx:GFP* transgenic fish and the extent of infiltration is quantified by counting the number of

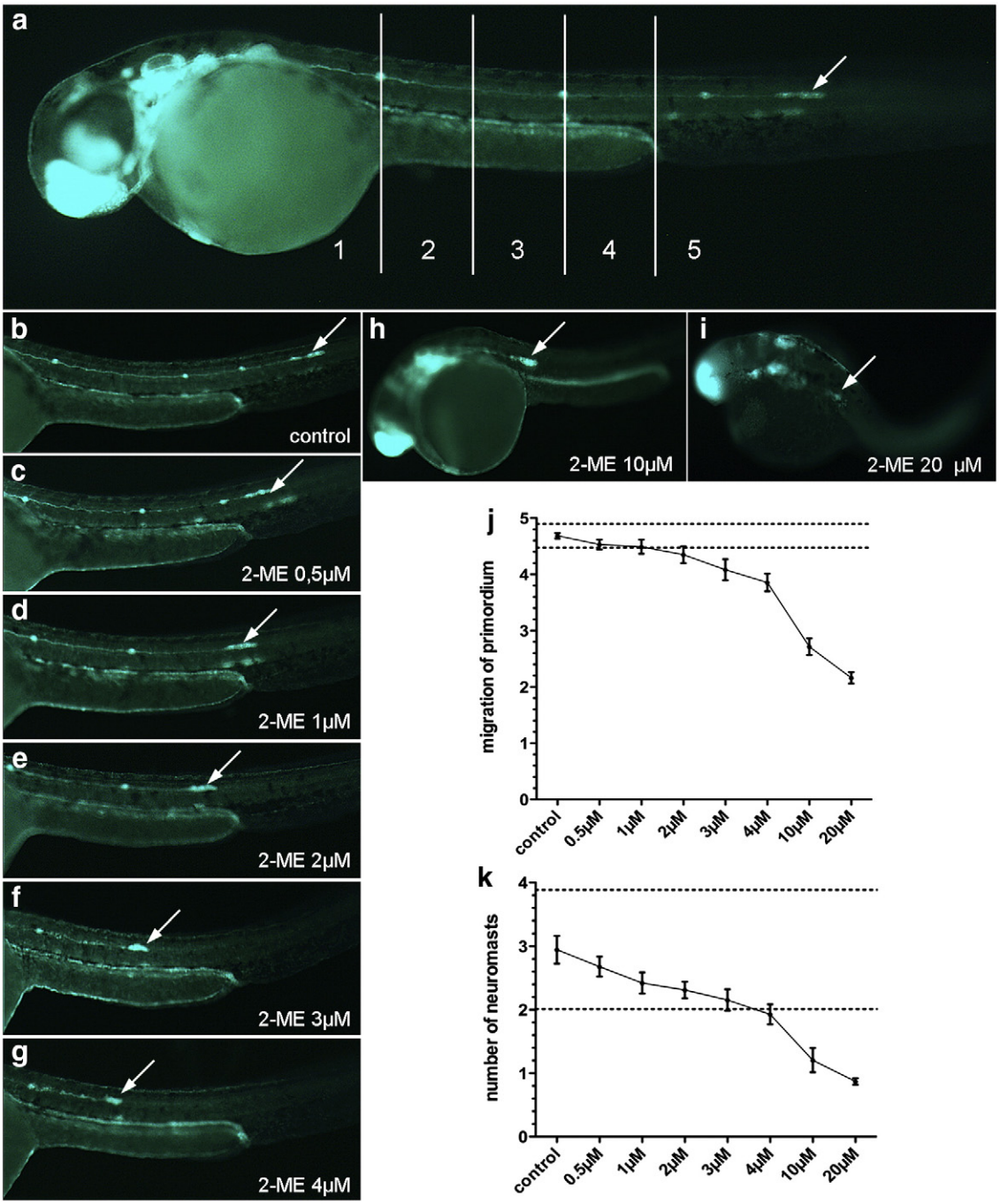

Fig. 3. 2ME inhibits collective cell migration *in vivo*. Larvae of the *cldnB:GFP* transgenic strain were incubated with 2ME or ethanol from 10 hpf and the position of the primordium was scored at 30 hpf by dividing the larvae in five sections (a). While control larva show normal primordium migration (b, arrow), 2ME treated larvae show primordium migration inhibition in a manner directly proportional to the concentration of 2ME used (c–i, arrows). Quantification of primordium position (j) shows significant inhibition of primordium migration in larvae that were exposed to 2 μM 2ME or higher ($p<0.001$). Furthermore, neuromast deposition was significantly decreased upon 2ME exposure. Quantification of deposited neuromasts (k) shows significantly less deposited neuromasts in larvae exposed to 4 μM 2ME or higher ($p<0.001$). Data are presented as average ± SE from at least 30 larvae per condition and three independent experiments were carried out. Comparisons were performed by using a Kruskal–Wallis nonparametric ANOVA with Dunn's post test. Dashed lines represent 95% confidence interval for migration of primordium (j) and number of deposited neuromasts (k), respectively.

labeled cells detected in this area 2 h after beginning the treatment (Fig. 4). In this case, we used 56 hpf larvae, which were incubated with 2ME for 1 h followed by treatment with copper sulphate for 40 min (without removing 2ME). In fish treated only with copper, neutrophils that are normally localized to the caudal hematopoietic tissue in the ventral trunk and tail (Fig. 4b), migrate to the horizontal midline and form clusters near lateral line neuromasts (Fig. 4a, arrowheads). In contrast, fish pre-exposed to 2ME show diminished neutrophil recruitment to damaged lateral line neuromasts (Fig. 4c–f). To exclude the possibility that 2ME might be protecting lateral line hair cells from copper-induced damage, we treated *brn3c:GFP* transgenic fish, in which hair cells are labeled with GFP (Xiao et al., 2005) with 2ME and subsequently with $CuSO_4$ in identical fashion as in the ChIN assay. We observed that under all concentrations of 2ME tested, there were no significant differences in hair cell viability between larvae treated with copper alone or with copper and 2ME, indicating there is no protection from damage to hair cells offered by 2ME (Supplementary Fig. 1). Furthermore, none of the concentrations of 2ME tested in this

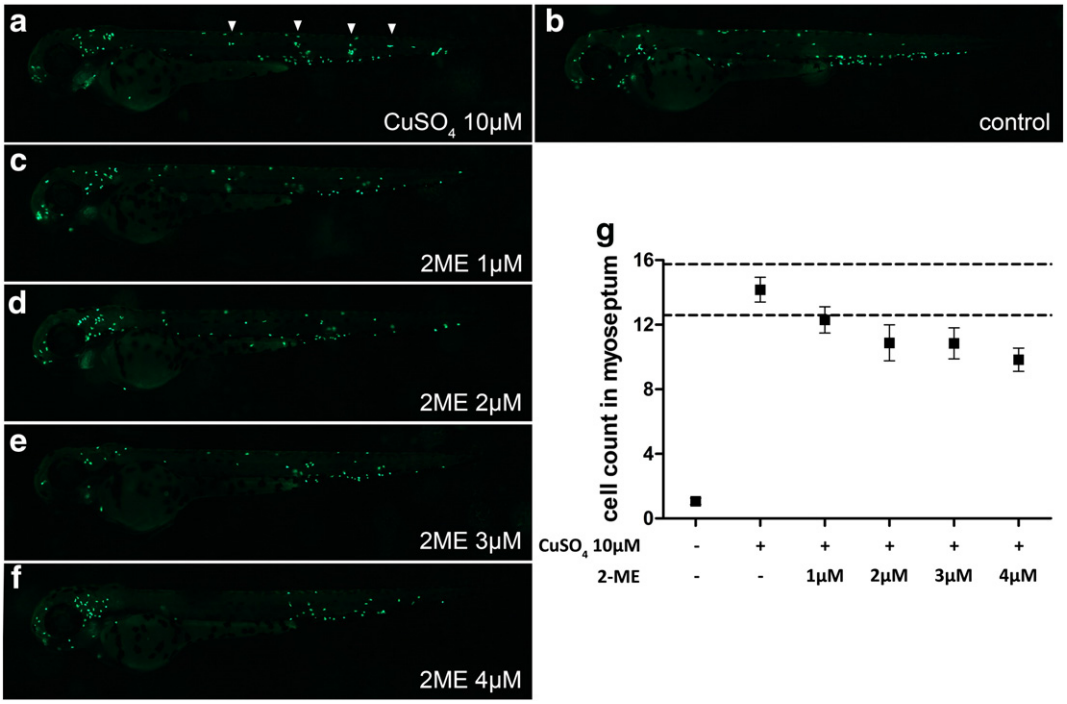

Fig. 4. 2ME inhibits leukocyte recruitment to damaged sensory organs. At 56 hpf *mpx:GFP* transgenic fish were incubated with 2ME for 1 h (with exception of control (b), incubated with ethanol), inflammation was induced by exposure to 10 μM $CuSO_4$ for 40 minutes, and GFP expressing leukocytes near damaged lateral line neuromasts were scored. In control larvae (b), leukocytes are mostly localized in ventral trunk and tail (the caudal hematopoietic tissue), while copper treated larvae display clusters of leukocytes recruited to the horizontal midline of the trunk and the tail (a, arrowheads). Larvae exposed to increasing concentrations of 2ME (c–f) exhibit a decreasing number of leukocytes along the posterior lateral line. Leukocyte quantification (g) shows significant reduction ($p<0.05$) in neutrophil recruitment to damaged lateral line neuromasts in larvae exposed to 2 μM 2ME and higher concentrations compared to controls exposed only to copper. Data are presented as average ± SE from 3 independent experiments in which at least 10 larvae per condition were analyzed. Comparisons were performed by using a Kruskal–Wallis nonparametric test. Dashed lines represent 95% confidence interval for recruited leukocyte average in copper treated larvae.

assay (with 1–2 h exposures) elicited noticeable toxic effects. Quantification of the number of labeled neutrophils localized in the horizontal midline of the trunk and tail (Fig. 4g) showed a significant reduction ($p<0.05$) in recruitment at 2.0 μM 2ME and higher. As with collective cell migration, individual cell migration is progressively inhibited with 2ME in a concentration dependent manner, reaffirming the general effect of this molecule on cell movements in diverse contexts. The widely reported differing modes of action of 2ME, even under the short exposure period used here, indicates a high sensitivity of this assay in comparison with the angiogenesis and cell migration assays.

4. Conclusions

We have demonstrated that the antiangiogenic and antimigratory effects of 2ME can be readily assayed in live zebrafish, introducing a new model system for testing steroid hormone based drugs *in vivo*. Moreover, we exemplify the versatility of the fish larvae as a convenient and robust screening tool for both single cell and collective cell migration assays.

Importantly, the observed effects are specific as no other noticeable developmental defects are associated with drug exposure if concentrations of less than 5.0 μM are used in long exposure times (20 h or more) whereas, in short exposures, concentrations of up to 20 μM were non-toxic. We were able to detect a specific effect of 2ME on blood vessels that form by angiogenesis, supporting the *in vitro* results obtained with mammalian endothelial cells that show that this drug is antiangiogenic. We further demonstrate that in the zebrafish, 2ME exposure in a concentration dependent manner is able to inhibit leukocyte recruitment to damaged lateral line neuromasts and that the lateral line primordium is retarded in its migration along the body, indicating that this metabolite has an effect on collective cell migration *in vivo* as well as the previously reported anti-tumorigenic activity. The effects shown here consolidate 2ME as a good candidate for the treatment of disorders that involve cell migration and inflammation.

Finally, we highlight the utility of the zebrafish larvae as an adequate substitute for cell culture assays that use mammalian cells suggesting that the molecular mechanisms governing cell migration in a variety of contexts are conserved between fish and mammals.

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