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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