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Chemical genomics reveals novel biological activity of 1,2,3-dithiazoles as potent and reversible melanin synthesis inhibitors

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Abstract

A chemical genomic screen of an *in-house* library of small molecule heterocycles was carried out using *Xenopus laevis* embryos. This led to the identification of *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-methoxyaniline (**1c**), which elicits loss of pigmentation in melanophores and the retinal pigment epithelium (RPE) of developing embryos, independent of developmental stage of initial exposure. The phenotype was reversible, since pigmentation returned upon compound removal while analysis of neural crest cell markers (Pax7) and melanophore markers (Dct/Xtrp2) revealed that both neural crest precursors, as well as fully differentiated melanophores were present in the dithiazole **1c** treated embryos. A subsequent focused structure-activity relationship (SAR) study, identified the more active 4-benzyloxy-*N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-aniline (**1l**) and the need for a chlorine substituent at the dithiazole C-4 position. Both the initial chemical genomic screen and the focused SAR highlighted the toxicity of (dithiazolylidene)-

aminoazines, and also of methoxyaniline (anisidine) analogues that hosted strongly electron withdrawing or electronegative substituents or acidic hydroxyl groups on the anisidine moiety. The study suggests that 1,2,3-dithiazoles can act as reversible melanin synthesis inhibitors, revealing a new biological activity for this class of compounds. The inhibition of melanin synthesis is medically relevant as a potential treatment of pigmentation disorders such as melasma.

1 Introduction

Chemical genomics is a rapidly evolving discipline that depends on both chemical synthesis and molecular biology.^{1,2} It entails high-throughput organic synthesis of small, non-peptide based molecules, which are phenotypically screened in model organisms to dissect complex biological pathways and processes.³⁻⁵

The discipline is complementary to classical and conditional genetic approaches, generating loss- or gain-of-function mutations in specific molecular targets, as a means of delineating the sequence of events in complex, multi-component biological processes, such as embryonic development and cell signaling.⁶ The former approach is gaining ground over the latter, as it offers advantages: a) technical simplicity, b) inexpensive reagents, c) low start-up costs, and d) unprecedented spatial and temporal control over molecular targets.⁷ Small molecules can be administered at any time during the cell cycle or at any developmental stage and at any desired location of the cell or organism, making this approach pivotal for studying biological mechanisms and drug discovery.⁷

Various model organisms have been used for phenotypic-based screening of compound libraries, including *Drosophila*, *C. Elegans*, *Zebrafish* and more recently *Xenopus*.⁸⁻¹¹ The latter, display

advantages since they represent the highest order vertebrate model used to date and are also easy to image and dissect, allowing reproducible phenotypic and biochemical readouts. Whole animal genomic screens are fundamental in identifying compounds that affect survival (toxicological studies), perturb a biological process of interest (*i.e.* pigment cell development and function) and aid with drug target discovery and validation.^{4,9,10,12-22}

Compounds that modulate pigment cell development and function include: a) 4-[(4-morpholinobutyl)thio]phenol [also known as (2-morpholinobutyl)-4-thiophenol, MoTP or 33N14] which specifically ablates embryonic melanophores (melanin producing cells),²³ and also affects melanogenesis by blocking neural crest cell (NCC) differentiation and melanophore multiplication,¹² b) phenylthiourea (PTU) that acts as a non-toxic melanin synthesis inhibitor by disrupting tyrosinase activity but not Dct and Tyrp1 function,²⁴ (*cf.* hydroquinone, Kojik acid and triazines),²⁵ c) Melanogenin, Oligomycin, Aurovertin that promote melanin synthesis,^{26,27} and d) β -amino-ethyl disulfides, such as cystamine, that affect pigmentation of human melanocytes and melanoma cells by reducing tyrosinase activity in a reversible manner.²⁸

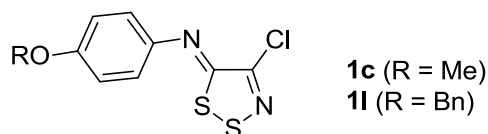
In the developing *Xenopus* embryo there are two main types of melanin producing pigment cells: a) neural crest cell derived melanophores,²⁹ and b) diencephalon derived retinal pigment epithelium (RPE) cells.³⁰ The primary melanophore function is melanin synthesis, which protects the skin from free radicals and UV radiation and also offers a survival advantage as it facilitates camouflage.^{31,32} The melanophores are committed to the melanogenic lineage before migration and this specification process is governed primarily by the transcription factors Mitf, Wnt and BMP, although other signaling pathways involving Kit, Endothelin 3 and the repressor FoxD3 also play a role.^{33,34} At developmental stages 24-30, melanoblasts (melanophore precursors), initiate active migration, in a progressive anterior to posterior wave, under the guidance of

various matrix metalloproteinases (MMPs),³⁵ and P-rax1, a Rac-specific Rho GTPase,³⁶ to form the lateral and dorsal pigment stripes in the embryo.^{37,38} The RPE, which functions as a signal centre for histogenesis, layer formation and maintenance of the overlying photoreceptors,³⁹ is also under the direct control of Mitf.^{40,41}

The delineation of the developmental pathway undertaken by pigment cells and the capability for intervention is medically relevant as numerous diseases can arise in case of a defect, including albinism (a congenital disorder attributed to absence or defect of tyrosinase, the enzyme responsible for melanin synthesis),⁴² piebaldism (an autosomal disorder leading to localized hypopigmentation),⁴³ vitiligo (autoimmune disease leading to destruction of melanocytes),⁴⁴ and melanoma (owed to uncontrolled growth of melanocytes).⁴⁴⁻⁴⁶

Recently, during a toxicological study of an *in-house* library of small molecule heterocycles {150 compds: descriptors [min-max (ave)]: molinspiration predicted LogP (miLogP) [0.415-6.668 (3.68)]; topological polar surface area (TPSA) [12.89-134.72 (55.53)]; number of atoms (natoms) [9-35 (18.84)]; molecular weight (mw) [157.18-459.55 (297.24)]; number of hydrogen bond acceptors (nON) [1-8 (4.03)]; number of hydrogen bond donors (nOHNH) [0-5 (0.44)]; number of violations (nViol) [0-1 (0.19)]; number of rotatable bonds (nrotb) [0-7 (2.21)]} using a chemical genomic screen in *Xenopus*, we serendipitously discovered that *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-methoxyaniline (**1c**) led to severe pigment loss on the developing embryo, which is a new biological activity for 1,2,3-dithiazoles. Early studies on the biological activity of [(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)amino]arenes focused on agrochemical properties such as antibacterial,⁴⁷⁻⁴⁹ antifungal,⁵⁰⁻⁵³ or herbicidal activities.⁵⁴ More recently, interesting antitumor activity,⁵⁵ and inactivation of the glutamine/amino acid transporter ASCT2⁵⁶ have been reported.

The 4-methoxyanilino dithiazole **1c** elicited loss of pigmentation in both melanophores and in the retinal pigment epithelium (RPE). The pigment loss was elicited irrespective of the developmental stage the compound was added and was reversible since pigmentation returned upon compound removal. Analysis of neural crest and melanophore markers revealed that both neural crest precursors, as well as fully differentiated melanophores were present in dithiazole **1c** treated embryos. The data together with the developmental stage independence of the phenotype and its reversibility suggested that compound **1c** inhibited melanin synthesis. We further performed a small structure-activity study (SAR), that identified the more active 4-benzyloxy-*N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)aniline (**1l**).



2 Results and Discussion

2.1 4-Methoxyanilino dithiazole **1c** induces severe pigment loss in melanophores of the dorsal pigment stripe and partial pigment loss in the RPE

A small library of [(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)amino]arenes was evaluated in a chemical genomics screen using *Xenopus laevis* as an *in vivo* model system (see Fig. 1), for their impact on survival and embryonic development, so as to obtain information regarding potential toxicity and biological activities of these compounds.

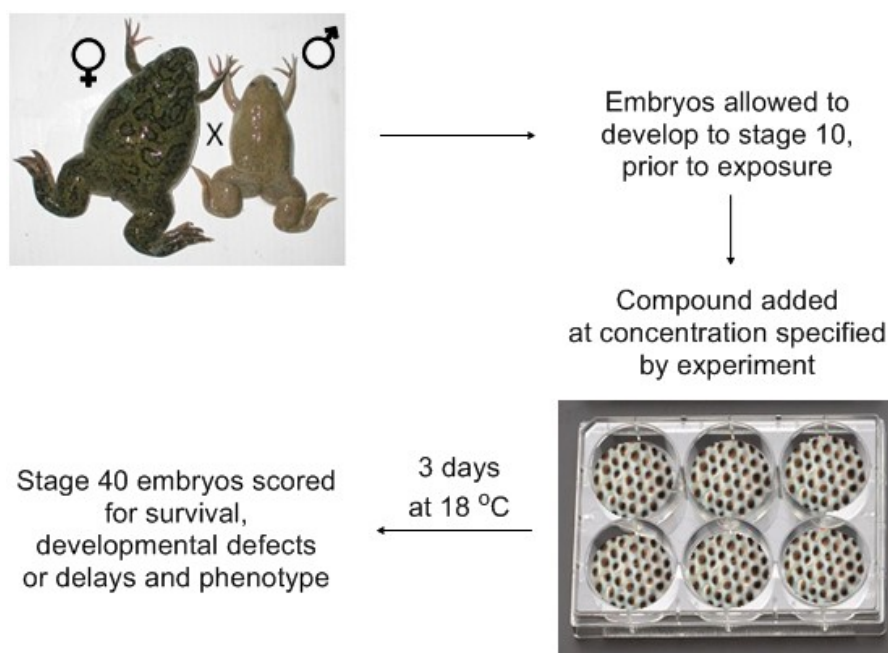


Fig. 1 Schematic representation of chemical genomic screening strategy using *Xenopus laevis* embryos as a model organism.

Stage 10 *Xenopus laevis* embryos, were cultured with increasing concentrations (5 and 10 μM) of the tested compound and assessed for survival and elicitation of a distinctive phenotype. From the compounds tested, the 4-methoxyanilino dithiazole **1c** induced a dramatic phenotype of severe pigment loss in the melanophores of the dorsal pigment stripe and the retinal pigment epithelium (RPE), when applied to embryos at concentrations as low as 10 μM (Fig. 2). The severity of the phenotype was dose dependent; doses $> 10 \mu\text{M}$ of **1c** led to increased toxicity (data not shown).

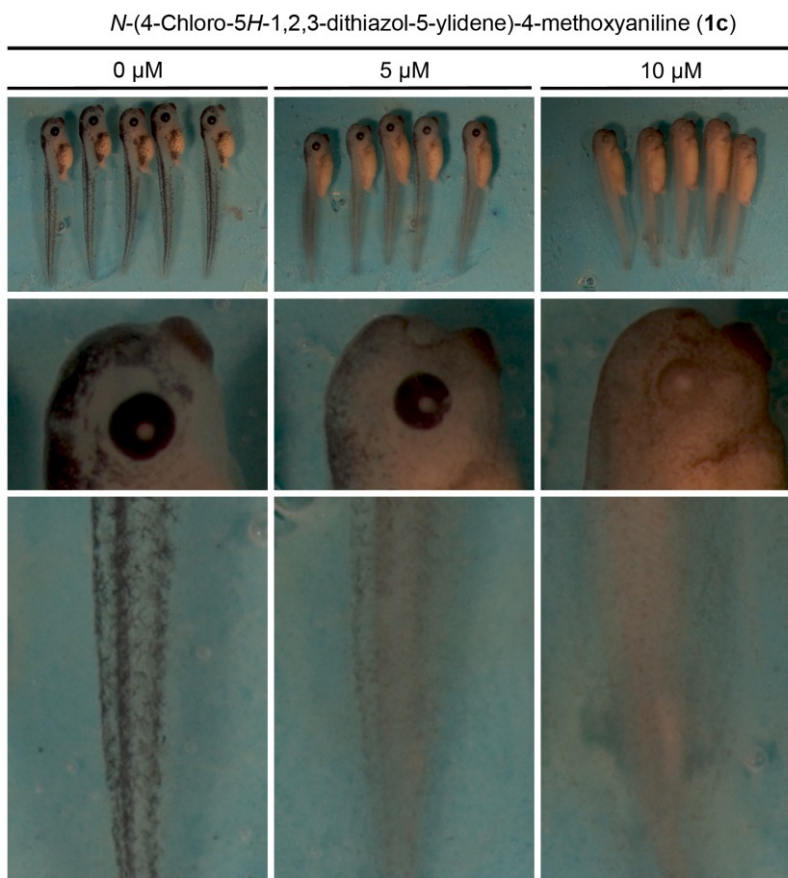


Fig. 2 Pigment loss attributed to the addition of the 4-methoxyanilino dithiazole **1c** (5 and 10 μ M) to stage 10 *Xenopus* embryos that were allowed to develop to stage 40.

2.2 4-Methoxyanilino dithiazole **1c** elicited pigment loss is reversible and can be elicited after melanophore differentiation

Pigment loss can be attributed to several factors including interference with melanophore differentiation, survival and inhibition of melanin synthesis. A small molecule that affects cell fate can lose its activity after melanophore differentiation and/or potentially lead to an irreversible defect, if for example the melanoblasts are pushed towards another fate. Therefore, to identify the underlying cause of the observed pigment loss the dithiazole **1c** (10 μ M) was applied at three different developmental stages 10 (gastrula), 21 (neurula), and 31 (tadpole). It

should be noted that by stage 31 melanoblasts differentiate into melanophores, migrate to the dorsal pigment stripe and begin melanin synthesis.³⁷ Embryos were allowed to develop to stage 40 and visually scored for pigmentation. The 4-methoxyanilino dithiazole **1c** (10 μ M) induced pigment loss in the melanophores of the dorsal pigment stripe and in the RPE, at all developmental stages examined, albeit less efficiently when the embryos were first exposed at stage 31 (Fig. 3). This suggested that the dithiazole acts directly on melanin synthesis rather than on melanophore differentiation.

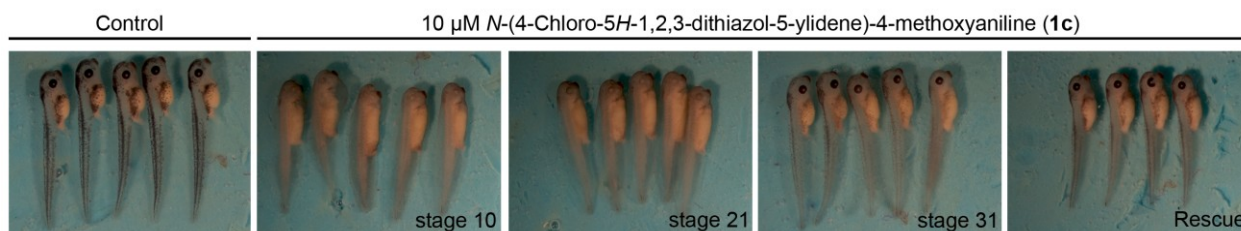


Fig. 3 The effect of 4-methoxyanilino dithiazole **1c** (10 μ M) when applied to *Xenopus* embryos at developmental stages 10, 21 and 31 and allowed to develop to stage 40. The last panel shows an image of rescued stage 21 exposed embryos (14-16 h after removal of compound **1c**).

Furthermore, the effects were reversible, and pigmentation returned in both neural crest derived melanophores and in the RPE, 14-16 h after removal of the compound **1c**, irrespective of the stage the embryos were originally exposed to the dithiazole **1c**. These results further support that compound **1c** acts as reversible enzyme inhibitor of the melanin synthesis pathway rather than interfering with melanophore survival and/or differentiation.

2.3 4-Methoxyanilino dithiazole **1c** does not affect expression of the neural crest marker PAX7 or the melanophore marker Dct (Xtrp2)

The Pax3 and Pax7 paralogs, are evolutionary conserved transcription factors, that have overlapping yet differential expression and roles in amphibian embryos. Both are widely used as neural crest markers⁵⁷ and it has been shown, using whole mount *in situ* hybridization and immunostaining, that both Pax3 and Pax7 are expressed in the melanophores of stage 45 embryos. Further analysis indicated a striking colocalization of Pax7 mRNA with eumelanin in melanosomes.⁵⁸ Therefore, to further characterize the biological activity of the dithiazole **1c** we examined the expression pattern and levels of Pax7 using immunofluorescence staining. Embryos were cultured in compound **1c** (10 μ M) from stage 10 and allowed to develop to stage 41, by which point they had already presented the pigment loss phenotype. As shown in Fig. 4 (top panels), both control embryos and embryos cultured in compound **1c** displayed normal expression levels of Pax7 protein and the expression patterns in treated and control tadpoles were indistinguishable. This showed that the pluripotent neural crest cell population, from which the melanophores are derived, was unaffected and developed, differentiated and migrated normally, further supporting that the dithiazole **1c** acts as a reversible melanin synthesis inhibitor.

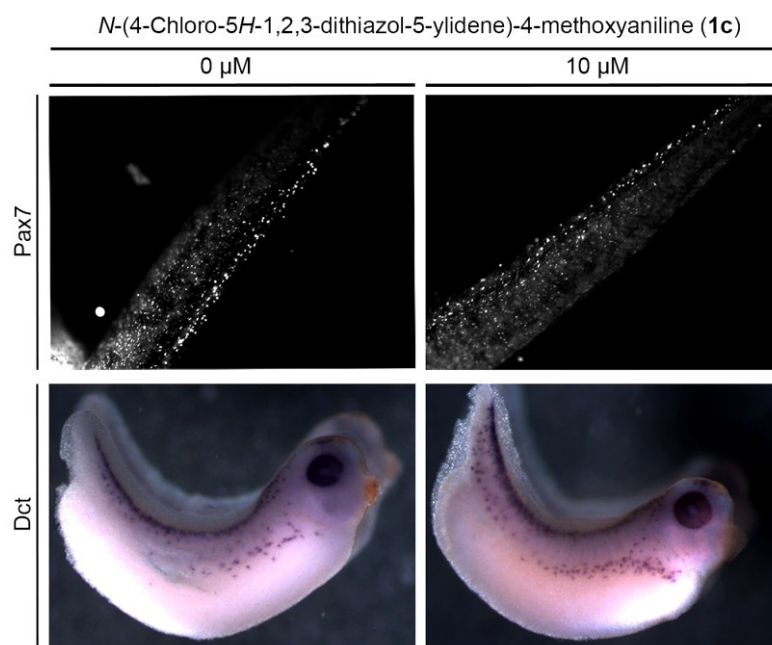


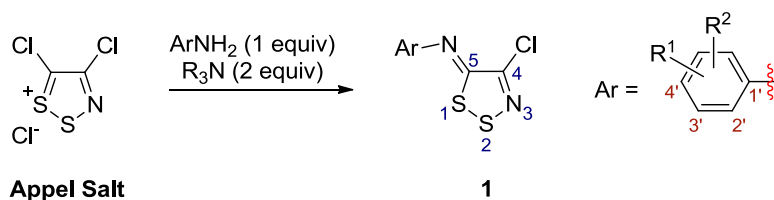
Fig. 4 Whole mount immunofluorescence and *in situ* hybridization (WISH) show similar expression of the neural crest cell marker Pax7 and the melanocyte marker Xtrp2 (Dct), respectively, between control embryos and the 4-methoxyanilino dithiazole **1c** treated embryos. (Top panels) Both control embryos and embryos cultured in compound **1c**, display normal expression levels of Pax 7 protein and the expression patterns in treated and control tadpoles at stage 40 are indistinguishable. (Bottom panels) Dct expression levels and pattern is unaffected in embryos cultured in the dithiazole **1c** to stage 32, compared to control embryos. This experiment was also performed using stage 40 embryos and the results were identical (data not shown). Experiments were repeated three times, with at least 10 embryos per condition.

Since the above data suggested that the neural crest was not affected by **1c**, we then examined the expression of a specific molecular marker of melanoblasts and differentiated melanophores Xtrp2 (Dct). Dct is a melanogenic enzyme with a central role in the melanin synthesis pathway. Dct possesses DOPAchrome tautomerase activity and isomerizes the pigmented intermediate

DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) rather than to 5,6-dihydroxyindole (DHI). This enzyme thus regulates a switch that controls the proportion of carboxylated subunits in the melanin biopolymer.⁵⁹ Melanophore specific expression of Dct is under the direct synergistic control of the transcription factors Sox10 and Mitf⁶⁰ and its expression can be detected by stage 25 in melanoblasts and the RPE. To examine if the dithiazole **1c** affects the RPE and melanophore lineages, embryos were cultured in **1c** (10 μ M) at stage 10 and allowed to develop to stage 32, by which point they clearly displayed loss of pigmentation in both the melanophores of the dorsal pigment stripe and the RPE compared to controls. Both control and treated embryos were then processed for whole mount *in situ* hybridization using a previously characterized Dct probe. As shown in Fig. 4 (bottom panels), Dct expression was unaffected in embryos cultured in compound **1c** suggesting that melanophore and RPE differentiation, proliferation and survival were unaffected by **1c**, providing further evidence that the 4-methoxy-anilino dithiazole **1c** is a melanin synthesis inhibitor.

2.4 Structure-activity relationship (SAR) study of 1,2,3-dithiazoles

In an effort to identify more active analogues, a small structure-activity relationship (SAR) study was performed on a series of 1,2,3-dithiazoles of general structure **1**. For the preparation of the 1,2,3-dithiazoles **1**, primary aromatic or heteroaromatic amines were reacted with 4,5-dichloro-1,2,3-dithiazolium chloride (Appel salt), followed by treatment with 2,6-lutidine (2 equiv) to give the corresponding [(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)amino]arenes **1** in moderate to excellent yields (Scheme 1).⁶¹⁻⁶³


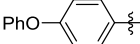
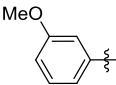
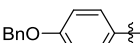
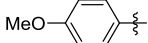
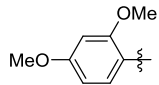
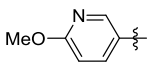
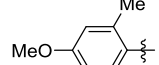
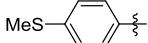
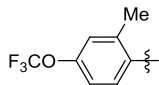
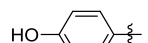
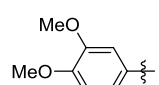
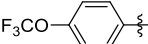
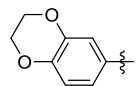
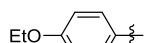
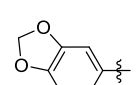
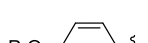
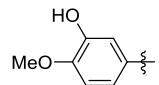
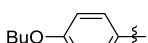
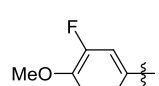


Scheme 1. Synthesis and general structure of 1,2,3-dithiazoles **1** studied in the SAR with dithiazole (blue) and arene (red) atom numbering.

In the initial chemical genomic screen the parent compound (Ar = Ph) and a series of 4' (*para*) substituted *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)anilines were studied and those with either inductively (*e.g.*, halogens, Ar = 4-HalC₆H₄; Hal = F, Cl, Br or I) and/or mesomerically electron withdrawing groups (*e.g.*, NO₂, Ar = 4-O₂NC₆H₄) did not induce pigment loss. The *p*-tolyl analogue that hosts an inductively electron releasing methyl group (Ar = 4-MeC₆H₄) also failed (data not shown). This suggested that the presence of the mesomerically electron releasing methoxy group was important. As such, in the focused SAR screen (see Table 1) we first investigated the position of the methoxy group: Analogues having the MeO group on the arene ring at position 2' (*ortho*) or 3' (*meta*) (Table 1, compounds **1a** and **1b**, respectively) showed no pigment loss and were therefore inactive. The introduction of an electronegative *sp*² ring nitrogen in the methoxyphenyl group to give the 2-methoxypyrid-3-yl **1d** led to toxicity; worthy of note was that in the initial chemical genomic screen the substitution of the aniline moiety by aminoazines (pyridines, pyrimidines and pyrazines) led to toxic compounds (data not shown). Next, the nature of the heteroatom linking the aryl and the alkyl group was investigated: Replacement of the 4-methoxy (MeO) group with the more lipophilic 4-methylthio (MeS) group gave the analogue **1e** that caused developmental delay and developmental defects (kinked tail phenotype). The role of the alkyl substituent on the ether oxygen was probed: When the methyl group attached to the oxygen atom was replaced by a hydrogen atom (H) to give the phenol **1f**,

or a trifluoromethyl group (F_3C) to give the benzotrifluoride **1g**, pronounced toxicity was observed; phenols are acidic ($\text{p}K_{\text{a}}$ 9.99),⁶⁴ while F_3C groups are strongly inductively electron withdrawing and lipophilic. Nevertheless, analogues of **1c** where the methyl group was replaced by larger alkyl chains *e.g.*, ethyl, *n*-propyl, *n*-butyl remained active (Table 1, compounds **1h**, **1i** and **1j**, respectively). Interestingly, displacement of the alkyl chain with a phenyl group, compound **1k**, led to high toxicity, however, when the phenyl group was separated from the oxygen atom by a methylene spacer, as in the benzyloxy (BnO) analogue **1l**, this led to enhanced pigment loss in comparison with **1c** (Fig. 5). As shown in the last panel of Fig. 5, the effects of the benzyloxy analogue **1l** were also reversible, and pigmentation returned in both neural crest derived melanophores and in the RPE, irrespective of the stage of initial exposure of the embryos (representative image shown from embryos initially exposed to compound **1l** at stage 21). Lastly, introduction of additional electron donating substituents at the 2' or 3' position of the arene ring of dithiazole **1c**, compounds **1m**, **1n** and **1p-r**, was, in some cases, detrimental to the activity observed. The data, tentatively showed that the activity decreased with increasing bulk at these positions. Introduction, however, of an acidic hydroxy group (3'-OH) or of an electron withdrawing and lipophilic fluorine atom (3'-F), analogues **1s** and **1t**, respectively, led to severe toxicity effects in accordance with the mono-substituted analogues **1f** and **1g**, further supporting that groups with acidic protons or strongly electronegative groups were associated with toxicity.

Table 1 Focused SAR study of dithiazoles **1a-t** at 5 and 10 μM concentrations (- = no pigment loss, + = minor pigment loss, ++ = moderate pigment loss, +++ = severe pigment loss, ++++ = complete pigment loss)

No.	Ar	Toxicity	Pigment loss		No.	Ar	Toxicity	Pigment loss	
			5 μM	10 μM				5 μM	10 μM
1a		Non Toxic	-	-	1k		Very toxic	-	-
1b		Non Toxic	-	-	1l		Non toxic	++++	++++
1c		Non Toxic	++	++++	1m		Non toxic	-	+
1d		Toxic	-	-	1n		Non toxic	++	+++
1e		Toxic	-	-	1o		Very toxic	-	-
1f		Toxic	-	-	1p		Mildly toxic at 10 μM	-	-
1g		Very Toxic	-	-	1q		Mildly toxic at 10 μM	+	++
1h		Non Toxic	+	++	1r		Mildly toxic at 10 μM	+++	+++
1i		Non Toxic	++	+++	1s		Very toxic	-	-
1j		Non Toxic	++	+++	1t		Very toxic	-	-

From this screen the most active compound was the 4-benzyloxyanilino dithiazole **1l**. A direct comparison of dithiazole **1l** with the 4-methoxyanilino dithiazole **1c** (active compound from

initial genomic screen) supported that the dithiazole **1l** treated embryos displayed a more severe pigment loss phenotype at lower doses and at all developmental stages examined compared to the 4-methoxyanilino dithiazole **1c** treated embryos (Fig. 5).

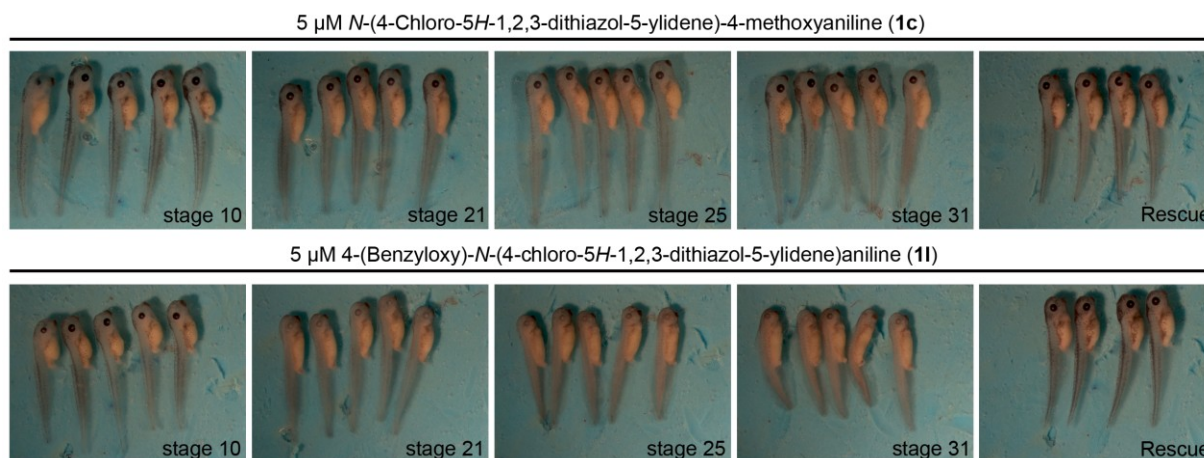
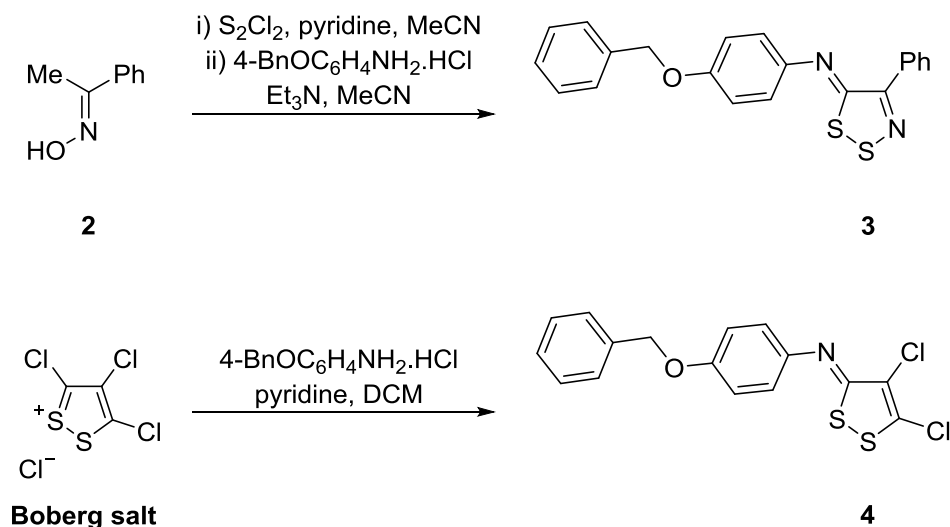


Fig.5 Direct comparison of the effect of the methoxyanilino and 4-benzyloxyanilino dithiazoles **1c** and **1l** (5 μ M), respectively, on *Xenopus* embryos at developmental stages 21, 25 and 31. The last panels on top and bottom row are representative images of embryos rescued (14-16 h after removal of dithiazoles **1c** and **1l**) after initial exposure to dithiazoles **1c** and **1l** at stage 21, showing the reversibility of the effect.

With the more potent structural motif for the arene part elucidated, structural modifications were introduced on the dithiazole part of the 4-benzyloxy analogue **1l**. These included replacing the dithiazole 4-chlorine substituent with a phenyl group to give compound **3** and also replacing the dithiazole ring nitrogen by a chlorocarbon to give compound **4**. Both compounds were synthesised starting from the oxime **2**,^{55,65} and Boberg salt,⁶⁶ respectively (Scheme 2).



Scheme 2. Synthesis of *N*-(4-phenyl-5*H*-1,2,3-dithiazol-5-ylidene)aniline (**3**) and *N*-(4,5-dichloro-3*H*-1,2-dithiol-3-ylidene)aniline (**4**).

Interestingly, replacing the dithiazole 4-chlorine substituent with a phenyl group gave the 4-phenyl-1,2,3-dithiazole **3** which displayed no phenotypic activity. Worthy of note was that the chlorine, a good nucleofuge, at the dithiazole C-4 position facilitates ring opening of the 1,2,3-dithiazole system.^{56,67} Replacing the chlorine by a group that is not a nucleofuge hampers this facile cleavage of the dithiazole ring.⁶⁸⁻⁷⁰ On the other hand, the 1,2-dithiole **4**, which maintains the nucleofuge chlorine at the C-4 position, did induce mild activity at 5 μM , while at 10 μM mild toxicity and some developmental defects were observed. These results tentatively suggest that a nucleofuge is needed at the dithiazole/dithiole C-4 position which implies that the mechanism of action may involve dithiazole or dithiole ring opening.⁵⁶

3 Conclusions

The 4-methoxyanilino and 4-benzyloxyanilino dithiazoles **1c** and **1l**, respectively, were identified in a chemical genomic screen as affecting pigmentation of melanophores of the dorsal pigment stripe and the retinal pigment epithelium (RPE) in developing *Xenopus* embryos. This biological activity of 1,2,3-dithiazoles has not previously been reported. Unlike other compounds shown to affect levels of melanophore pigmentation *in vivo*, at doses $> 40 \mu\text{M}$,^{28,71} compounds **1c** and **1l** elicit a phenotype of severe pigment loss at extremely low doses ($< 10 \mu\text{M}$), with higher doses being increasingly toxic. In addition to potency, these compounds can have an effect even at later developmental stages (up to stage 31) and do so reversibly. Reversibility is an extremely important feature of melanin synthesis inhibitors to ensure that their molecular targets are not permanently inactivated and thus avoid cytotoxicity and disruption of other pathways in which they might be involved. Finally, these compounds inhibit melanin synthesis without affecting the survival, differentiation and migration of neural crest cells, melanophores or RPE, as indicated by unaltered expression levels of Pax7 and Dct following exposure to the 4-methoxyanilino dithiazole **1c**. In conclusion, 1,2,3-dithiazoles comprise a new class of potent and reversible melanin synthesis inhibitors. Further work is needed to examine cross-species activity, to determine the mechanism of action and to identify the molecular target of these compounds.

4 Experimental Procedures

4.1 General synthetic methods and materials

Reactions were protected from atmospheric moisture by CaCl_2 drying tubes. Anhydrous Na_2SO_4 was used for drying organic extracts, and all volatiles were removed under reduced pressure. All reaction mixtures and column eluents were monitored by TLC using commercial glass backed

thin layer chromatography (TLC) plates (Merck Kieselgel 60 F₂₅₄). The plates were observed under UV light at 254 and 365 nm. The technique of dry flash chromatography was used throughout for all non-TLC scale chromatographic separations using Merck Silica Gel 60 (less than 0.063 mm).⁷² Melting points were determined using a PolyTherm-A, Wagner & Munz, Kofler-Hotstage Microscope apparatus or a TA Instruments DSC Q1000 with samples hermetically sealed in aluminum pans under an argon atmosphere, using heating rates of 5 °C/min. Solvents used for recrystallization are indicated after the melting point. UV spectra were obtained using a Perkin-Elmer Lambda-25 UV/vis spectrophotometer and inflections are identified by the abbreviation “inf”. IR spectra were recorded on a Shimadzu FTIR-NIR Prestige-21 spectrometer with a Pike *Miracle* Ge ATR accessory and strong, medium and weak peaks are represented by s, m and w respectively. ¹H and ¹³C NMR spectra were recorded on a Bruker Avance 300 machine (at 300 and 75 MHz, respectively) or on a Bruker Avance 500 machine (at 500 and 125 MHz, respectively). Deuterated solvents were used for homonuclear lock and the signals are referenced to the deuterated solvent peaks. Low resolution (EI) mass spectra were recorded on a Shimadzu Q2010 GCMS with direct inlet probe. MALDI-TOF MS were conducted on a Bruker AUTOFLEX III time-of-flight (TOF) mass spectrometer. 4,5-Dichloro-1,2,3-dithiazolium chloride (Appel salt),⁶³ 3,4,5-trichloro-1,2-dithiazolium chloride (Boberg salt),⁷³ *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-2-methoxyaniline (**1a**),⁴⁸ *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-3-methoxyaniline (**1b**),⁶¹ *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-methoxyaniline (**1c**),⁶¹ *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-(trifluoromethoxy)-aniline (**1g**),⁵⁶ *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-phenoxyaniline (**1k**),⁵⁶ *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-3,4-dimethoxyaniline (**1p**),⁷⁴ *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-2,3-dihydrobenzo[*b*][1,4]dioxine-6-amine (**1q**),⁷⁵ *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-

ylidene)benzo[*d*][1,3]dioxol-5-amine (**1r**),⁵⁶ and *N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)-3-hydroxy-4-methoxyaniline (**1s**)⁵⁶ were prepared according to literature procedures. For the newly synthesized compounds **1d-f**, **1h-j**, **1l-o** and **1t** analytical, physicochemical and spectroscopic data are reported below.

4.2 Synthesis of 4-chloro-1,2,3-dithiazoles (General procedure)

To a stirred solution of 4,5-dichloro-1,2,3-dithiazolium chloride (Appel salt) (100 mg, 0.48 mmol) in dichloromethane (4 mL) at *ca.* 20 °C the corresponding aminoarene (0.48 mmol) was added. After 1 h, to the reaction mixture was added dropwise 2,6-lutidine (111.2 μ L, 0.96 mmol). After a further 2 h the reaction mixture was adsorbed onto silica and purified by dry flash chromatography to afford the corresponding [(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)amino]arene **1**.

***N*-(4-Chloro-5*H*-1,2,3-dithiazol-5-ylidene)-6-methoxypyridin-3-amine (**1d**).** Obtained as yellow needles (113 mg, 91%), mp 117-119 °C (from *c*-hexane); *R*_f 0.48 (*n*-hexane/*t*-BuOMe, 70:30); (Found: C, 36.76; H, 2.56; N, 16.01. C₈H₆ClN₃OS₂ requires C, 37.00; H, 2.33; N, 16.18%); λ_{max} (DCM) 244 (log ϵ 3.07), 300 (2.74), 378 inf (2.92), 394 (2.93), 411 inf (2.84); ν_{max} /cm⁻¹ 1599m, 1562m, 1553m, 1537w, 1520w, 1506w, 1483s, 1470m, 1441w, 1429m, 1375s, 1296s, 1281m, 1254m, 1231m, 1182m, 1153m, 1128s, 1028s, 1009m, 945w, 916m, 862s, 816s, 804s, 770s, 748m; δ_{H} (500 MHz; CDCl₃) 8.23 (1H, d, *J* 3.0, *ArH*), 7.58 (1H, dd, *J* 9.0, 3.0, *ArH*), 6.85 (1H, d, *J* 8.5, *ArH*), 3.98 (3H, s, CH₃O); δ_{C} (125 MHz; CDCl₃) 162.5 (s), 157.3 (s), 148.3 (s), 140.2 (s), 138.6 (d), 131.8 (d), 111.6 (d), 53.9 (q); MALDI-TOF MS (*m/z*) 262 (MH⁺+1, 45%), 260 (MH⁺, 100).

***N*-(4-Chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-methylthioaniline (1e).** Obtained as yellow needles (122 mg, 98%), mp 127-128 °C (from *c*-hexane); R_f 0.55 (*n*-hexane/DCM, 40:60); (Found: C, 39.41; H, 2.46; N, 10.28. $C_9H_7ClN_2S_3$ requires C, 39.34; H, 2.57; N, 10.19%); λ_{max} (DCM) 262 (log ϵ 3.09), 354 inf (2.76), 398 inf (2.92), 411 (2.94), 431 inf (2.83); ν_{max}/cm^{-1} 3003w, 2920w, 1589m, 1568m, 1545m, 1504m, 1489m, 1479m, 1429m, 1400m, 1321w, 1298w, 1265w, 1221m, 1179m, 1142m, 1111m, 1092m, 1005m, 968m, 857m, 945m, 860s, 833m, 824m, 775m; δ_H (500 MHz; $CDCl_3$) 7.33 (2H, d, J 8.5, *ArH*), 7.21 (2H, d, J 8.5, *ArH*), 2.52 (3H, s, CH_3S); δ_C (125 MHz; $CDCl_3$) 157.3 (s), 148.2 (s), 147.5 (s), 137.1 (s), 127.5 (d), 120.5 (d), 15.9 (q); m/z (EI) 276 ($M^{+}+2$, 39), 274 (M^{+} , 79), 259 (4), 229 (10), 227 (21), 210 (6), 181 (27), 175 (17), 166 (26), 149 (100), 134 (15), 123 (11), 116 (33), 108 (31), 104 (9), 90 (11), 82 (8), 77 (11), 64 (31).

***N*-(4-Chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-hydroxyaniline (1f).** Obtained as orange plates (63 mg, 54%), mp (DSC) onset: 130.0 °C, peak max: 136.9 °C (from *c*-hexane/1,2-DCE); R_f 0.56 (DCM/*t*-BuOMe, 90:10); (Found: C, 39.38; H, 1.97; N, 11.33. $C_8H_5ClN_2OS_2$ requires C, 39.27; H, 2.06; N, 11.45%); λ_{max} (DCM) 251 (log ϵ 3.10), 280 inf (2.70), 398 (2.87), 414 inf (2.80); ν_{max}/cm^{-1} 3121m (OH), 1605m, 1585m, 1547m, 1504m, 1495m, 1441m, 1364m, 1364m, 1346w, 1269m, 1231m, 1215m, 1153m, 1107m, 1011w, 935w, 887s, 858m, 829s, 812m, 768m; δ_H (500 MHz; $CDCl_3$) 7.23 (2H, d, J 8.5, *ArH*), 6.92 (2H, d, J 9.0, *ArH*), 4.97 (1H, s, OH); δ_C (125 MHz; $CDCl_3$) 156.0 (s), 154.2 (s), 148.4 (s), 143.4 (s), 121.9 (d), 116.3 (d); m/z (EI) 246 ($M^{+}+2$, 21%), 244 (M^{+} , 54), 227 (4), 209 (2), 183 (9), 180 (3), 151 (16), 145 (21), 125 (4), 122 (3), 119 (100), 96 (3), 15 (93), 91 (11), 70 (4), 64 (26).

***N*-(4-Chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-ethoxyaniline (1h).** Obtained as yellow needles (119 mg, 91%), mp 106-107 °C (from *c*-hexane); R_f 0.48 (*n*-hexane/DCM, 40:60); (Found: C,

43.98; H, 3.26; N, 10.39. $C_{10}H_9ClN_2OS_2$ requires C, 44.03; H, 3.33; N, 10.27%; $\lambda_{\max}(\text{DCM})$ 251 (log ϵ 3.05), 309 (2.57), 402 (2.88), 419 inf (2.80); $\nu_{\max}/\text{cm}^{-1}$ 3032w, 2978w, 2928w, 1605m, 1576m, 1551m, 1526w, 1501m, 1481m, 1454w, 1439w, 1393m, 1296m, 1244s, 1225m, 1171m, 1134m, 1115m, 1043m, 1005w, 957w, 918m, 858s, 833m, 822m, 791m, 760m; δ_H (500 MHz; $CDCl_3$) 7.27 (2H, d, J 8.5, ArH), 6.98 (2H, d, J 8.5, ArH), 4.07 (2H, q, J 7.0, CH_2O), 1.44 (3H, t, J 7.0, CH_3); δ_C (125 MHz; $CDCl_3$) 157.7 (s), 155.4 (s), 148.5 (s), 142.8 (s), 121.8 (d), 115.2 (d), 63.7 (t), 14.8 (q); m/z (EI) 274 ($M^+ + 2$, 25%), 272 (M^+ , 60), 245 (12), 243 (27), 227 (6), 209 (3), 183 (3), 179 (10), 173 (14), 151 (26), 147 (50), 122 (14), 119 (100), 102 (4), 91 (18), 70 (6), 64 (34).

***N*-(4-Chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-*n*-propoxyaniline (1i).** Obtained as yellow needles (125 mg, 91%), mp 71-72 °C (from *c*-hexane); R_f 0.67 (*n*-hexane/*t*-BuOMe, 70:30); (Found: C, 46.14; H, 3.96; N, 9.77. $C_{11}H_{11}ClN_2OS_2$ requires C, 46.07; H, 3.87; N, 9.77%); $\lambda_{\max}(\text{DCM})/\text{nm}$ 251 (3.14), 309 (2.66), 343 inf (2.71), 382 inf (2.96), 402 (2.99), 424 inf (2.89); $\nu_{\max}/\text{cm}^{-1}$ 2972w, 2943w, 2913w, 2878w, 1605m, 1574m, 1562m, 1531w, 1499m, 1472m, 1391w, 1288w, 1250s, 1219m, 1171w, 1136s, 1115m, 1047m, 1013s, 970w, 953w, 941w, 912w, 899w, 860s, 831s, 802m, 766s; δ_H (500 MHz; $CDCl_3$) 7.27 (2H, d, J 9.0, ArH), 6.98 (2H, d, J 9.0, ArH), 3.96 (2H, t, J 6.8, CH_2O), 1.83 (2H, qt, J 7.0, 7.0, CH_2), 1.05 (3H, t, J 7.3, CH_3); δ_C (125 MHz; $CDCl_3$) 157.9 (s), 155.4 (s), 148.5 (s), 142.8 (s), 121.8 (d), 115.2 (d), 69.8 (t), 22.6 (t), 10.5 (q); m/z (EI) 288 ($M^+ + 2$, 23%), 286 (M^+ , 56), 244 (37), 183 (12), 151 (27), 145 (19), 119 (100), 93 (13), 64 (30).

4-*n*-Butoxy-*N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)aniline (1j). Obtained as yellow cotton fibres (124 mg, 86%), mp 61-62 °C (from *c*-hexane); R_f 0.71 (*n*-hexane/*t*-BuOMe, 70:30); (Found: C, 48.03; H, 4.30; N, 9.28. $C_{12}H_{13}ClN_2OS_2$ requires C, 47.91; H, 4.36; N, 9.31%);

λ_{\max} (DCM)/nm 252 (3.16), 309 (2.68), 343 inf (2.72), 382 inf (2.98), 402 (3.01), 425 inf (2.91); ν_{\max} /cm⁻¹ 2963w, 2940w, 2872w, 1607m, 1570w, 1562w, 1501m, 1470w, 1395w, 1296w, 1252s, 1175w, 1121s, 1096s, 1036m, 1007m, 930m, 903s, 856m, 829m, 800w, 762m; δ_{H} (500 MHz; CDCl₃) 7.27 (2H, d, *J* 9.0, Ar*H*), 6.98 (2H, d, *J* 9.0, Ar*H*), 4.00 (2H, t, *J* 7.0, CH₂O), 1.79 (2H, tt, *J* 7.0, 7.0, CH₂), 1.51 (2H, qt, *J* 7.8, 7.8, CH₂), 0.99 (3H, t, *J* 7.5, CH₃); δ_{C} (125 MHz; CDCl₃) 157.9 (s), 155.3 (s), 148.5 (s), 142.7 (s), 121.8 (d), 115.2 (d), 68.0 (t), 31.3 (t), 19.2 (t), 13.8 (q); *m/z* (EI) 302 (M⁺+2, 26%), 300 (M⁺, 51), 244 (59), 227 (11), 183 (18), 151 (28), 145 (23), 119 (100), 93 (16), 64 (35), 57 (20).

4-Benzoyloxy-*N*-(4-chloro-5*H*-1,2,3-dithiazol-5-ylidene)aniline (1l). Obtained as yellow plates (158 mg, 98%), mp 109-111 °C (from *c*-hexane/1,2-DCE); *R_f* 0.48 (*n*-hexane/DCM, 40:60); (Found: C, 53.71; H, 3.25; N, 8.25. C₁₅H₁₁ClN₂OS₂ requires C, 53.81; H, 3.31; N, 8.37%); λ_{\max} (DCM) 251 (log ϵ 3.20), 256 inf (3.18), 307 (2.67), 401 (2.98), 418 inf (2.90); ν_{\max} /cm⁻¹ 3038w, 2866w, 1603m, 1576m, 1562m, 1535w, 1495m, 1466m, 1454m, 1416w, 1381m, 1325w, 1304m, 1294m, 1275w, 1242s, 1227m, 1177m, 1157w, 1130m, 1111m, 1082w, 1030w, 1007m, 993m, 959w, 922m, 856s, 835s, 799m, 766s; δ_{H} (500 MHz; CDCl₃) 7.45 (2H, d, *J* 7.5, Ar*H*), 7.41 (2H, dd, *J* 7.5, 7.5, Ar*H*), 7.35 (1H, dd, *J* 7.3, 7.3, Ar*H*), 7.28 (2H, d, *J* 9.0, Ar*H*), 7.07 (2H, d, *J* 9.0, Ar*H*), 5.10 (2H, s, CH₂O); δ_{C} (125 MHz; CDCl₃) 157.4 (s), 155.7 (s), 148.4 (s), 143.3 (s), 136.6 (s), 128.6 (d), 128.1 (d), 127.5 (d), 121.7 (d), 115.7 (d), 70.3 (t); *m/z* (EI) 336 (M⁺+2, 4%), 334 (M⁺, 10), 245 (13), 243 (32), 154 (4), 150 (11), 122 (5), 91 (100), 65 (18).

***N*-(4-Chloro-5*H*-1,2,3-dithiazol-5-ylidene)-2,4-dimethoxyaniline (1m).** Obtained as orange cotton fibres (119 mg, 86%), mp 70-71 °C (from *n*-hexane at *ca.* -20 °C); λ_{\max} (DCM)/nm 258 inf (2.91), 346 inf (2.60), 381 inf (2.67), 412 (2.72), 442 inf (2.59); ν_{\max} /cm⁻¹ 1599s, 1580s, 1558w, 1499s, 1468w, 1452m, 1431w, 1414w, 1306s, 1263s, 1211s, 1155s, 1117s, 1038s, 1026s, 984w,

959w, 924m, 856s, 829s, 800w, 789w, 770s, 752m, 733m; δ_{H} (500 MHz; CDCl_3) 7.16 (1H, d, J 8.5, ArH), 6.57 (1H, d, J 2.5, ArH), 6.54 (1H, dd, J 8.5, 2.5, ArH), 3.87 (3H, s, CH_3O), 3.84 (3H, s, CH_3O); δ_{C} (125 MHz; CDCl_3) 159.5 (s), 157.9 (s), 151.9 (s), 147.8 (s), 132.7 (s), 119.5 (d), 104.3 (d), 99.9 (d), 55.8 (q), 55.6 (q); m/z (EI) 290 ($\text{M}^+ + 2$, 39%), 288 (M^+ , 100), 195 (33), 188 (87), 180 (14), 174 (14), 163 (67), 152 (24), 137 (11), 134 (38), 120 (19), 107 (14), 93 (11), 79 (20), 64 (30), 51 (17).

***N*-(4-Chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-methoxy-2-methylaniline (1n).** Obtained as orange needles (93 mg, 71%), mp 86-87 °C (from *c*-hexane); R_f 0.62 (*n*-hexane/*t*-BuOMe, 70:30); (Found: C, 43.93; H, 3.39; N, 10.21. $\text{C}_{10}\text{H}_9\text{ClN}_2\text{OS}_2$ requires C, 44.03; H, 3.33; N, 10.27%); λ_{max} (DCM)/nm 254 (3.03), 309 inf (2.54), 349 inf (2.67), 382 inf (2.81), 405 (2.84), 431 inf (2.73); ν_{max} /cm⁻¹ 3013w, 2965w, 2941w, 2924w, 2839w, 1607m, 1591w, 1578w, 1489m, 1450w, 1431w, 1373w, 1306s, 1281m, 1250s, 1221s, 1165m, 1146m, 1109s, 1049m, 991w, 928w, 922w, 854s, 820m, 802m, 775m, 762s, 723m; δ_{H} (300 MHz; CDCl_3) 7.15 (1H, d, J 8.7, ArH), 6.86 (1H, d, J 2.7, ArH), 6.80 (1H, dd, J 8.6, 2.9, ArH), 3.82 (3H, s, CH_3O), 2.31 (3H, s, CH_3); δ_{C} (75 MHz; CDCl_3) 158.1 (s), 155.4 (s), 148.4 (s), 142.3 (s), 133.9 (s), 116.8 (d), 116.6 (d), 111.5 (d), 55.4 (q), 18.1 (q); m/z (EI) 274 ($\text{M}^+ + 2$, 30%), 272 (M^+ , 64), 257 (10), 237 (9), 181 (10), 179 (20), 173 (36), 147 (100), 136 (13), 132 (17), 117 (18), 104 (19), 91 (21), 77 (63), 64 (46), 51 (32).

***N*-(4-Chloro-5*H*-1,2,3-dithiazol-5-ylidene)-2-methyl-4-trifluoromethoxyaniline (1o).**

Obtained as yellow prisms (41 mg, 26%), mp 49-50 °C (from *c*-hexane); (Found: C, 36.62; H, 1.94; N, 8.65. $\text{C}_{10}\text{H}_6\text{ClF}_3\text{N}_2\text{OS}_2$ requires C, 36.76; H, 1.85; N, 8.57%); λ_{max} (DCM)/nm 274 (log ϵ 3.54), 372 (2.93); ν_{max} /cm⁻¹ 2955w, 2924w, 2853w, 1607w, 1591w, 1570w, 1485m, 1381w, 1250s, 1200s, 1157s, 1138w, 1107m, 1040w, 1009w, 976w, 891w, 878m, 847m, 822m, 793m,

773s, 723m; δ_{H} (500 MHz; CDCl_3) 7.16 (1H, s, ArH), 7.13-7.10 (2H, m, ArH), 2.28 (3H, s, CH_3); δ_{C} (125 MHz; CDCl_3) 159.0 (s), 148.6 (s), 147.8 (s), 146.9 (q, $^3J_{\text{CF}}$ 1.3), 132.5 (s), 123.7 (d), 120.5 (q, $^1J_{\text{CF}}$ 255.0), 119.6 (d), 117.2 (d), 17.7 (q); m/z (EI) 328 ($\text{M}^{+}+2$, 25%), 326 (M^{+} , 50), 291 (5), 265 (6), 241 (10), 227 (37), 201 (100), 169 (13), 147 (12), 136 (10), 132 (15), 125 (17), 116 (12), 109 (22), 104 (12), 83 (19), 77 (33), 69 (64), 64 (62), 51 (21).

***N*-(4-Chloro-5*H*-1,2,3-dithiazol-5-ylidene)-4-fluoro-3-methoxyaniline (1t).** Obtained as yellow prisms (86 mg, 65%), mp 87-88 °C (from *c*-hexane); R_{f} 0.43 (*n*-hexane/*t*-BuOMe, 70:30); (Found: C, 38.95; H, 2.12; N, 10.17. $\text{C}_9\text{H}_6\text{ClFN}_2\text{OS}_2$ requires C, 39.06; H, 2.19; N, 10.12%); λ_{max} (DCM)/nm 248 (log ϵ 3.17), 306 inf (2.63), 340 inf (2.73), 382 (2.97), 399 (2.98), 424 inf (2.84); ν_{max} /cm $^{-1}$ 2970w, 2938w, 2907w, 2843w, 1616m, 1572m, 1564m, 1541w, 1514s, 1468w, 1454m, 1447m, 1429w, 1315s, 1279s, 1227s, 1188m, 1161s, 1109s, 1022s, 964m, 905w, 866s, 856s, 799s, 781m, 766w, 748s; δ_{H} (500 MHz; CDCl_3) 7.11-7.02 (3H, m, ArH), 3.92 (3H, s, CH_3O); δ_{C} (125 MHz; CDCl_3) 157.1 (s), 152.5 (d, $^1J_{\text{CF}}$ 247.0), 148.3 (s), 146.3 (d, $^2J_{\text{CF}}$, 10.9), 146.4 (d, $^3J_{\text{CF}}$ 8.0), 115.9 (d, $^3J_{\text{CF}}$ 3.5), 113.8 (d, $^4J_{\text{CF}}$ 2.6), 109.1 (d, $^2J_{\text{CF}}$ 19.6), 56.4 (q); m/z (EI) 278 ($\text{M}^{+}+2$, 43%), 276 (M^{+} , 45), 263 (12), 245 (12), 183 (14), 168 (23), 151 (100), 140 (13), 136 (23), 125 (19), 108 (22), 96 (11), 85 (29), 71 (42), 64 (21), 57 (56).

4-Benzoyloxy-*N*-(4-phenyl-5*H*-1,2,3-dithiazol-5-ylidene)aniline (3). Pyridine (237 mg, 0.24 mL, 3 mmol) was added dropwise at -5 to 0 °C to a stirred solution of acetophenone oxime **2** (135 mg, 1 mmol) and disulfur dichloride (0.16 mL, 2 mmol) in acetonitrile (10 mL) under argon. The mixture was stirred at 0 °C for 15 min, and a mixture of 4-benzyloxyaniline hydrochloride (235 mg, 1 mmol) and triethylamine (101 mg, 1 mmol) in acetonitrile (2 mL) was added. The mixture was stirred at this temperature for another 0.5 h. Pyridine (158 mg, 0.16 mL, 2 mmol) was added and the reaction mixture was stirred at *ca.* 20 °C for 1 h. Then the mixture

heated at reflux for 5 h, filtered, and the solvent was evaporated under vacuum. The residue obtained was adsorbed onto silica and chromatographed (*n*-hexane/DCM) to give the *title compound 3* as yellow needles (55 mg, 15%), mp (DSC) onset: 65.8 °C, peak max: 68.2 °C, decomp. onset: 69.3 °C, peak max: 69.7 °C (from *c*-hexane); R_f 0.63 (*n*-hexane/DCM, 40:60); (Found: C, 66.93; H, 4.36; N, 7.51. $C_{21}H_{16}N_2OS_2$ requires C, 67.00; H, 4.28; N, 7.44%); λ_{max} (DCM) 276 (log ϵ 3.58), 231 (3.66); ν_{max}/cm^{-1} 3068w, 3034w, 2907w, 2864w, 1903m, 1486m, 1454m, 1439m, 1331m, 1288m, 1244s, 1207m, 1173m, 1165m, 1111m, 1065m, 1020m, 828w, 912w, 868w, 843m, 327m, 810m, 781m, 770m; δ_H (500 MHz; acetone- d_6) 8.20 (2H, dd, J 8.0, 1.5, ArH), 7.54-7.48 (5H, m, ArH), 7.41 (2H, dd, J 7.5, 7.5, ArH), 7.34 (1H, dd, J 7.3, 7.3, ArH), 7.25 (2H, d, J 9.0, ArH), 7.16 (2H, d, J 9.0, ArH), 5.17 (2H, s, CH_2O); δ_C (125 MHz; acetone- d_6) 164.2 (s), 160.7 (s), 157.9 (s), 146.8 (s), 138.3 (s), 134.0 (s), 131.1 (d), 130.0 (d), 129.3 (d), 129.0 (d), 128.7 (d), 128.5 (d), 121.8 (d), 116.7 (d), 70.8 (t); MALDI-TOF MS (m/z) 377 ($M^+ + 1$, 100%), 345 (18), 285 (24), 91 (4).

4-Benzoyloxy-*N*-(4,5-dichloro-3*H*-1,2-dithiol-3-ylidene)aniline (4). A solution of pyridine (151 mg, 1.92 mmol) in DCM (10 mL) was added dropwise at *ca.* 20 °C to a stirred mixture of Boberg salt (154 mg, 0.64 mmol) and 4-benzyloxyaniline hydrochloride (172 mg, 0.73 mmol) in DCM (10 mL). The reaction mixture was stirred at *ca.* 20 °C for 2 h, filtered, washed with 10% HCl (2 \times 10 mL) and water (2 \times 10 mL), dried (Na_2SO_4), and evaporated. The residue was adsorbed onto silica and chromatographed (*n*-hexane/DCM) to give the *title compound 4* as orange needles (70 mg, 26%), mp (DSC) onset: 104.1 °C, peak max: 105.0 °C, decomp. onset: 136.1 °C, peak max: 147.3 °C (from *c*-hexane); R_f 0.38 (*n*-hexane/DCM, 40:60); (Found: C, 52.22; H, 2.95; N, 3.86. $C_{16}H_{11}Cl_2NOS_2$ requires C, 52.18; H, 3.01; N, 3.80%); λ_{max} (DCM) 312 (log ϵ 2.70), 365 (2.76); ν_{max}/cm^{-1} 3034w, 2928w, 2914w, 2866w, 1601m, 1593m, 1576m, 1501s, 1496m, 1454m,

1379m, 1287m, 1242m, 1231S, 1184m, 1168w, 1159w, 1113m, 1080w, 1009w, 997m, 978m, 962w, 943w, 922w, 872m, 858s, 835m, 820w, 808w, 799m, 758s; δ_{H} (500 MHz; CDCl_3) 7.45 (2H, d, J 7.0, ArH), 7.40 (2H, dd, J 7.5, 7.5, ArH), 7.34 (1H, dd, J 7.3, 7.3, ArH), 7.06 (2H, d, J 9.0, ArH), 7.02 (2H, d, J 9.0, ArH), 5.07 (2H, s, CH_2O); δ_{C} (125 MHz; CDCl_3) 158.2 (s), 156.8 (s), 142.99 (s), 142.95 (s), 136.8 (s), 128.6 (d), 128.1 (d), 127.5 (d), 122.7 (s), 121.3 (d), 115.9 (d), 70.3 (t); MALDI-TOF MS (m/z) 372 ($\text{M}^+ + 5$, 17%), 370 ($\text{M}^+ + 3$, 78), 368 ($\text{M}^+ + 1$, 100), 340 (15), 301 (33), 277 (51), 90 (41).

4.3 General biological methods and materials

University of Cyprus Animal Ethics Guidelines. Research using animals is rigorously defined and controlled by the Animal Protection and Welfare Act that was enacted in 1994, and is carried out under licenses granted by the Animal Health and Welfare Division, of the Veterinary Services Department of the Ministry of Agriculture, Natural Resources and the Environment of the Republic of Cyprus.

Stock solutions. Compounds were dissolved in DMSO to make a stock solution of 1 mM concentration and then diluted in 0.1X Marc's Modified Ringer's (MMR) for the indicated working concentrations (usually 5 or 10 μM).

Embryos. *Xenopus laevis* embryos from induced spawning⁷⁶ were staged according to Nieuwkoop and Faber.⁷⁷ *Xenopus* oocytes were fertilized *in vitro* and dejellied using 1.8% cysteine-HCl, pH 7.8, then maintained in 0.1X MMR until they reach the required stage for use in the experiments. Five to ten *Xenopus laevis* embryos of the appropriate stage were arrayed in individual wells of a 6-well plate in either 0.1X MMR alone (control embryos) or supplemented with the reported concentrations of the tested compound. The embryos were allowed to develop

and assessed for survival and elicitation of a distinctive phenotype. The embryos were rinsed multiple times in fresh media and freshly solubilized compound was added every day, since the compound stability *i.e.* rates of compound uptake, distribution and metabolism within embryos, was not known, so as to exclude the possibility of compound degradation and ensure activity. All experiments were repeated three times with at least 10 embryos for each condition.

Quantification of pigmentation loss phenotype. Embryos were visually scored for loss of pigmentation. Embryos were all initially photographed on a stereomicroscope under the same conditions and then visually scored by two individuals one at a time after setting criteria for each category: - = no pigment loss (no pigment change compared to controls on either the melanophores or the RPE), += minor pigment loss (minor pigment reduction in melanophores, no visible change in RPE), ++ = moderate pigment loss (moderate pigment reduction in melanophores and the RPE), +++ = severe pigment loss (severe pigment reduction on melanophores and the RPE), ++++ = complete pigment loss (no visible pigment on either the melanophores or the RPE)

Rescue experiments. To test if pigmentation in the developing embryos would return after compounds were removed the following experiment was performed. Briefly, 5-10 embryos (Developmental stages 10, 21 and 31) were cultured in 0.1X MMR (3 mL), supplemented with compound **1c** or **1l** (5 or 10 μ M) in a 6 well plate. The embryos were allowed to present the pigmentation loss phenotype and develop to stage 40. At this point, the media was removed; the embryos were washed extensively and then incubated with 0.1X MMR. Pigment levels were visually assayed after 14 h.

Whole mount immunofluorescence. *Xenopus laevis* embryos were fixed in 3.7% formaldehyde in MEMFA (2 h at room temperature) and the vitelline envelope was manually removed with forceps. Permeabilization of embryos was carried out several ways: 1) overnight in 1X PBS supplemented with 0.5% Triton, and 1% DMSO (PBDT), 2) 2 h in 1X PBS supplemented with 0.2% SDS, 3) for 4 h in 1X PBS supplemented with 0.2% SDS or 4) for 25 min in 10 µg/mL Proteinase K. Embryos were then blocked for 2 h in 1X PBS with 0.5% Triton, 5% BSA and 1% Normal Goat serum. Primary antibody staining followed. Embryos were incubated with mouse anti-*Xenopus* Pax7 antibody overnight at 4 °C at a dilution of 1:100 (in block solution). Embryos were then washed (3 × 10 min) in PBDT and incubated for 2 h at room temperature with Cy3 conjugated anti-mouse IgG antibody at 1:500 dilution in fresh block solution. After incubation, embryos were washed in PBDT (3 × 10min). For negative control experiments, embryos were blocked and then incubated with secondary conjugates only. Clearing of embryos was performed by immersion of the embryos in two parts benzyl benzoate (BB) and one part benzyl alcohol (BA) after methanol dehydration (Murray's Clearing Medium, BB:BA). The refractive index of BB:BA closely matches the refractive index of yolk thereby rendering *Xenopus* embryos nearly transparent.

In vitro transcription. Anti-sense Digoxigenin (DIG) labelled Xtrp2/Dct (in pGEMT) probe was synthesized by *in vitro* transcription from NcoI linearized plasmid using RNA polymerase SP6 or T3 and ribonucleotide mixture which results in RNA transcripts containing Dig-UTP (Roche). The manufacturer's protocol was followed with a modification in the total reaction volume which was scaled down to 20 µL. RNA probes from these reactions was purified using isopropanol/LiCl precipitation.

Whole-mount *in situ* hybridization (WISH). DIG labeled Xtrp2/Dct RNA probe (transcribed as described above) was used to perform *in situ* hybridization using the protocol reported by Harland RM⁷⁸ with some modifications. Methanol was substituted with ethanol and 4% paraformaldehyde in PBS was used to fix the embryos instead of formaldehyde. After proteinase K treatment (5 min) embryos were blocked with 0.1% BSA, 10% Sheep serum in 0.1% Tween in 1 X PBS (PBT) solution and then washes were performed in PBS. After blocking, embryos were re-fixed for 1 h in 4% paraformaldehyde followed by prehybridization at 65 °C (6 h). Subsequently the RNA probe was added to the fixed embryos and incubated at 65 °C (12 h). Several washes using 0.2X SSC at 65 °C followed. After the last wash the embryos were incubated in Rnase and then blocked with 2% BMB Blocking Reagent + MAB for 1 h and then transferred to a new vial which contained 1 mL of a 1:2000 dilution Anti-DIG AP conjugates in blocking solution for 4 h at room temperature. After the incubation the embryos are washed extensively with MAB (12 h) and AP is added, followed by the substrate BM purple. The reaction stops with addition of MAB. Finally embryos were washed in PBT (4 × 30 min) at room temperature. Embryos were then cleared in BB:BA (as described above). The chromogenic signal remains localized after clearing and this allows data acquisition from different planes within the embryo without the need for sectioning.

Imaging. The embryos were imaged on a Zeiss Axio Imager Z1 using a Zeiss Axiocam MR3, the Axiovision software 4.7. Optical sectioning was achieved using a Zeiss Apotome structured illumination system.

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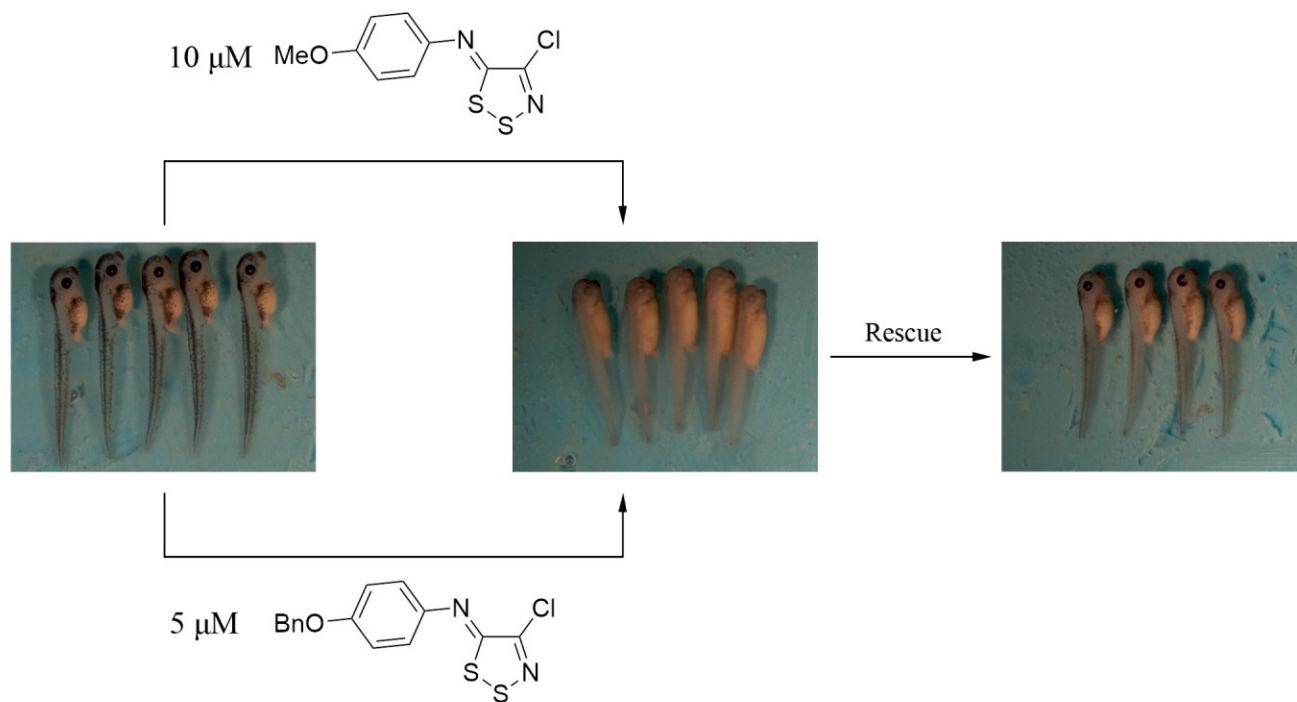
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Graphical Abstract



Text Caption

A new biological activity is identified for 1,2,3-dithiazolimines: reversible inhibition of melanine synthesis in *Xenopus laevis* embryos.