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Chemical Genetics

Cardiosulfa, a Small Molecule that Induces Abnormal Heart Development in Zebrafish, and Its Biological Implications**

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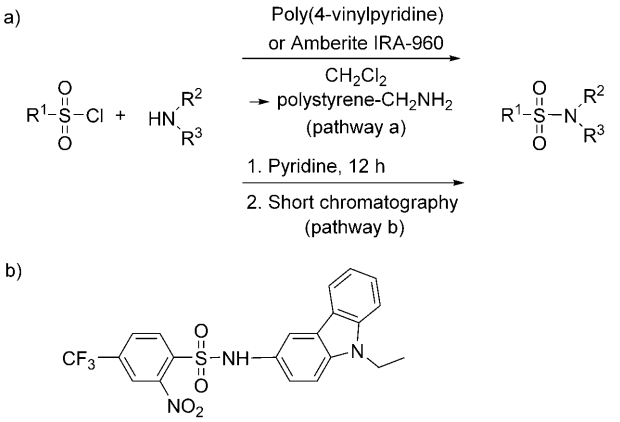
Heart disease is one of the most common causes of human death. As a result, a major goal in understanding this disease is the elucidation of mechanisms and pathways that are directly related to the human heart function. The zebrafish has emerged as a powerful model vertebrate for the assessment of heart development owing to the fact that its heart is nearly identical to that of a human embryo at three weeks of gestation.[1] The hearts of vertebrate embryo, including zebrafish, consist of two chambers, an anterior ventricle and a posterior atrium, each having a characteristic shape, size, and function that contribute to efficient circulation. Owing to the transparency of embryonic zebrafish, heart function during developmental stages can be visually detected by using microscopy. Furthermore, significant defects in cardiac form or function are easily observed in live embryos, because embryonic zebrafish can survive in the absence of circulating blood during early developmental stages.

Implementation of a conventional genetic approach, in combination with phenotypic screening, serves as a common approach to dissecting developmental processes and characterizing protein functions.[2] However, there are drawbacks associated with this methodology, including relatively long experimental times and premature organism death resulting from gene mutations or deletions. The forward chemical genetic approach that employs small molecules to temporarily regulate protein function in zebrafish has certain advantages over genetic methods owing to its simplicity and ability to conditionally regulate the activities of gene products.[3]

To date, small molecules that affect developmental processes in zebrafish have been identified by employing the forward chemical genetic approach and used to gain an understanding of the functions of gene products involved in vertebrate developmental events.[4] However, a more intense effort is required to uncover new chemical modulators. As part of a recent investigation aimed at this goal, we have prepared and screened a sulfonamide library designed to identify small molecules that perturb heart development in zebrafish. Herein, we describe the development of a novel, sulfonamide-based small molecule that induces abnormal heart morphology during zebrafish development.

Sulfonamides have great potential for use as bioactive molecules because they display a diverse range of biological activities, including antihypertensive, antidiabetic, and antibacterial activities.[5] As a result, by using solution-phase chemistry, we have constructed a novel sulfonamide library comprising members with diverse substitution patterns. The goal was to identify compounds that induce distinctive phenotypic changes of the zebrafish heart during development. The library was prepared by reacting excess sulfonyl chlorides with primary and secondary amines in the presence of either poly(4-vinylpyridine) or amberite IRA 960 as polymer-supported bases (Scheme 1 a, pathway a). After the coupling reactions are complete, excess sulfonyl chlorides



Scheme 1. a) Preparation of a sulfonamide library by using solution-phase chemistry (substituents R^1, R^2, and R^3 are given in the Supporting Information, Scheme S1). b) Structure of cardiosulfa.

were removed by adding aminomethylated polystyrene resin to the reaction mixture.[6] Amines that have poor solubilities in CH_2Cl_2 or poor reactivities were reacted with excess sulfonyl chlorides in dry pyridine, and the target products were isolated by column chromatography (Scheme 1 a, pathway b).[7] These synthetic pathways produced about 300 sulfonamides in good yields and high purities.

The sulfonamide library was screened for phenotypic changes of the zebrafish heart during development by using a 96-well plate format.[8] Zebrafish embryos (three embryos per well) were exposed to 10 μM sulfonamides at 28 °C, and their phenotype was visually observed by using a dissecting microscope over a five-day post-fertilization (dpf) period. One

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compound, named "cardiosulfa" (Scheme 1b), was found to induce impaired heart morphology, severe pericardial edema, and severe yolk edema (Figure 1a,b). Cardiosulfa showed pronounced effects on cardiac development at concentrations of 5–30 μM. Zebrafish embryos exposed to these concentrations of the substance displayed narrow and elongated ventricle and atrium within an enlarged pericardial sac. In addition, peripheral blood flow was reduced in the exposed embryos. Although serious heart deformation is observed in the exposed embryos, they remain alive up to 7 dpf.

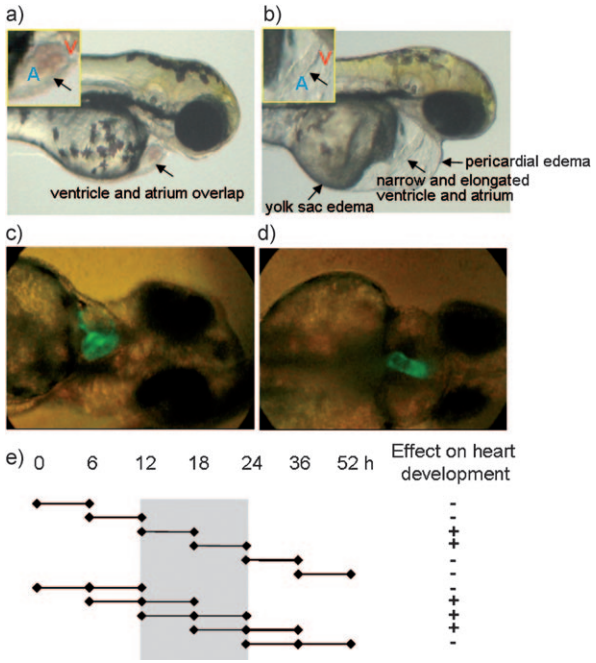

Figure 1. Effects of cardiosulfa on zebrafish heart development. a,b) Three-day-old zebrafish embryo untreated (a) and treated (b) with 20 μM cardiosulfa. Arrows in the insets indicate normal and abnormal heart morphology; V = ventricle, A = atrium. c,d) Tg(*cmlc2:GFP*) transgenic zebrafish embryo (three days old) untreated (c) and treated (d) with 20 μM cardiosulfa. Heart marked with green fluorescent protein (GFP). e) Effect of heart development upon exposure of embryos to 20 μM cardiosulfa during the times indicated by bars; thereafter, the embryos were transferred to the fresh media. Embryos were observed at 96 hours post-fertilization (hpf).

To further examine cardiovascular malformations, Tg(*cmlc2:GFP*) transgenic zebrafish embryos were treated with 20 μM of cardiosulfa. Because *cmlc2* (cardiac myosin light chain 2) is expressed throughout the heart tube,[9] the heart morphology of transgenic zebrafish can be clearly visualized by using fluorescence microscopy.[10] As shown in Figure 1c,d, serious heart deformation with a narrow and elongated ventricle and atrium was observed in the exposed embryos at 72 hpf (hours post-fertilization). The heart rates of treated embryos were similar to those of untreated ones at 48 hpf (untreated embryo/treated embryo = 102:96 beats per minute (bpm)), but it lowers as development proceeds (at 72 hpf, untreated/treated = 128:80 bpm; at 96 hpf, untreated/treated = 140:40 bpm).

Temporal control experiments with cardiosulfa were performed to identify the developmental stage at which this sulfonamide induces abnormal heart formation in zebrafish. In these experiments, cardiosulfa was added or washed away at various time points during development. When cardiosulfa was added at initiation and washed away before 12 hpf, normal heart morphology was observed (Figure 1e). Similarly, embryos exposed to cardiosulfa after 24 hpf did not show any apparent effects on heart development. However, embryos exposed to this sulfonamide between 12 and 24 hpf exhibited heart deformation. The results, obtained from temporal control experiments, indicate that a critical stage for heart development occurs between 12 and 24 hpf.[4c]

Several cardiosulfa analogues that contain a nitro and/or trifluoromethyl group at the *ortho*, *meta*, and *para* positions of the benzene ring were prepared using solution-phase chemistry to explore structure–activity relationships (Supporting Information, Table S1). Each analogue (concentrations of 10, 20, and 30 μM) was added to zebrafish embryos at the initiation of the experiments. Analogues with a single substituent at the *ortho* and *para* position (**2**, **5**, **7**; Supporting Information, Table S1) induced abnormal heart development although they were slightly less effective than cardiosulfa. In contrast, *meta*-substituted analogues (**3**, **4**, **6**; Supporting Information, Table S1) did not perturb heart development. Based on this limited analysis, it seems that substitution at the *meta* position abolishes the abnormal heart formation effect during zebrafish development.

The status of sarcomeric proteins in the heart were examined by staining cardiosulfa-exposed zebrafish embryos with two monoclonal antibodies, MF-20 and S46, that are commonly employed to identify cardiovascular cells and to screen mutated hearts (whole-mount immunostaining). Whereas MF-20 recognizes a sarcomeric myosin heavy chain present in both the ventricle and atrium, S46 binds to an atrium-specific sarcomeric myosin heavy chain in zebrafish.[11] Both antibodies were found to stain the atrium and ventricle in cardiosulfa-treated embryos, suggesting that sarcomeric proteins are present at normal levels throughout heart development in the treated zebrafish (Supporting Information, Figure S1).

The expression pattern of heart-related genes in whole embryos was examined by using digoxigenin-labeled antisense RNA probes (whole-mount in situ hybridization). This technique enables the detection of specific genes in morphologically preserved embryos. Two zebrafish cardiac myosin genes, *cmlc2* and ventricular myosin heavy chain (*vmhc*), were used to distinguish two populations of myocardial precursors at an early stage. Whereas *cmlc2* is expressed throughout both chambers, *vmhc* is expressed throughout the ventricle but not in the atrium.[9] The results obtained from these experiments show that by 48 hpf, expression patterns of two genes are nearly identical in both the untreated and treated embryos (Figure 2a), suggesting that cardiosulfa does not affect early heart development in zebrafish.

As experiments of whole-mount in situ hybridization are difficult to perform at late developmental stages, expression patterns of heart-related proteins after 48 hpf were analyzed by using Tg(*cmlc2:GFP*) transgenic embryos. By 54 hpf, the

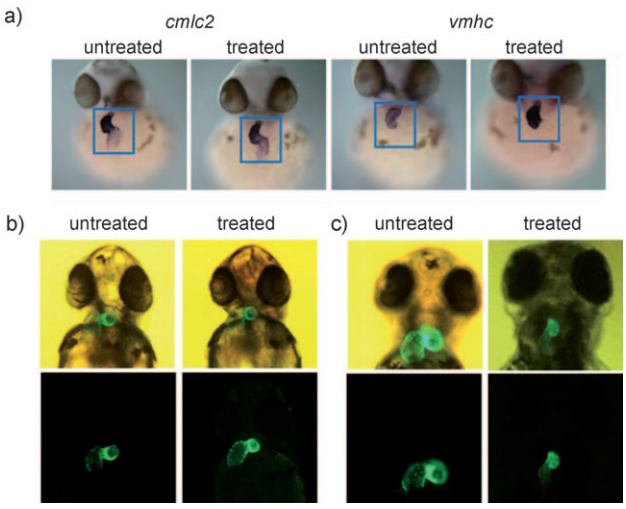

Figure 2. Expression of heart-specific genes and proteins in whole embryos. a) *cmcl2* and *vmhc* expression analyzed by whole-mount in situ hybridization at 48 hpf. b) Views of Tg(*cmlc2:GFP*) transgenic zebrafish embryos at 54 hpf and c) 60 hpf. Embryos were exposed to 20 μM cardiosulfa at the initiation of experiments.

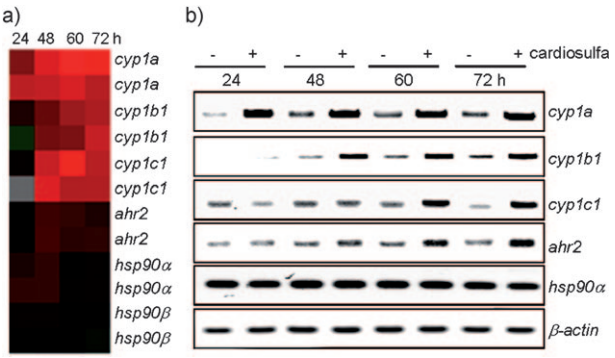

Figure 3. Gene expression profiling of 24 h-, 48 h-, 60 h-, and 72 h-old zebrafish embryos. a) Data represent increased (red) or decreased (green) gene expression that has changed more than twofold at various time points after exposure to 20 μM cardiosulfa at the initiation of experiments. Relative gene expression was obtained using zebrafish 44 K DNA chips. b) RT-PCR data for untreated and treated embryos exposed to 20 μM cardiosulfa at the initiation of experiments.

hearts of embryos exposed to cardiosulfa are phenotypically normal (Figure 2b). However, at 60 hpf, the exposed embryo is a narrow and elongated tube even though no significant change takes place in the expression patterns of heart-related proteins. These results demonstrate that cardiosulfa does not induce changes in the expression patterns of heart-related genes, such as *cmlc2* and *vmhc*, by 54 hpf, but that it does cause abnormalities in the heart at later stages of development.

Understanding the mode of action of cardiosulfa should lead to important insights into the mechanisms involved in heart development and disease states. We carried out gene expression profiling by using zebrafish 44 K DNA chips to assess the effects of cardiosulfa on heart development. Gene expression profiles were obtained on 24 h-, 48 h-, 60 h-, and 72 h-old zebrafish after early exposure of the embryos to 20 μM of this compound. It was observed that known housekeeping genes, such as β-actin, were not affected by cardiosulfa treatment. Importantly, this sulfonamide induces a high expression level of genes involved in aryl hydrocarbon receptor (AhR)-mediated signaling pathways. For example, cardiosulfa causes the remarkable expression of *cyp1a* (cytochrome P450 1A), *cyp1b1* (cytochrome P450 1B1) and *cyp1c1* (cytochrome P450 1C1), members of the aryl hydrocarbon receptor (AhR) gene battery (Figure 3a and Supporting Information, Table S2).[12] Furthermore, aryl hydrocarbon receptor 2 gene (*ahr2*) displays a subtle but significant induction in the exposed embryos. However, gene expression of Hsp90, which is associated with AhR, is not affected by cardiosulfa.[13]

To obtain additional evidence for the cardiosulfa-promoted changes in expression levels of these genes, reverse-transcription–polymerase chain reactions (RT-PCR) using treated and untreated embryos were carried out. The results of RT-PCR measurements of the genes *cyp1a, cyp1b1, cyp1c1, ahr2*, and *hsp90* were generally consistent with the results arising from microarray analysis (Figure 3b). As it is known that activation of AhR-mediated signaling pathways that leads to expression of genes such as *cyp1a* and *cyp1b1* produces cardiovascular malformations in fish, birds, and mice,[14] we surmised that the effects of cardiosulfa on heart development might occur by an AhR-mediated mechanism.

To gain more insights into the mode of action of cardiosulfa, two experiments using (−)-epigallocatechin-3-gallate (EGCG) and AhR2-morpholino (MO) antisense were carried out. Because Hsp90 is essential for the activation of AhR-mediated transcription of *cyp1a* and *cyp1b1* genes, an inhibitor of Hsp90, such as EGCG, suppresses AhR-mediated signaling pathways.[15] Consequently, it is expected that administration of an inhibitor for Hsp90 would cause normal heart development in cardiosulfa-exposed embryos.

The effect of EGCG on heart development in zebrafish embryos was determined. The results show that 150 μM of this compound does not affect zebrafish development (Figure 4c). Next, embryos were exposed to both 150 μM EGCG and 15 μM cardiosulfa at the initiation of the experiments; impaired heart development was not observed in embryos exposed to both compounds (Figure 4d). Changes of the expression of AhR target genes in embryos treated with cardiosulfa and EGCG were then examined by RT-PCR. Expression level of these genes in embryos treated with both compounds was significantly reduced in comparison with that in embryos treated only with cardiosulfa (Supporting Information, Figure S2).

The observed rescue of heart phenotype by EGCG may provide some evidence for heart deformation induced by cardiosulfa possibly by an AhR-mediated mechanism. However, as EGCG is known to have other functions than inhibition of Hsp90,[16] we also performed knockdown experiments using AhR2-MO antisense to more directly examine the effect of cardiosulfa on an AhR2-mediated signaling pathways. AhR2-MO was microinjected into the zebrafish embryos at the initiation of experiments; embryos treated with AhR2-MO did not show any effect on heart development (Figure 5c). Importantly, abnormal heart morphology

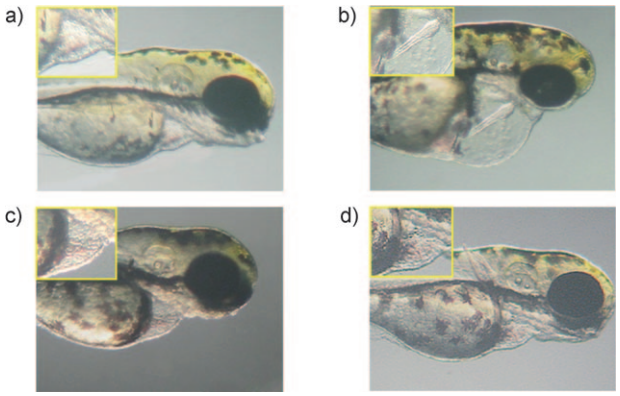

Figure 4. Rescue of heart phenotype by EGCG. Three-day-old embryonic zebrafish a) untreated, b) exposed to 15 μM cardiosulfa, c) exposed to 150 μM EGCG, and d) exposed to both 150 μM EGCG and 15 μM cardiosulfa.

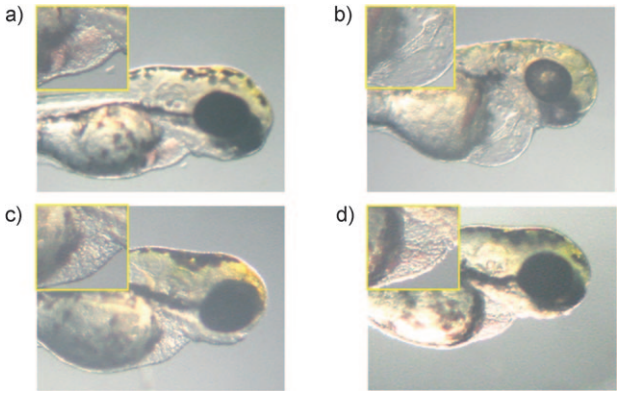

Figure 5. Effect of AhR2-MO on heart development of embryos treated with cardiosulfa. Three-day-old embryonic zebrafish a) after exposure to vehicle, b) exposed to 15 μM cardiosulfa, c) after exposure to AhR2-MO, and d) after exposure to both AhR2-MO and 15 μM cardiosulfa.

was not observed in embryos exposed to both substances (Figure 5d). The results obtained from RT-PCR show that expression of *ahr2* and *cyp1a* in embryos treated with AhR2-MO or both substances is suppressed in comparison with that in embryos treated only with cardiosulfa (Supporting Information, Figure S3). The results obtained from experiments using EGCG and AhR2-MO suggest that the effect of cardiosulfa on abnormal heart development is likely to be a consequence of its activation of AhR2. Alternatively, it is possible that cardiosulfa activates xenobiotic metabolizing enzymes owing to its toxicity, and this may be responsible for heart deformation. Further work will focus on understanding detailed mechanism of abnormal heart development elicited by cardiosulfa.

In conclusion, we have identified a novel small molecule that impairs zebrafish cardiovascular development and function, and evokes a striking edema response in the pericardial and yolk sac. This compound can be used to unveil mechanism and pathways relevant to heart disease. The observations made in this investigation build upon recent findings that illustrate the value of chemical biology approaches as tools for the elucidation of differentiation and development processes.[17]

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