Small molecule developmental screens reveal the logic and timing of vertebrate development

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Much has been learned about vertebrate development by random mutagenesis followed by phenotypic screening and by targeted gene disruption followed by phenotypic analysis in model organisms. Because the timing of many developmental events is critical, it would be useful to have temporal control over modulation of gene function, a luxury frequently not possible with genetic mutants. Here, we demonstrate that small molecules capable of conditional gene product modulation can be identified through developmental screens in zebrafish. We have identified several small molecules that specifically modulate various aspects of vertebrate ontogeny, including development of the central nervous system, the cardiovascular system, the neural crest, and the ear. Several of the small molecules identified allowed us to dissect the logic of melanocyte and otolith development and to identify critical periods for these events. Small molecules identified in this way offer potential to dissect further these and other developmental processes and to identify novel genes involved in vertebrate development.

Mutagenesis followed by phenotypic screening has proven to be a powerful approach for identifying genes involved in developmental processes. By altering a gene's sequence and modulating its function, mutagenesis can result in a permanent, heritable change in the phenotype of an organism. By binding to specific proteins, small molecules also can modulate gene product functions and result in changes in an organism's phenotype in a nonheritable manner. For example, the small molecule cyclopamine causes cyclopia in the developing vertebrate (1, 2). The ability to modulate function specifically and rapidly makes small molecules especially useful tools for studying processes like development in which the timing of protein function is critical. Currently, systematic methods for identifying small molecules that alter specific developmental processes are limited, so the number of useful, small molecule developmental probes remains small.

As a step toward dissecting specific developmental processes, we have performed a screen for small molecules that affect vertebrate development. It has been estimated by mutagenesis studies that modulation of any one of about 2,500 gene products will result in a specific, visible developmental defect. Therefore, we chose to perform this screen using whole embryos to maximize the number of potential targets that could be screened simultaneously. Furthermore, by using whole embryos for our screen, we hoped to eliminate candidate small molecules that had nonspecific effects on cell or organism viability and to select for small molecules to which the embryo is permeable. The zebrafish *Danio rerio* was selected for this screen because of its small embryo size, large clutch sizes, and permeability to small molecules, and because it develops *ex utero* as a transparent embryo, offering visual access to most systems throughout development (3, 4). Furthermore, because development occurs *ex utero*, maternal effects caused by the small molecules will not influence embryonic development. Some of these zebrafish attributes formed the basis for the highly successful genetic screens that provided many insights into zebrafish development (5, 6). Here, we demonstrate that these attributes also facilitate the identification of small molecules that modulate vertebrate development and show that these small molecules can be used to determine the timing of critical developmental events.

Methods

A large number of fertilized eggs was generated by group matings of wild-type Ekwill and AB zebrafish strains as described (7). Synchronized embryos were collected and arrayed by pipette, three embryos per well, in 96-well plates containing 200 μl of embryo buffer (7) supplemented with 40 units of penicillin G and 40 μg of streptomycin. Synthetic small molecules from the DiverSet E were obtained from Chembridge Corporation, San Diego, and prepared as 5 mg/ml stock solutions in dimethyl sulfoxide. Aliquots (100 nL) of the stock solutions were robotically pin transferred to 384-well plates containing 50 μl of fish water (7) per well. The fish water samples containing the small molecules were then added to the arrayed zebrafish embryos at the 8–16 cell stage. Embryos were grown at 28.5°C and examined visually with a dissecting microscope at 1, 2, and 3 days post fertilization (dpf). Phenotypes were recorded only when exhibited by every embryo in a well. Embryos were photographed live or after fixation with 4% paraformaldehyde in PBS. Phenotype-inducing small molecules were further tested on TL and Tübingen zebrafish strains, where they induced similar phenotypes with the exception of 31N3 (see *Results*), which was inactive in these strains.

Results and Discussion

Synchronously fertilized zebrafish eggs were collected and arrayed in 96-well plates (three embryos per well) containing embryo buffer. Pure samples of 1,100 synthetic small molecules selected randomly from a small molecule library were added to the embryo buffer to a concentration of $\approx$1 μM. Treated embryos were examined visually under a dissecting microscope for developmental defects 1, 2, and 3 dpf. Four systems that differ significantly between vertebrates and invertebrates were selected for the screen—namely the central nervous system, the cardiovascular system, pigmentation, and the ear. Careful visual examination of all of these systems limited the number of compounds that could be screened by one person to about 400 compounds per day. Many more compounds could theoretically be screened by using a more specific visual screen or by using transgenic fish possessing a reporter gene. In this screen, approximately 2% of the small molecules screened were lethal at very early stages of development or caused general necrosis of the embryos at $\approx$1 μM concentration. In addition to these small
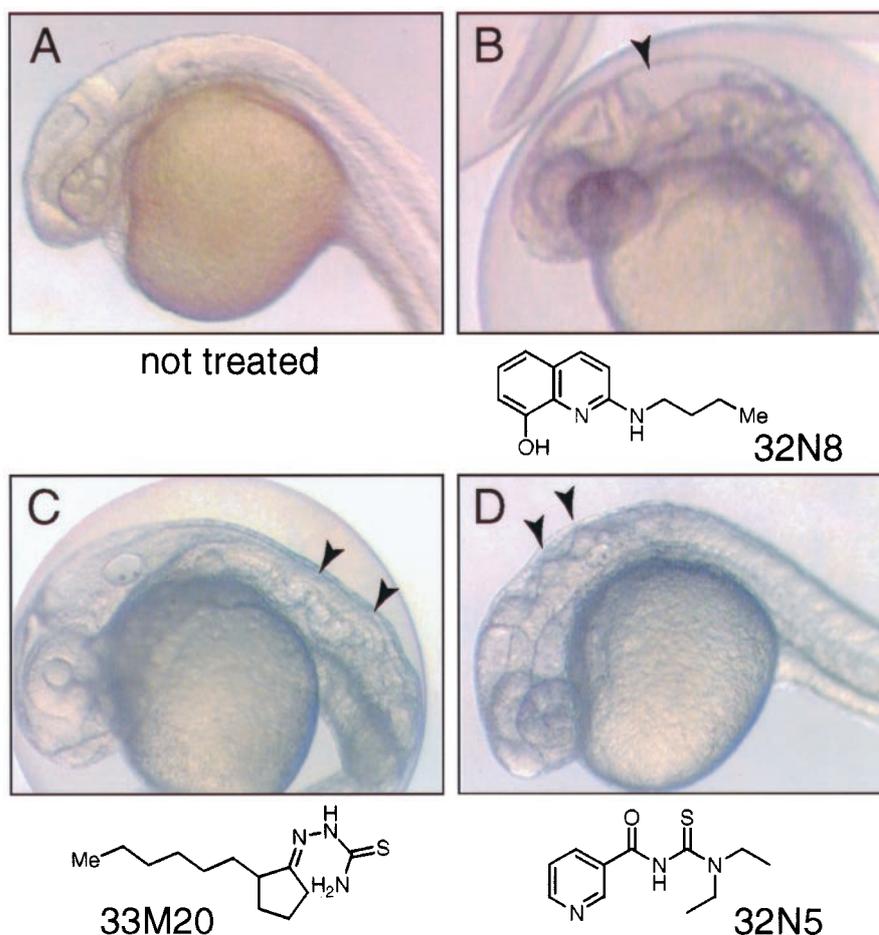

DEVELOPMENTAL BIOLOGY

CHEMISTRY

Fig. 1. Small molecules alter development of the central nervous system. (*A*) Untreated zebrafish embryo. (*B*) Embryo treated with the small molecule 32N8 at a concentration of 2 μg/ml. The enlarged hindbrain ventricle is indicated by an arrowhead. (*C*) Embryo treated with the small molecule 33M20 (2 μg/ml). The folds in the notochord are indicated by arrowheads. (*D*) Embryo treated with the small molecule 32N5 (2 μg/ml). Hindbrain projections are indicated by arrowheads. All embryos were photographed 25 hpf.

molecules, approximately 1% of the small molecules screened affected a specific aspect of one of the systems examined.

Many structures of the zebrafish central nervous system are visible 24 h post fertilization (hpf) (ref. 8; Fig. 1*A*), and we have identified several compounds that affect the morphology of the central nervous system. The small molecule 32N8 causes a dramatic increase in the size of the hindbrain ventricle (Fig. 1*B*). 33M20 causes elongation of the notochord, forcing it to buckle along the axis of the trunk (Fig. 1*C*). The small molecule 32N5 causes sawtooth-like projections to form along the hindbrain that resemble the duplicated neural folds of *atlantis* mutants (9) (Fig. 1*D*). In each case, all of the embryos exposed to a given small molecule exhibited the phenotype described. Small molecules that affect the cardiovascular system were also identified. For example, 31J6 (Fig. 2*A*) increases the ratio of atrium to ventricle contractions to 2:1, instead of the usual ratio of 1:1. This phenotype resembles the human cardiovascular condition known as second-degree atrioventricular heart block. The small molecule 32P6 causes two edematous pericardial sacs to form, one on each side of the body as occurs in zebrafish mutants with the condition cardia bifida (10, 11), instead of a single heart forming in the middle (Fig. 2*B*). However, in the case of 32P6-treated fish, a beating heart is present only in the left pericardial sac. Circulation is absent from 32P6-treated fish, and the heart ventricle is reduced in size.

The pigment cells that form the characteristic zebrafish stripes are derived from neural crest cells that are specified to become melanocytes, migrate to proper locations in the body, differentiate, and produce pigment (12, 13). Zebrafish also possess pigment-producing cells in the pigmented retinal epithelium (PRE) that are not derived from the neural crest (12). We have identified small molecules that disrupt pigment production as well as a small molecule that may affect specification of neural crest cells into melanocytes. The small molecule 31B4 prevents pigmentation throughout the body and in the PRE (Fig. 3*A*). When 31B4-treated fish are transferred to fresh water, pigment appears in cells in the expected numbers and locations after a few hours (Fig. 3*C*), suggesting that 31B4 affects general pigment production rather than specification or migration of melanocytes. 31B4 is structurally related to phenylthiourea (PTU), a tyrosinase inhibitor (14) that is used routinely to inhibit pigment production in zebrafish. However, 31B4 is about 20 times more potent than PTU (data not shown). Therefore, it is likely that 31B4 is a potent inhibitor of tyrosinase.

The small molecule 33N14 also inhibits pigmentation in the zebrafish body, but it does not prevent pigmentation of the non-neural crest-derived PRE (Fig. 3*B*). Unlike 31B4-treated fish, 33N14-treated fish transferred to fresh water 3 dpf develop only a few pigment-producing cells. These few cells are in appropriate locations and appear to produce normal amounts of pigment, but the number of pigment-producing cells remains
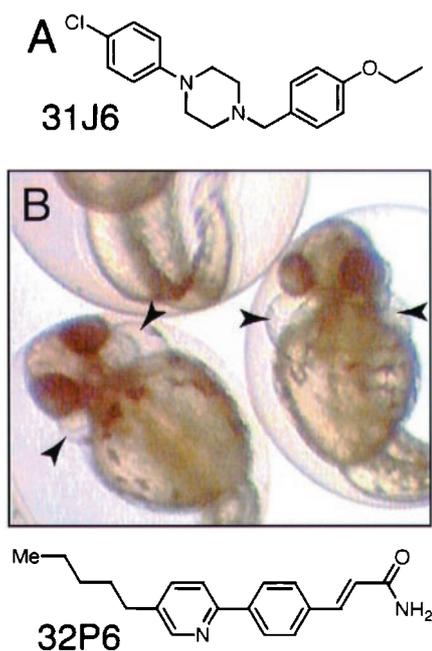
Fig. 2. Small molecules alter development of the heart. (*A*) The structure of the small molecule 31J6. (*B*) Ventral view of embryos treated with the small molecule 32P6 (2 μg/ml). The two putative pericardial sacs are indicated by arrowheads. Embryos were photographed 76 hpf.

dramatically reduced (Fig. 3*D*). Therefore, it appears that 33N14 blocks the specification of neural crest cells into melanocytes or their proliferation, but does not inhibit the migration of mela-

nocytes nor the production of pigment itself. When 33N14 is removed before 2 dpf, normal numbers of melanocytes form, suggesting that melanocyte specification/proliferation can occur up to this point in development, but is limited thereafter (data not shown).

We identified one compound that specifically affects ear development. As shown in Fig. 4, embryos treated with the small molecule 31N3 possess clearly visible otic vesicles but do not develop otoliths within the otic vesicles. Otoliths are small, bony structures that are physically linked to bundles of hair cells in the ear. These structures move in response to gravity, inertia, and sound, allowing the zebrafish to sense vibrations and maintain balance. Aside from the absence of otoliths, embryos treated with 31N3 appear normal. Other structures of the inner ear, including the otic vesicle and semicircular canals, develop normally. However, 31N3-treated fish are unable to maintain balance and often swim on their sides or upside down.

We took advantage of the temporal control afforded by small molecules to define the developmental stage at which 31N3 inhibits otolith development. By adding or washing away 31N3 at various time points during development, we demonstrated that a critical stage for development of otoliths occurs between 14 and 26 hpf (Fig. 4*C*). When 31N3 is added at the initiation of the experiment but washed away before 14 hpf, otolith development occurs normally. Similarly, adding 31N3 after 26 hpf has no apparent effect on otolith formation. By waiting until 14 hpf to add 31N3, we were also able to identify embryos that develop one otolith, often unilaterally, instead of the two otoliths that normally form on each side (Fig. 4*E*). This finding suggests that each otolith develops independently and that, occasionally, the cells that give rise to an individual otolith are no longer sensitive to the effects of 31N3 by 14 hpf. When 31N3 was added a few hours earlier at 8 hpf, no otoliths were observed in any of the fish.
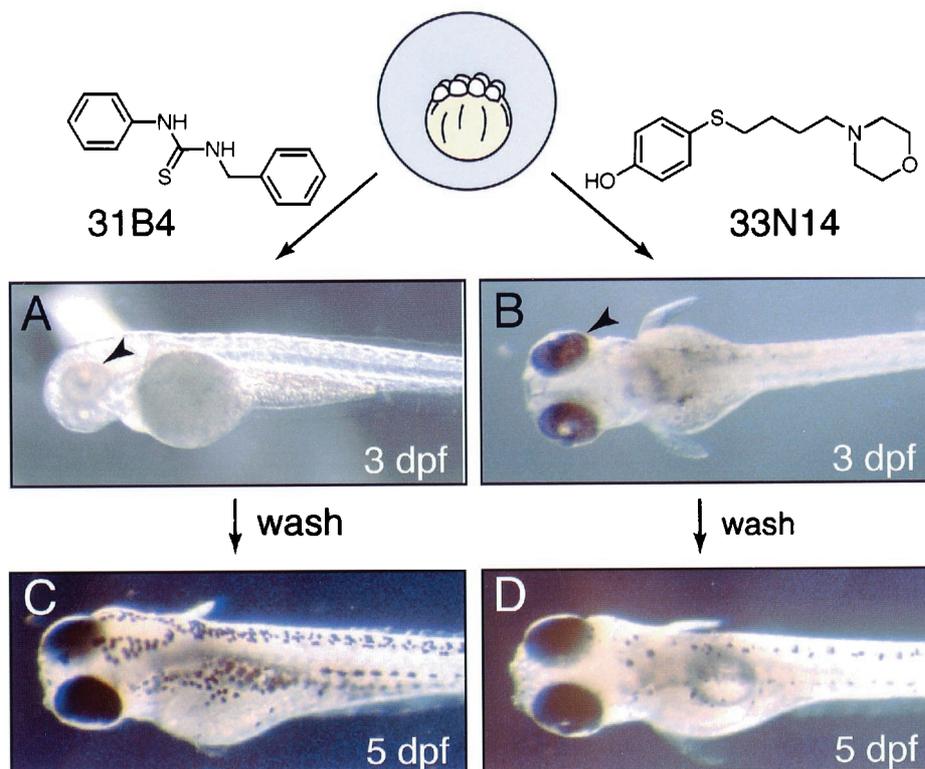

Fig. 3. Small molecules affect specific stages of pigment cell development. Zebrafish embryos were treated with 2 μg/ml of the small molecules 31B4 (*A*) or 33N14 (*B*) during the 3 dpf, after which the embryos were transferred to fresh water and allowed to grow for an additional 2 days in the absence of the small molecules (31B4 and 33N14 in *C* and *D*, respectively). The PRE is indicated by arrowheads.
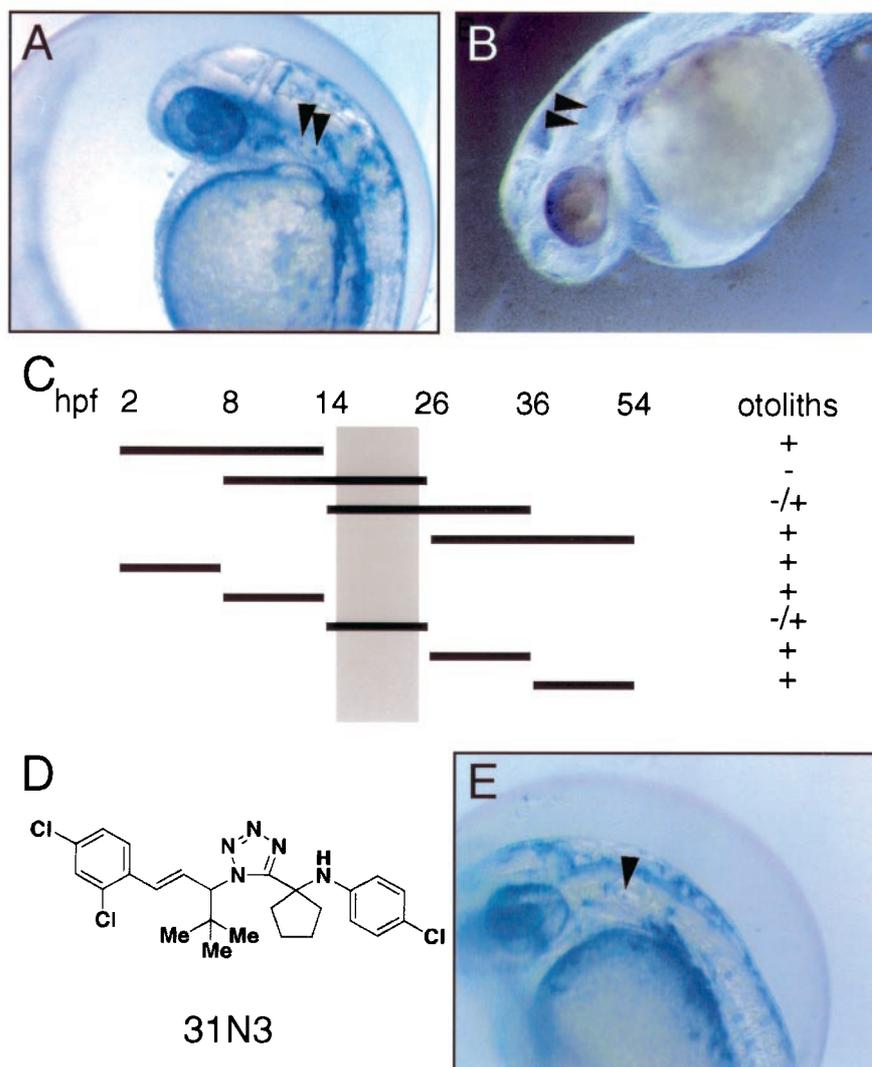
Fig. 4. The small molecule 31N3 alters ear development between 14 and 26 hpf. (*A*) Untreated zebrafish embryo photographed 36 hpf. The two otoliths are indicated with arrowheads. (*B*) Embryo treated with 31N3 from 2 to 36 hpf and then photographed. The expected locations of the two otoliths are indicated by arrowheads. (*C*) Embryos were treated with 31N3 at a concentration of 2 μg/ml during the times indicated by black bars, after which the embryos were transferred to fresh fish water. The presence of otoliths was scored at 54 hpf. (*D*) The structure of the small molecule 31N3. The olefin is *trans* as confirmed by nuclear magnetic resonance, coupling constant 15.6 Hz. (*E*) Embryo treated with 31N3 from 14 to 26 hpf and then allowed to develop in the absence of 31N3 until 36 hpf. The embryo was photographed 36 hpf. The single otolith is indicated by an arrowhead.

Thus, the critical event affected by 31N3 that allows initiation of otolith formation seems to occur at about 14 hpf. Moreover, the reversibility of small molecule-mediated gene product modulation provides information that is not obtainable through the use of genetic mutants.

Some of the small molecules identified in this study produce phenotypes that are similar to those caused by genetic mutations. For example, the heart contractility of fish treated with the small molecule 31J6 is similar to that of *breakdance* mutants (11). Also, like 31N3-treated fish, *keinstein* and *empty ear* mutants do not form otoliths (15, 16). In such cases, the small molecule targets are likely to be products of the mutated genes or other proteins involved in the same biological pathway, allowing epistasis-like analyses to be performed. In other cases, small molecules may be able to disrupt functions that are not disruptable by traditional genetic methods. For example, genetic screens are unlikely to detect loss of gene function when more than one copy or functional isoform of that gene exists, whereas small molecules can disrupt products from all copies of a given gene.

The small molecules identified by this approach are likely to vary in the specificity with which they modulate particular gene products. Until the target of a given small molecule is identified, it will be difficult to demonstrate conclusively its specificity for that target. However, phenotypic specificity and reproducibility over a broad concentration range are suggestive of high molecular specificity for a given gene product. Small molecules with poor specificity would be expected to cause a broad range of developmental defects, especially at high concentrations. In contrast, the small molecule 31N3 completely blocks formation of otoliths with no apparent effect on other systems. Furthermore, we have tested the effects of 18 compounds that are structurally related to 31N3 but possess minor chemical modifications. None of the related compounds affects otolith formation (data not shown), suggesting a specific, sensitive interaction between 31N3 and its target. The small molecule 32P6 also appears to be specific as judged by its phenotypic reproducibility. 32P6 causes virtually the same phenotype when used at a concentration of 6 μM as it does when used at a concentration

of 6 nM ($ED_{50} \approx 2$ nM), suggesting that it disrupts a specific developmental process at least 1,000 times more potently than it interferes with other visible developmental processes. Therefore, the potency of these compounds, their sensitivity to structural modification, and the apparent absence of general developmental defects suggest that many of the small molecules identified in this screen have specific molecular targets.

The results of this study indicate that large-scale developmental screens can identify small molecules that disrupt developmental events with specificity approaching that of genetic mutation. This "chemical genetic" approach (17–20) is rapid, inexpensive, requires no long-term breeding, and can, in theory, target every gene product in the vertebrate genome through a variety of physiological and behavioral screens. More important, the small molecules identified make instantaneous, temporal control of protein function possible. This temporal control can provide insight into the timing of developmental events and the nature of the modulated processes, as illustrated here for otolith development and neural crest specification. Recent advances in

diversity-oriented organic synthesis will likely yield a rich bounty of small molecule modulators of developmental processes in the near future (20).

The targets of the small molecules identified by these screens can be discovered by a variety of techniques (21). For example, the small molecules can be covalently linked to resins to produce affinity reagents that can be used for biochemical purification and sequencing of the target proteins. Identifying the targets of small molecule developmental modulators in this way should provide insight into developmental processes at the molecular level and contribute to the process of linking vertebrate genes with their functions.

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