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Toxic Effects of Cephalosporins with Specific Functional Groups as Indicated by Zebrafish Embryo Toxicity Testing

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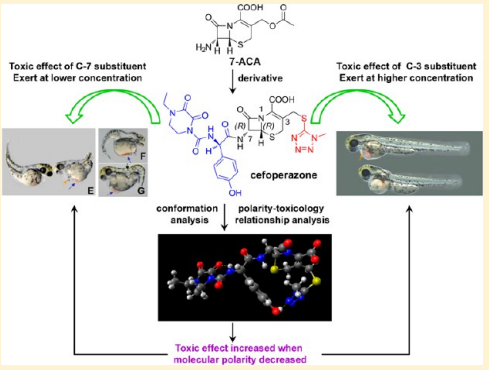
Supporting Information

ABSTRACT: Cephalosporins, derivatives of 7-aminocephalosporanic acid (7-ACA), are potent antibacterial agents. The toxicity prediction of these compounds is of considerable importance in new drug development. Zebrafish embryo toxicity testing was thought to be suitable for evaluation of the toxic properties of cephalosporins. Here, five kinds of cephalosporins and their isomers were used for investigation of the toxic functional groups of cephalosporins and for further evaluation of the efficacy of zebrafish embryo toxicity testing. Computational chemistry methods were also used to study the conformations of the stereoisomers of cephalosporins in aqueous solution to explore the relationship between the stereoisomers and the experimental results of toxicity tests on zebrafish embryos. Our results suggest that both the C-7 and C-3 substituents of cephalosporins are toxic functional groups. The toxic functional groups increase the toxic reaction of 7-ACA and can induce variable abnormal phenotypes in zebrafish embryo toxicity testing. The embryonic toxicities of cephalosporins were involved in organogenesis, mainly in the development of the cranial nerve, cardiovascular system, notochord and abdomen, and pigment formation; those tissues and organs are derived from ectoderm, mesoderm, and endoderm. The theoretical calculations showed a strong negative correlation between topological polar surface area (TPSA) values and the toxic effect, which indicated that molecular polarity may be crucial to the toxic effects of the isomers of cephalosporins. The concept of toxic functional groups may help us understand the safety differences of cephalosporins.



1. INTRODUCTION

The discovery of β-lactam antibiotics has had a profound impact on human health by enabling rapid treatment of patients with bacterial infections. Cephalosporins, which are β-lactam antibiotics, are potent antibacterial agents. They have maintained their glamour among medicinal chemists for over 60 years. Extensive research has produced four generations of cephalosporins that have already seen clinical use.[1] The fifth generation, which includes ceftaroline and ceftobiprole, has been launched,[2] and some new candidates are undergoing clinical evaluations.[3,4] Cephalosporins are a kind of derivatives from 7-aminocephalosporanic acid (7-ACA). Chemical alterations are used to design new compounds that may overcome the emergence of drug-resistant bacteria and the economic and regulatory challenges of antibiotic research. The principal strategy in the design of new cephalosporins is structural modifications at C-3 and C-7, which impart lipophilic and basic properties.[5,6] The structure-bioactivity relationships of cephalosporin side chains have also been explored.[5−8] However, few reports have described toxic functional groups of cephalosporins in detail, even though the toxicity of these new drugs is of considerable importance.

Improving drug candidate safety by design based on physicochemical properties is challenging and competitive for new drug development.[9] Zebrafish have recently been used in toxicity assessment.[10−13] Our previous study indicated that zebrafish embryo toxicity testing may be suitable for investigations of the toxic functional groups of cephalosporins.[13] Results showed that the 2-mercapto-5-methyl-1,3,4-thiadiazole (MMTD), the 3′-side chain of cefazolin sodium (CFZL), and cefazedone sodium (CFZD) have a close relationship with the teratogenic effects of these agents on the embryonic development of zebrafish. Compounds with MMTD structures mainly interfere with the development of tissues and organs derived from embryonic ectoderm and mesoderm. For these reasons, MMTD was once considered a toxic functional group. Here, five kinds of cephalosporins and their isomers were used for further evaluation of the efficacy of the zebrafish embryo toxicity testing and for investigations of the toxic functional groups of the cephalosporins; also, three kinds of cephalosporins were used for verification.

2. MATERIALS AND METHODS

2.1. Samples. The reference standards of cefoperazone and its 7-epimer ((6*R*,7*S*)-cefoperazone); cefaclor and its Δ-3 isomer; cefepime, ceftriaxone, cefotaxime, and their (*E*)-isomers; ceftizoxime, cefmenoxime, cefmetazole, 7-aminocephalosporanic acid (7-ACA), 1-methyl-1*H*-tetrazole-5-thiol (MTT), and *N*-methyl-2-pyrrolidone

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(NMPD) were obtained from the National Institutes for Food and Drug Control (NIFDC). The batch and content/purity information of the reference standards are provided in Table S1 in the Supporting Information.

2.2. Preparation of Test Solution. All test samples were dissolved in water and diluted to stock solutions, and if necessary, an appropriate amount of Na_2CO_3 solution was added to help solutes dissolve. Then, the solutions were diluted with artificial seawater (Instant Ocean sea salts, Tropical Marine, Tianjin Casic Marine Biotechnology Co., Ltd., China) into a series of test solutions based on the results of the pre-experiments.

2.3. Zebrafish Embryo Toxicity Testing.[13] Zebrafish (*Danio rerio Tuebingen*) were reared in the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Beijing Union Medical College. Thirty embryos at the 50% epiboly stage (midgastrula) were immersed in 3−4 mL of test solutions in a dish with a diameter of 20 mm until three days postfertilization (3 dpf). Three or four doses of each compound were administered to different groups. The zebrafish control liquid containing the same feeding water without the study compounds used for bathing the 30 embryos was used as a normal control. The embryos were incubated in a standard environment, and their development was observed daily.[14] The rates of abnormal embryonic development, rates of mortality, and number of surviving embryos were statistically analyzed from the third day on. Each experiment was repeated at least three times. The concentration of 50% teratogenic rate (ED_{50}) and the concentration of 50% death rate (LD_{50}) were calculated if necessary.

2.4. Theoretical Study on Molecular Conformational Analysis in an Aqueous Environment. *2.4.1. Molecular Mechanical Computation.* The initial 3D chemical structures were obtained from the PubMed online compound database. Structures were modified manually if necessary. Conformer generation and minimization were performed with Accerlys Discovery Studio 2.5.5, and the "best" algorithm was used to generate possible conformers. For each structure, the relative energy threshold between conformers was limited to 50 kcal/mol, and a maximum of 1023 conformers were generated. Then conformer minimization was performed using the CHARMm force field with the generalized Born with molecular volume (GBMV) implicit solvent model. The dielectric constant of water was fixed to 80.0. After conformation minimization, conformers with the lower energies and characteristic frameworks were selected as global minima candidates for further quantum chemical optimization.

2.4.2. Quantum-Mechanical Study. Quantum-mechanical geometry optimization and thermochemistry calculations were performed with the ORCA 2.9.1 program.[15−17] The density functional theory (DFT) method BP86 with Grimme's latest London-dispersion correction[18,19] (BP86-D_3) and Ahlrich's new triple-ζ valence (def2-TZV) plus polarization basis sets[20] were used. All the conformers were first optimized at the def2-TZVP(-df) basis set level to locate the most stable conformers. Then, these conformers were reoptimized at the def2-TZVP(-f) basis set level, and harmonic vibrational frequencies were used to verify that all the structures were minima on the potential energy surface. These computations took advantage of the resolution of the identity (RI) approximation with the auxiliary def2-TZVP/J Coulomb fitting basis set[21] to accelerate the calculation process.

The def2-TZVPP basis set was used for final single-point energy calculation for thermochemistry of the minima structures. A COSMO (conductor-like screening model) solvation model[22] with a dielectric constant of 80.0 was used throughout the quantum-mechanical study to represent an aqueous environment. The geometries were visualized using Avogadro software.[23]

2.4.3. Calculation of Molecular Polarity. The molecular polarity of the minima structures were computed using topological polar surface area (TPSA) methodology with Molden 5.0.[24]

3. RESULTS AND DISCUSSION

3.1. Toxic Effects of Cefaclor and Its Δ-Isomer. Δ-Isomers are impurities found in cephalosporins,[25] and they are discussed in official compendia. Although the acid forms of cephalosporins are chemically stable, the naturally occurring Δ^3 double bond of the dihydrothiazing ring easily isomerizes to the Δ^2 position resulting in a loss of biological activity when the 4-carboxylic group is esterified or otherwise altered.[26,27] Cefaclor Δ-3 isomer with a Δ^2 double bond in its dihydrothiazing ring is a common impurity of cefaclor (Figure 1). It is produced during the synthesis process. Cefaclor and its Δ-3 isomer showed similar rates of teratogenicity with ED_{50} values of 1.6 mmol/L and 1.2 mmol/L, respectively (Figure 2a). They also produced similar abnormal phenotypes. For example, they caused smaller heads and eyes, twisted notochords, shortened body lengths, bent body axes, swollen abdomens, blood island congestion, and blurred and darkened body color (Figure 2b−c). The behavioral responses of 5 dpf larvae to outside stimulation were attenuated in a concentration-dependent manner. A lethality comparison showed that the toxicity of cefaclor (LD_{50} = 8.3 mmol/L) was a little greater than that of its Δ-3 isomer (LD_{50} = 14.1 mmol/L) (Figure 2a).

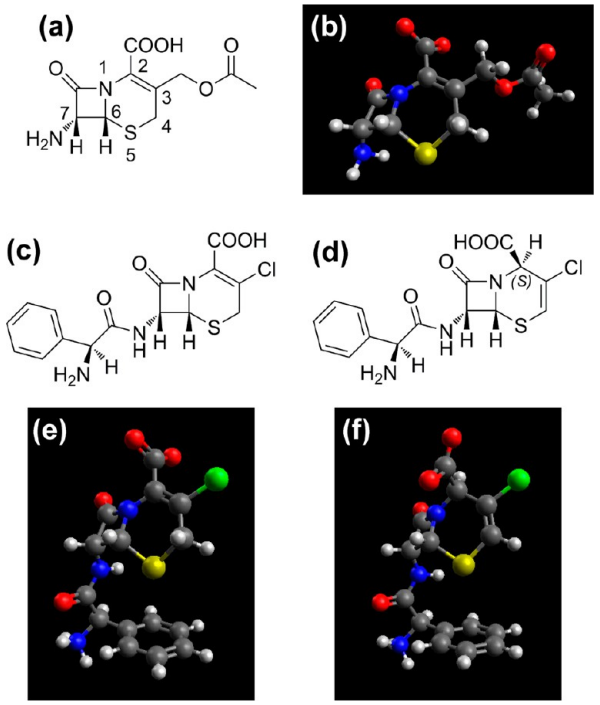

Figure 1. Structures of (a) 7-ACA, (c) cefaclor, and (d) its Δ-3 isomer; and the most stable conformations of (b) 7-ACA, (e) cefaclor, and (f) Δ-3 cefaclor.

The toxicity of 7-ACA, the lead compound of cephalosporins, was tested to determine its contribution to the toxicity of cephalosporins on embryonic development. It showed no significant difference between the WT group and 7-ACA group at 3.5 mmol/L; but when the concentration increased beyond 17.5 mmol/L, teratogenicity increased quickly and reached a peak at 52.5 mmol/L with 30% lethality. All embryos died at 70 mmol/L of 7-ACA at 3 dpf (Figure 2a). Phenotypes showed many of the same features of those produced by cefaclor and its isomer, except occasional yolk invagination with blood island congestion and cardiovascular teratogenicity at high concentrations (Figure 2d). These results suggest that the toxicity of 7-ACA during embryonic development is far less pronounced than that of either type of cefaclor. The substituent at the C-7 position of cefaclor is a toxic functional group that increases the toxic reaction to 7-ACA.

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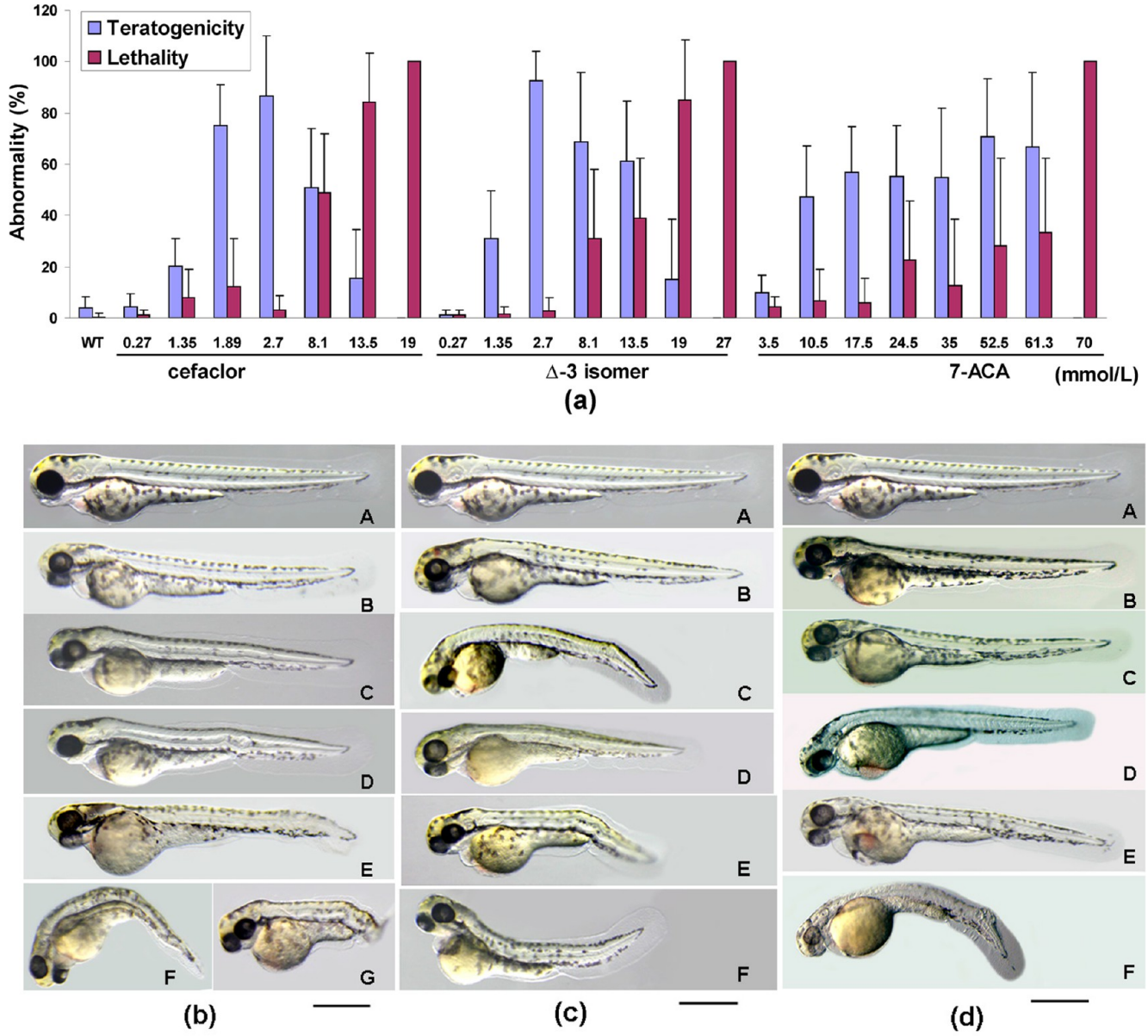

Figure 2. Toxicity of cefaclor, its Δ-3 isomer, and 7-ACA in zebrafish embryo testing. Zebrafish embryos were treated with cefaclor, its Δ-3 isomer, or 7-ACA from 6 hpf to 72 hpf. Then, photos were taken at 3 dpf. (a) Comparison of the toxicity results of cefaclor, its Δ-3 isomer, and 7-ACA on zebrafish embryonic development. The data are 3-dpf statistics. The teratogenic rates do not include the fatality rate. WT means wild type, which here represents untreated normal controls. (b) Abnormal phenotypes caused by cefaclor. A, WT, an untreated control; B−G, cefaclor at 1.4 mmol/L (B, C), at 1.9 mmol/L (D, E), at 2.7 mmol/L (F), and at 8.1 mmol/L (G). The scale indicates 550 μm. (c) Abnormal phenotypes caused by the cefaclor Δ-isomer. A, WT, an untreated control; B−F, cefaclor Δ-isomer at 1.4 mmol/L (B), at 2.7 mmol/L (C, D), and at 8.1 mmol/L (E, F). The scale indicates 550 μm. (d) Abnormal phenotypes caused by 7-ACA. A, WT, an untreated control; B−F, 7-ACA at 3.5 mmol/L (B), at 10.5 mmol/L (C), at 17.5 mmol/L (D,E), and at 24.5 mmol/L (F). The scale indicates 570 μm.

3.2. Toxic Effects of Cefoperazone and Its 7-Epimer.

Epimerization of cephalosporins at C-7 can take place during synthesis.[28] These epimers can also be degraded during alkaline hydrolysis.[29] Cefoperazone ((6*R*,7*R*)-cefoperazone) (Figure 3a) has a different structure from cefotaxime, ceftriaxone, and cefepime both at the C-7 and C-3 substituents. However, part of its C-7 substituent is similar to that of cefaclor. The 7-epimer of cefoperazone ((6*R*,7*S*)-cefoperazone) (Figure 3b) is described as an impurity in official compendia. The toxic effects of cefoperazone and its 7-epimer were compared for their teratogenicity and fatality on the development of zebrafish embryos. The ED_{50} of cefoperazone on teratogenicity was at about 28 mmol/L and that of the (6*R*,7*S*)-cefoperazone was at about 7 mmol/L. The death rates at 3 dpf were less than 10% in all groups treated with cefoperazone including at the highest concentration of 28 mmol/L; but in the groups treated with (6*R*,7*S*)-cefoperazone, the death rates were about 10% at 7 mmol/L and increased depending on the treated doses (Figure 4a). The abnormal phenotypes of the treated groups (Figure 4b−d) are mainly as follows. Embryos treated with 5−7 mmol/L of cefoperazone presented deformation such as serious twisting of the notochord, small heads and eyes, and short and bent body axes. However, when the concentration reached 14 mmol/L, two types of malformation occurred (Figure 4b). One type was similar to the phenotype described above and much like those exposed to cefaclor. The other one presented only a slightly short body and

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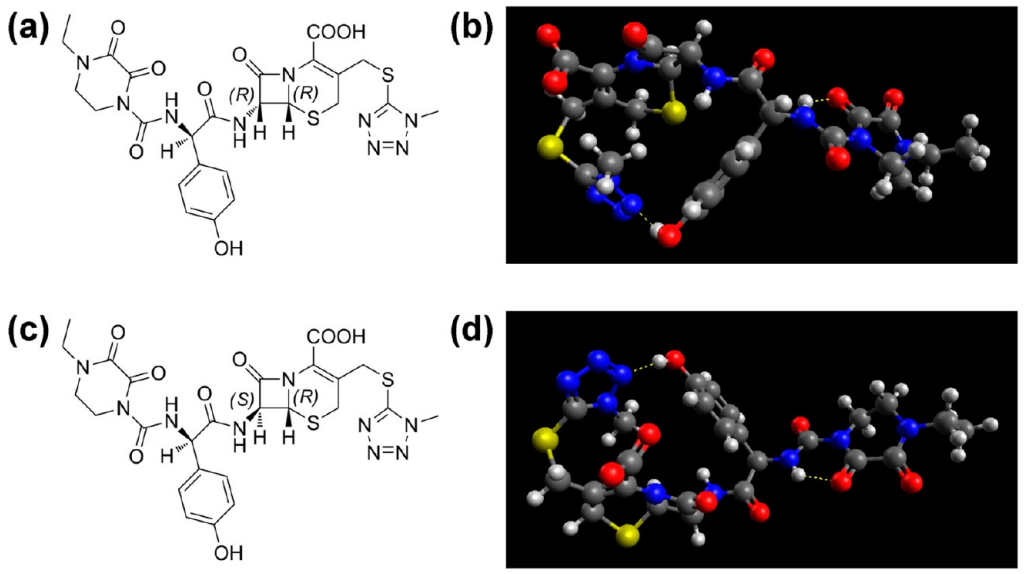

Figure 3. Structures of (a) cefoperazone ((6*R*,7*R*)-cefoperazone) and (c) (7*S*)-cefoperazone ((6*R*,7*S*)-cefoperazone); (b) the most stable conformations of cefoperazone and (d) (7*S*)-cefoperazone. The intramolecular hydrogen bonds are indicated by dashed lines.

cardinal vein congestion near the heart but no twisted notochord. At 4−5 dpf, the second type of surviving larvae maintained an inclined position, and only a few of them displayed active responsive potency on touching stimulation. They also showed opaque trunks and abdomens with cardinal vein congestion, heart abnormalities, and swelling of the pericardial sac. A few of the larvae died suddenly at 4 dpf, and almost all of them were dead at 5 dpf (Figure 4b). (6*R*,7*S*)-Cefoperazone produced nearly the same phenotype as cefoperazone (Figure 4c). Their C-3 substituents, MTT, also had some impact on embryonic development, such as delayed growth, weak heartbeats, small and abnormal heart structures, anterior-constricted yolk extension, and opaque yolks. At 4−5 dpf, nearly all the larvae died (Figure 4d). This was similar to the second type of deformed phenotype exposed to cefoperazone. These results suggest that there are at least two toxic functional groups, the C-7 substituent and the C-3 substituent, within the cefoperazone molecule. The two toxic groups showed different abnormal phenotypes of toxicity. At lower concentrations, the toxicity of cefoperazone was inferred to be driven by the C-7 side chain. At higher concentrations, both groups exerted their toxic effects separately and exclusively.

3.3. Toxic Effects of *syn*-/*anti*-Isomers of Cephalosporins with 2-Amino-5-thiazolyl Residues at C-7. The introduction of the 2-amino-5-thiazolyl ring in the C-7 side chain was a major advance in cephalosporin research. The *syn*-methoxyimino groups are responsible for the high stability of cephalosporins against β-lactamase hydrolysis by broad-spectrum β-lactamase. Cefotaxime was the first cephalosporin to have a *syn*-/(*Z*)-methoxyimino residue at C-7, and its C-3 chain is an acetoxymethyl moiety. The C-3 substituent is mainly responsible for the pharmacokinetic properties, like highlighted with ceftriaxone, which possesses a triazine substituted ring or cefodizime, which has a 3′,5′-disubstituted thiazolyl ring.[30] The C-3 moiety is also responsible for antibacterial activity.[31] All of the oximino cephalosporins on the market have methoxyimino groups in the *syn*/(*Z*) configuration. Using UV light irradiation, Iorio et al.[32] observed that *syn*/*anti* isomerization of *N*-oxime function occurs until the two isomers reach equilibrium. The (*E*)-isomer is less active than the (*Z*)-isomer.[31] However, few comparative reports have mentioned the toxic characteristics of the (*Z*)-/(*E*)-isomers. Cefotaxime, ceftriaxone, and cefepime (Figure 5a) have the same C-7 substituent but different C-3 substituents. Although the influence of each of the paired (*Z*)-/(*E*)-isomers on zebrafish embryonic phenotypes was basically the same (Figures 6−8), all the (*E*)-isomers were more toxic than the (*Z*)-isomer (Figures 6a, 7a, and 8a).

Comparison of the toxic effects of the (*Z*)- and (*E*)- isomers of cefotaxime on the development of zebrafish embryos (Figure 6): The ED_{50} of cefotaxime (the (*Z*)-isomer) on teratogenicity appeared at about 30 mmol/L, but that of the (*E*)-isomer appeared at about 6 mmol/L. No embryo death attributable to cefotaxime was observed during the first three days post-fertilization, but the mortality rate of embryos was about 10% in the group treated with the (*E*)-isomer at the concentration of about 10 mmol/L and increased obviously with the treated doses (Figure 6a). The abnormal phenotypes observed in the groups treated with cefotaxime (Figures 6b) were mainly as follows: The embryos had swollen abdomens and pericardial sacs, deformed heart structures lacking blood cells, blood island congestion, and yolk invagination. Even at the concentration of 48 mmol/L, the teratogenesis intensity did not become more severe than that at the lower doses though the deformity rate reached 100%. However, in the (*E*)-cefotaxime exposed groups, the abnormal phenotype was more serious, such as with the abdomens expanding until diabrosis, the central axis being thin and short, the brain and eyes being small, and the heart being so badly deformed that it was nearly invisible under a microscope. The structure difference between the (*Z*)- and (*E*)-cefotaximes probably is a decisive reason for their levels of teratogenesis.

Comparison of the toxic effects of the (*Z*)- and (*E*)- isomers of ceftriaxone (Figure 7): The ED_{50} of ceftriaxone (the (*Z*)-isomer) on teratogenicity was about 17 mol/L, but that of the (*E*)-isomer was less than 3.4 mol/L. As the concentration of the test solution increased, the teratogenic rates decreased, but the death rates increased at 3 dpf in both the (*Z*)- and (*E*)-isomer groups. The LD_{50} values of ceftriaxone and the (*E*)-isomer were about 24 mmol/L and 5.1 mmol/L, respectively (Figure 7a). The abnormal phenotypes of ceftriaxone treated groups were mainly as follows: the embryos presented deformations similar to those

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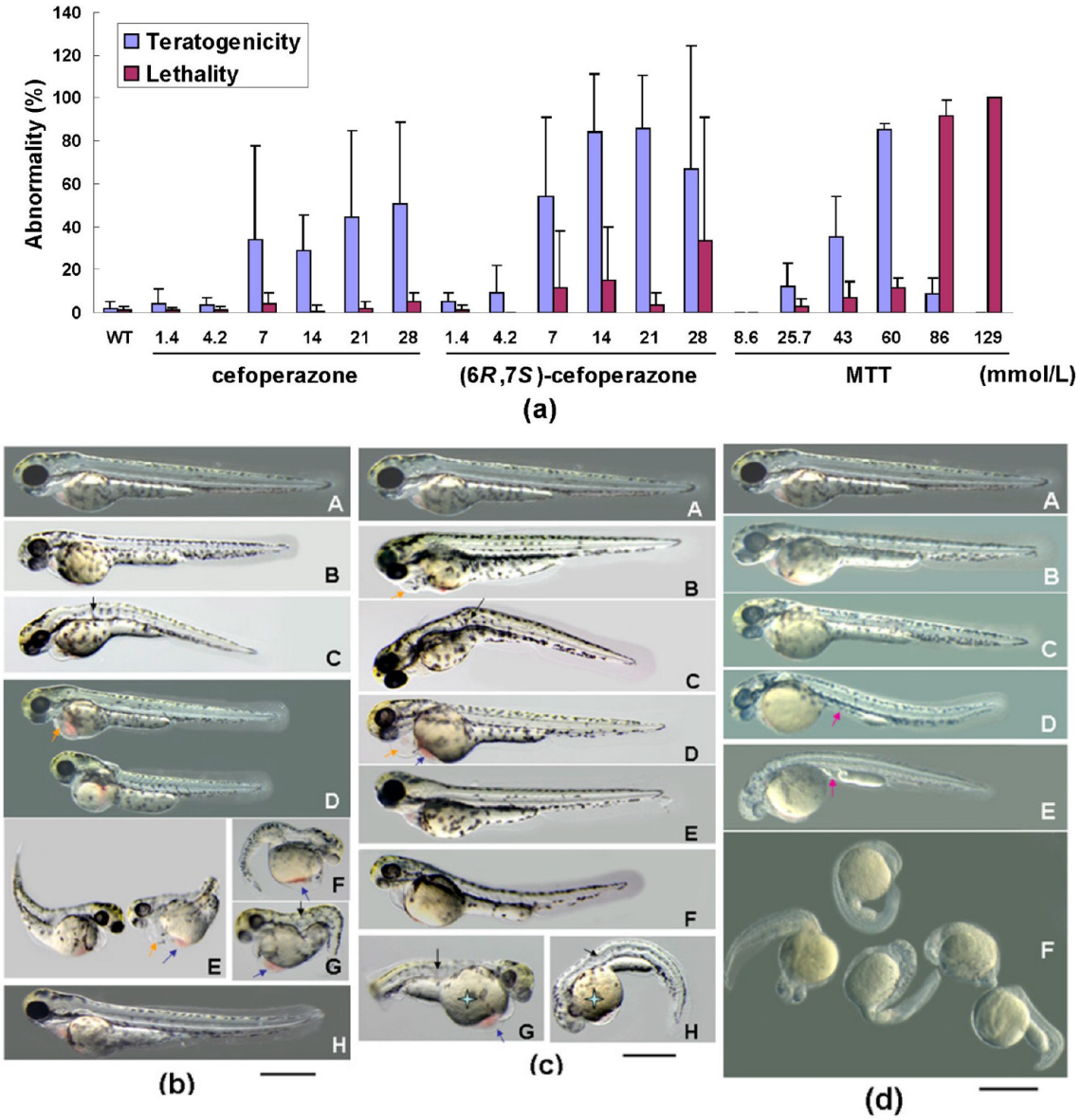

Figure 4. Toxicity of (6*R*,7*R*)-cefoperazone, (6*R*,7*S*)-epimer, and their C-3 substituent, 1-methyl-1*H*-tetrazole-5-thiol (MTT), in zebrafish embryo testing. (a) Comparison of the toxicity results of (6*R*,7*R*)-cefoperazone, (6*R*,7*S*)-epimer, and MTT on zebrafish embryonic development. The data are 3-dpf statistics. The teratogenic rates do not include the fatality rate. (b–d) Effects of (6*R*,7*R*)-cefoperazone, (6*R*,7*S*)-cefoperazone, and MTT on the embryonic development of zebrafish. Embryos were exposed to the studied compounds separately from 6–72 hpf. Photos were taken at 3 dpf. In b, A, WT, an untreated control; B–H, abnormal phenotypes caused by (6*R*,7*R*)-cefoperazone at 4.2 mmol/L (B), at 7 mmol/L (C), at 14 mmol/L (D,E), and at 21 mmol/L (F,G,H). The scale represents 580 μm. In c, A, WT, an untreated control; B–H, abnormal phenotypes caused by (6*R*,7*S*)-cefoperazone at 4.2 mmol/L (B), at 7 mmol/L (C), at 14 mmol/L (D,E), and at 21 mmol/L (F), and at 28 mmol/L (G,H). The scale represents 570 μm. In d, A, WT, an untreated control; B–F, abnormal phenotypes caused by MTT at 8.6 mmol/L (B), at 25.7 mmol/L (C), at 43 mmol/L (D,E), and at 60 mmol/L (F). The scale represents 580 μm. Orange arrows indicate swelling of the pericardial sac. Blue arrows show blood island congestion near the heart. Black arrows show S-twisted notochords. Purple arrows show defective constriction of the yolk extension.

of cefotaxime treated embryos, such as swollen abdomens, vessel congestion near the heart that lacked blood redness, slight swelling of the pericardial sac, and small heads and eyes. When the concentration reached 25.5 mmol/L, the body became opaque and the axis bent, and by chance, individual head hemorrhage occurred (Figure 7b). Embryos exposed to (*E*)-ceftriaxone at 3.5 mmol/L showed abnormalities similar to those of the ceftriaxone group, but when the concentration exceeded 5 mmol/L, teratogenesis became more severe and was similar to the embryonic phenotype of (*E*)-cefotaxime treatment, for example, the hearts were too small to see, the yolk expanded until diabrosis, body length was shortened, and the heads and eyes were smaller, but the notochord did not display an S-twist (Figure 7c). Examination of the triazine ring, the C-3 side chain of ceftriaxone, showed that no teratogenesis took place at a concentration of 6.3 mmol/L. However, 12.5 mmol/L triazine ring caused nearly 100% teratogenesis (Figure 7a). Though there was no significant lethality at 25 mmol/L at 3 dpf, after hatching the larvae died quickly at 4–5 dpf. The abnormal phenotype is very similar to the one described above, such as big and ball-like abdomens, small eyes, and blood island congestion. Within range of the used concentration, body length did not shorten (Figure 7d). This is similar to the toxic effects of high doses of ceftriaxone on zebrafish embryos, but it is distinct from cefotaxime toxicity to an extent.

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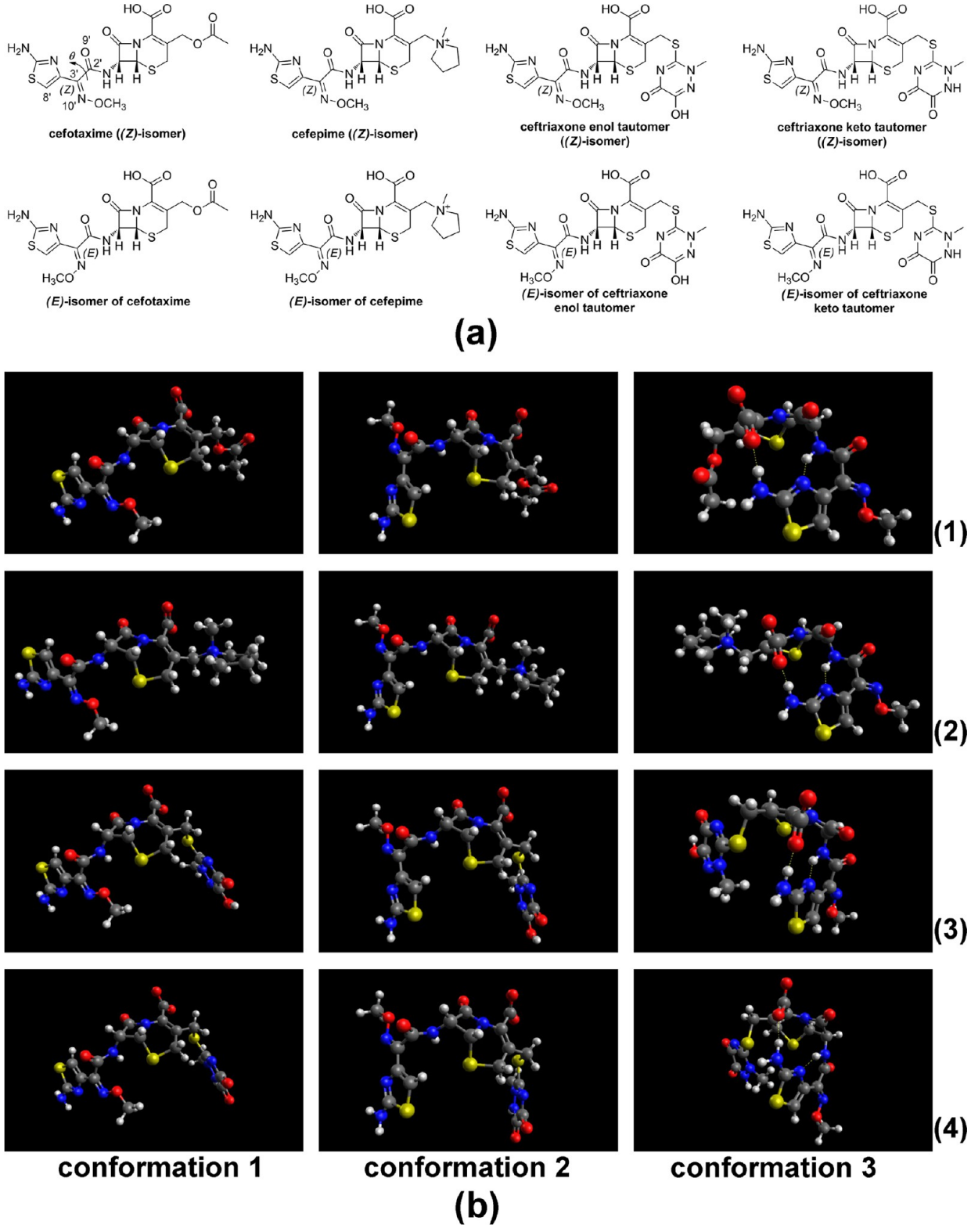

Figure 5. (a) Structure of cephalosporins with 2-amino-5-thiazolyl residues at C-7 and their most stable conformations. The dihedral angel θ (<(O9′-C2′-C3′-N10′)) defines the dihedral angel between the carbonyl of amide and methoxyimino. (b) Conformation 1 and conformation 2 mean the two most stable conformations of the (*Z*)-isomer, and conformation 3 means the most stable comformations of the (*E*)-isomer. (1) Cefotaxime, (2) cefepime, (3) ceftriaxone enol tautomer, and (4) ceftriaxone keto tautomer. The hydrogen bonds are indicated by dashed lines.

Comparison of the toxic effects of the (*Z*)- and (*E*)- isomers of cefepime (Figure 8): The ED_{50} of cefepime (the (*Z*)-isomer) on teratogenicity was about 18 mmol/L, but that of the (*E*)-isomer was 14.4 mmol/L. The death rates at 3 dpf were less than 5% in all of the cefepime treated groups, but about 30% high in 12.6 mmol/L of the (*E*)-isomer treated groups. The rates of increase were found to depend on the doses (Figure 8a). The abnormal phenotypes of cefepime treated groups (Figure 8b)

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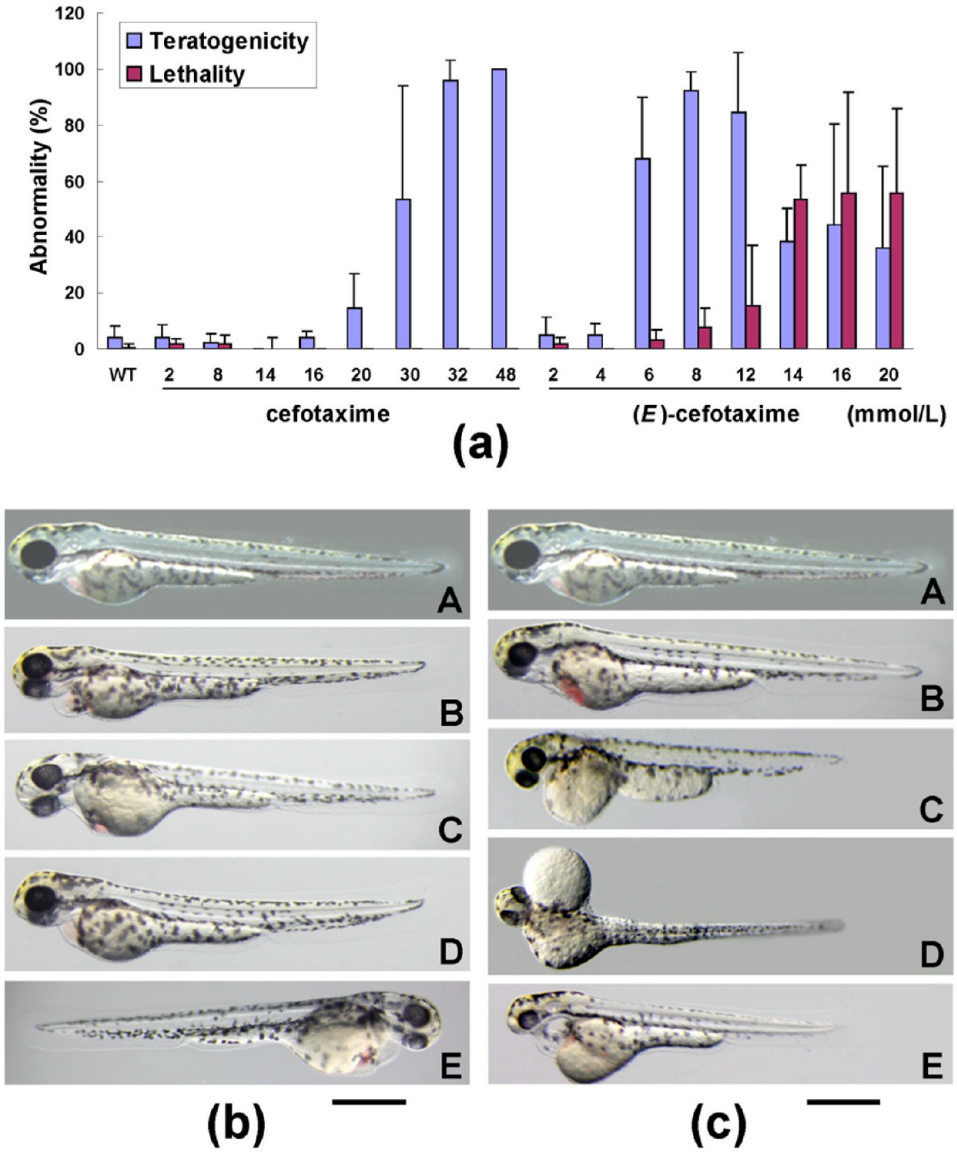

Figure 6. Toxicity of (*Z*)- and (*E*)-isomers of cefotaxime in zebrafish embryo testing. (a) Comparison of the toxicity results of (*Z*)- and (*E*)-isomers of cefotaxime on zebrafish embryonic development. The data are 3-dpf statistics. The teratogenic rates do not include the fatality rate. WT means wild type, which here means untreated normal controls. (b) Effects of (*Z*)-isomers of cefotaxime on the embryonic development of zebrafish (3 dpf). In b, A, WT, an untreated control; B−E, abnormal phenotypes caused by cefotaxime at 14 mmol/L (B), at 20 mmol/L (C, D), and at 30 mmol/L (E). The scale represents 570 μm. (c) The abnormal phenotypes (3 dpf) treated with the (*E*)-isomer of cefotaxime. In c, A, WT, an untreated control; B−E, abnormal phenotypes caused by (*E*)-cefotaxime at 6 mmol/L (B), at 8 mmol/L (C), and at 14 mmol/L (D, E). The scale represents 570 μm.

were mainly as follows: When exposed to cefepime concentrations below 12.6 mmol/L, the embryos grew slowly and had shorter body lengths and slight swelling of the pericardial sac. Most of these abnormalities were improved significantly at 4−5 dpf, but some of the embryos kept lying on their sides, indicating delayed development. When the cefepime concentration increased to 12.6−18 mmol/L, the embryos had dark, spherical abdomens, slightly small heads, eyes, and bodies, and swollen pericardial sacs with blood island congestion. After hatching, the larvae displayed a weak response to tail-touch stimulation, but this improved at 4−5 dpf. At 23.5 mmol/L, embryo death occurred, and the malformation became more severe (Figure 8b). (*E*)-Cefepime caused abnormal phenotypes similar to those caused by cefepime. Embryos exposed to (*E*)-cefepime at 9 mmol/L presented evident vein congestion close to the heart where the yolk was entrapped, shortened stem length, and poor response to tail-touch stimulation at 3 dpf. This teratogenesis was concentration-dependent. At concentrations of 18 mmol/L, all treated embryos were deformed (Figure 8c), and 80% death occurred at 3 dpf (Figure 8a). Teratogenesis of NMPD, the C-3 side chain of cefepime, was observed (Figure 8d). The embryos exposed to NMPD between 2.6 and 4.4 mmol/L displayed phenotypes similar to those caused by cefepime. At NMPD concentrations of 8.8 mmol/L, this malformation became more severe. The zebrafish bodies were significantly shortened and bent, the heart atria and ventricle developed into tubular shape without erythrocytes, blood island congestion and pericardiac sac edema formed, the trunk was nearly colorless, the escape response to tail-touching was retarded, and all of the fish died at 5 dpf. In a group of embryos exposed to NMPD at 26.4 mmol/L, embryonic development was arrested at about 1 dpf. The head and eyes were defective, the whole body was

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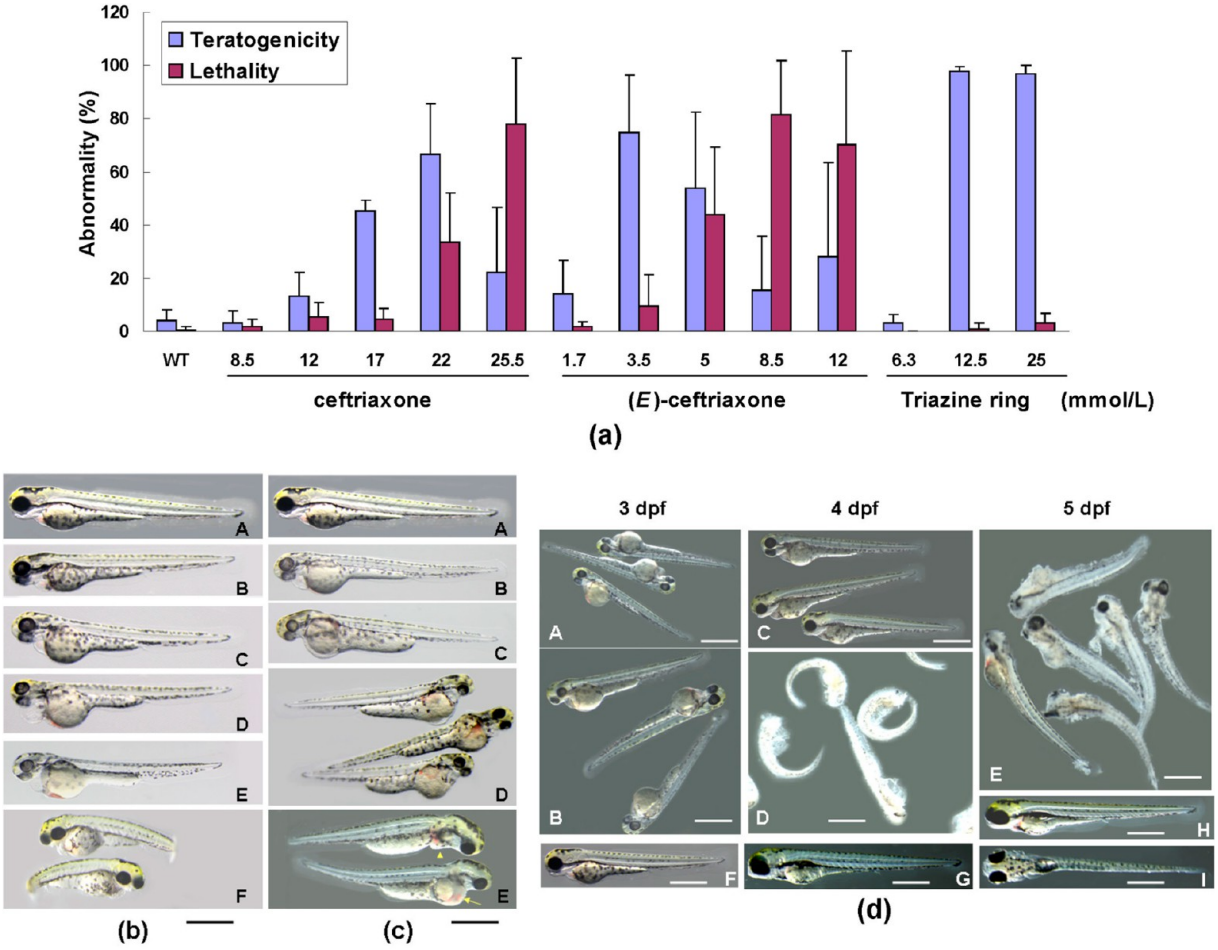

Figure 7. Toxicity of (*Z*)- and (*E*)-isomers of ceftriaxone and their C-3 substituent, triazine ring, in zebrafish embryos. (a) Comparison of the toxicity on the embryonic development in zebrafish. The data are 3 dpf statistics. The teratogenic rates do not include the fatality rate. WT means wild type, which here represents untreated normal controls. (b) Effects of (*Z*)-isomers of ceftriaxone on the embryonic development of zebrafish. In b, A, WT, an untreated control; B−F, abnormal phenotypes caused by ceftriaxone at 8.5 mmol/L (B), at 12 mmol/L (C), at 17 mmol/L (D), and at 25.5 mmol/L (E, F). The scale presents 660 μm. (c) (*E*)-isomers of ceftriaxone on the embryonic development of zebrafish. In c, A, WT, an untreated control; B−E, abnormal phenotypes caused by (*E*)-ceftriaxone at 3.5 mmol/L (B), at 5−8.5 mmol/L (C), and at 8.5−12 mmol/L (D, E). The yellow arrowhead indicates yolk diabrosis. The yellow arrow indicates a damaged yolk that is going to go through diabrosis. The scale presents 700 μm. (d) Effects of the triazine ring on zebrafish embryonic development. In d, A−E, abnormal phenotypes caused by 12.5 mmol/L triazine ring at 3 dpf (A), 4dpf (C), and at 5 dpf (E), and by 25 mmol/L triazine ring at 3 dpf (B) and 4 dpf (D). F−I, WT embryos at 72 hpf (F), at 96 hpf (G), at 120 hpf with the left lateral view (H), and with the dorsal view (I). The scale presents 710 μm in F, 660 μm in G, 700 μm in H, and 680 μm in I.

colorless like an opaque broad bean, no classic heart was formed, somites were aligned in parallel, yolk extension was deficient, and the trunk and tail were thin, short, and twisted (Figure 8d). The embryos all died at 4 dpf. All of the phenotypes resembled toxic reactions to high concentrations of cefepime (data not shown).

3.4. Theoretical Studies on the Toxicity Differences of Cephalosporin Stereoisomers. To explore the relationship between cephalosporin's stereosiomers and experimental results of toxicity tests on zebrafish embryos, computational chemistry methods were used to study the conformations of the stereoisomers in aqueous solution. The carboxyl groups of cephalosporin dissociate into carboxylate ions in near-neutral water. For this reason, all of the compounds were treated as anions except cefaclor, cefepime, and their stereoisomers, which have additional cation nitrogen centers.

The conformers obtained from computation studies that had the lowest energy indicated that cefaclor and Δ-3 cefaclor had very similar structural patterns, especially the side chain at C-7 position (Figure 1). These results are in excellent agreement with those of a previous X-ray crystallography study on cefaclor.[33] The main structural difference between these two stereoisomers is the orientation of the carboxylic group, which was caused by double bond migration. These structural features are consistent with the toxic effects of cefaclor and Δ-3 cefaclor in zebrafish embryo testing. In this way, we propose that the substituent at the C-7 position of cefaclor and Δ-3 cefaclor is the main toxic functional group.

For cefoperazone and (6*R*,7*S*)-cefoperazone, the substituents at the C-3 and C-7 positions were more complex than other cephalosporins, leading to a wide variety of conformers. However, the most stable conformations of cefoperazone and (6*R*,7*S*)-cefoperazone still shared some common features. Both conformers contained two intramolecular hydrogen bonds at the same sites, one in the substituent moiety of C-7 position and the other between the phenolic hydroxyl of C-7 substituent and tetrazolium of C-3 substituent (Figure 3). Also, cefoperazone and its epimer, (6*R*,7*S*)-cefoperzone, could roughly be considered as "mirror images" of each other with respect to their conformations (Figure 3). Because the substituents at the C-7 position adopted a more extended form and the substituents at the C-3 position

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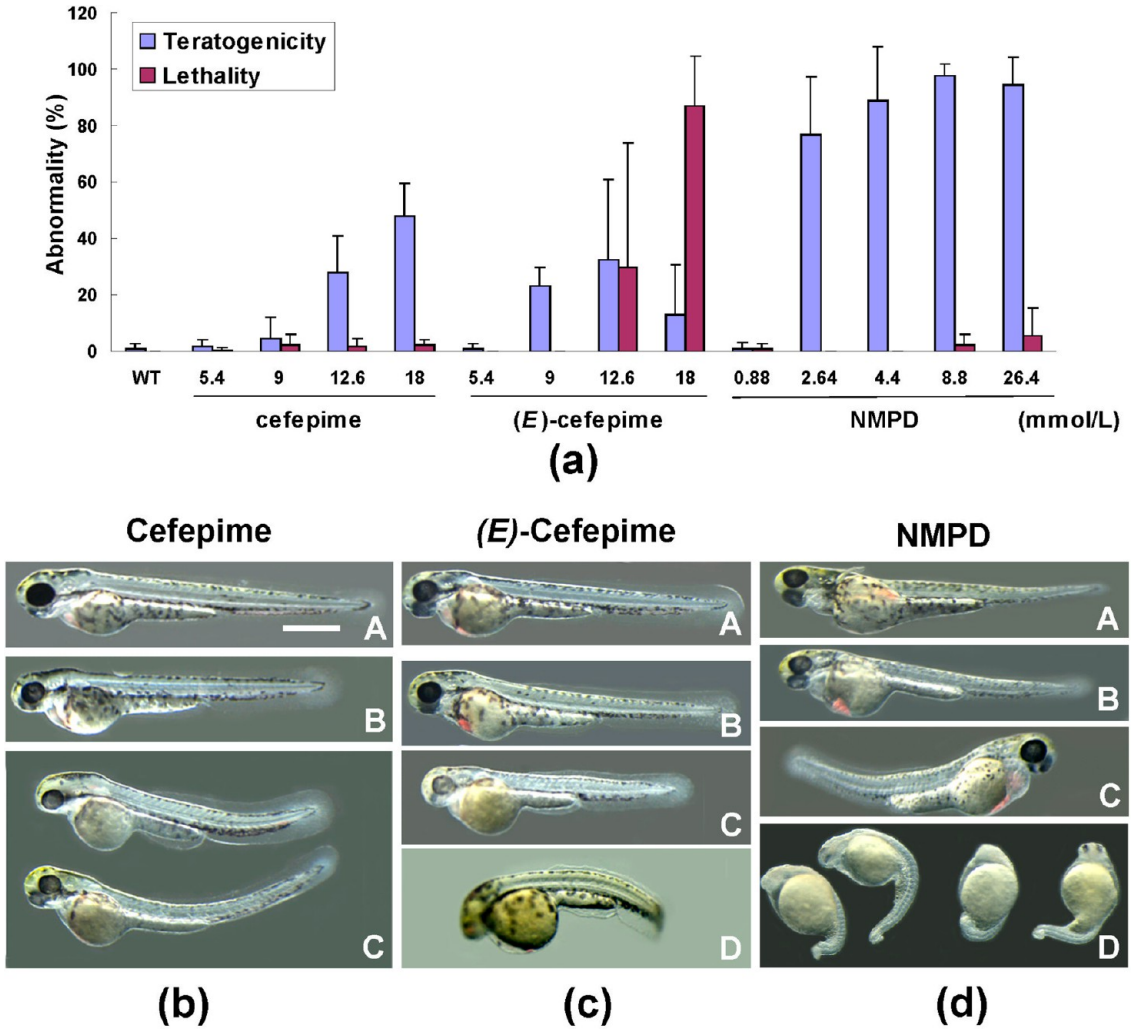

Figure 8. Toxicity of (*Z*)- and (*E*)-isomers of cefepime and their C-3 substituent, *N*-methyl-2-pyrrolidone, in zebrafish embryo testing. (a) Comparison of the toxicity results of (*Z*)- and (*E*)-isomers of cefepime on zebrafish embryonic development. The data are 3 dpf statistics. The teratogenic rates do not include the fatality rate. WT means wild type, which here represents untreated normal controls. (b) Abnormal phenotypes of zebrafish embryos caused by (*Z*)-isomers of cefepime. In b, A, WT, an untreated control; (B, C), abnormal phenotypes caused by cefepime at 12.6 mmol/L (B) and at 23.5 mmol/L (C). (c) Abnormal phenotypes of zebrafish embryos caused by (*E*)-isomers of cefepime. In c, A–D, abnormal phenotypes caused by (*E*)-cefepime at 5.4 mmol/L (A), at 9 mmol/L (B), and at 12.6 mmol/L (C), and at 18 mmol/L (D). (d) Effects of *N*-methyl-2-pyrrolidone on zebrafish embryonic development. In d, A–D, abnormal phenotypes caused by *N*-methyl-2-pyrrolidone at 2.6 mmol/L (A), at 4.4 mmol/L (B), at 8.8 mmol/L (C), and at 26.4 mmol/L (D). The scale in b-A represents 570 μm for all the images in (b–d).

adopted a folded form, the side chain at the C-7 position had less steric hindrance. Thus, the substituents at the C-7 position are more likely to behave as toxic functional groups. This is quantitatively in accordance with the experimental toxicity results. Specifically, at lower concentrations, the toxicity of cefoperazone was driven by the C-7 side chain. At higher concentrations, both functional groups exhibited toxic activity.

Cefotaxime, cefepime, and ceftriaxone share the same substituent at the C-7 position. The structural differences are located at the C-3 position. Ceftriaxone and (*E*)-ceftriaxone contain unique substituents at the C-3 position, which probably show keto–enol tautomerism. Therefore, the study of both tautomers is merited. These compounds and their (*E*)-isomers provide a good opportunity to investigate the influence of substituents at the C-7 and C-3 positions on toxicity. Theoretical studies indicate that the side chain at position C-7 of cefotaxime, cefepime, and ceftriaxone has two conformations (Figure 5b). In conformation 1, the dihedral angle θ (<(O9′-C2′-C3′-N10′)) (Figure 5a) between the carbonyl of amide and methoxyimino is about −105°, and in conformation 2, the methoxyimino moiety is oriented toward the β-latam ring, with a dihedral angle θ of about 86° (Table S2, Supporting Information). The differences in energy between these two conformations are all less than 0.5 kcal/mol (Table S3, Supporting Information), indicating that both conformations are stable in aqueous solution. Again, previous X-ray crystallography data of cefmenoxime hemihydrochlorid,[34] ceftizoxime,[35] and ceftizoxime monohydrochloride monohydrate[35] were consistent with these computational results. The three (*E*)-isomers also had similar conformations (conformation 3) (Figure 5b). These were significantly different from conformations 1 and 2. The side chain at the C-7 position of (*E*)-isomers adopted a quasi-planar form, and the dihedral angle θ became much smaller (Table S2, Supporting Information). Another distinct feature is the formation of two medium-strength intramolecular hydrogen bonds: The distal amine group of the C-7 side chain formed a hydrogen bond with the carboxylic group on the six-member ring, and the amide nitrogen of the C-7 side chain formed a bond with the nitrogen atom in the thiazole ring (Figure 5b; Table S4,

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Supporting Information). These two hydrogen bonds made the conformation of (*E*)-isomers 1−3 kcal/mol lower in energy than others, for example, conformations similar to conformation 1 and 2 (Table S3, Supporting Information). Thus, the hydrogen bonds stabilized conformation 3 of (*E*)-isomers. In addition, the substituents at the C-3 position were oriented toward the C-7 side chain in (*E*)-isomers, while in the (*Z*)-isomer form, the C-3 substituents tend to direct away from the C-7 side chain. This phenomenon may have contributed to the weak interactions (e.g., van der Waals interactions and $\pi-\pi$ interactions for ceftriaxone) between C-3 and C-7 side chains in (*E*)-isomers. All of these features forced the (*E*)-isomers to adopt a more folded conformation than the (*Z*)-isomers. We concluded that the differences in toxicity between these (*Z*)- and (*E*)-isomers stemmed from the different conformations that they assumed in aqueous solution. The substituents at the C-7 position were found to have a greater chance of exhibiting toxic activity than substituents at the C-3 position. This was because the side chains at the C-3 position were more likely to assume a folded conformation.

Molecular polar surface area (PSA) has proved to be a very good descriptor of molecular polarity. It is also closely correlated with passive transport through the cellular membrane, including intestinal absorption and blood−brain barrier permeation.[36] Topological polar surface area (TPSA) is a fast and reliable means of calculating molecular PSA. It produces practically the same results as the conventionally more sophisticated PSA algorithm.[37] Our toxicity test on zebrafish embryos have shown that cefotaxime, cefepime, ceftriaxone, and their stereoisomers have the same type of toxic action. However, the toxic effects were quite different, and the toxic effects of cefaclor, cefoperazone, and their stereoisomers are very similar. A more in-depth investigation is required to interpret their toxic behavior. Our study has demonstrated a strong negative correlation between TPSA values and the toxic effect of the stereoisomers. The absolute TPSA values, TPSA differences between stereoisomers, and the relative sizes of differences in TPSA are listed in Table 1. Both the absolute and relative TPSA differences of cefotaxime, cefepime, and ceftriaxone are clearly much larger than those of their (*E*)-isomers, indicating that they are more polar than (*E*)-isomers. However, the TPSA values of cefaclor, cefoperazone, and their stereoisomers are not very different from each other. These results are consistent with the data obtained from our toxicity test on zebrafish embryos. Specifically, the toxic effects on zebrafish embryos become more pronounced when the TPSA values of these stereoisomers decrease. For (*E*)-isomers of cefotaxime, cefepime, and ceftriaxone, molecular polarity decreased markedly when a hydrogen bond was formed between the amine group on the C-7 side chain and carboxylic group, forcing the entire molecule to adopt a packed conformation with a hydrophobic nature. This may increase the membrane permeability of the (*E*)-isomers and finally give rise to more potent toxic activity. Instead, the dissimilar conformations of cefoperazone and (6*R*,7*S*)-cefoperazone made little difference with respect to toxic effects because they had almost identical polarity properties, as indicated by our TPSA study. In summary, molecular polarity seems to be crucial to the toxic effects of these compounds.

3.5. Experimental Verification. The above results suggest that both the C-7 and C-3 substituents of cephalosporins are toxic functional groups and that the molecular polarity may relate to the toxic action of the cephalosporins. To verify our inference, the toxicity of three cephalosporins, namely, ceftizoxime, cefmenoxime, and cefmentazole (Figure 9), with different C-7 and C-3 substituents was examined in the zebrafish model, and the theoretical calculations on the different cephalosporins were also carried out.

Table 1. TPSA Values of Cephalosporins and Their Stereoisomers

comformation	TPSA (Å^2)	ΔTPSA (Å^2)[a]	ΔTPSA/TPSA (%)
cefotaxime conformation 1	183.344	25.310	13.8
cefotaxime conformation 2	188.758	30.724	16.3
(*E*)-cefotaxime conformation 3	158.034	0.0	
cefepime conformation 1	151.828	15.948	10.5
cefepime conformation 2	157.453	21.573	13.7
(*E*)-cefepime conformation 3	135.880	0.0	
ceftriaxone keto tautomer conformation 1	218.102	16.082	7.4
ceftriaxone keto tautomer conformation 2	222.809	20.789	9.3
(*E*)-ceftriaxone keto tautomer conformation 3	202.020	0.0	
ceftriaxone enol tautomer conformation 1	217.936	20.162	9.3
ceftriaxone enol tautomer conformation 2	223.084	25.310	11.3
(*E*)-ceftriaxone enol tautomer conformation 3	197.774	0.0	
cefaclor conformation	120.483	3.895	3.2
Δ-3 cefaclor conformation	116.588	0.0	
cefoperazone conformation	215.972	8.414	3.9
(6*R*,7*S*)-cefoperazone conformation	207.558	0.0	
7-ACA	120.494		
ceftizoxime conformation 1	154.221	0.0	
ceftizoxime conformation 2	158.893	4.672	2.9
cefmenoxime conformation 1	195.541	0.0	
cefmenoxime conformation 2	200.577	5.036	2.5
cefmentazole	165.194		

[a]For each stereoisomer group, the conformation with the lowest TPSA is assigned zero.

Ceftizoxime and cefotaxime share the identical substituent at the C-7 position. Theoretical calculation indicated that the side chain at position C-7 of both drugs had the same conformations (Figure 9a). According to our theoretical suggestion, ceftizoxime should have a phenotype similar to that of cefotaxime, but the toxicity effects should be stronger since ceftizoxime has no C-3 substituent, which lead it to be less polar than cefotaxime. The zebrafish model was used to confirm the following deduction (Figure 10): The ED_{50} of ceftizoxime on teratogenicity appeared at about 4.5 mmol/L, and the LD50 was about 17.8 mmol/L during the first three days post-fertilization. Both the deformity rate and the mortality rate increased remarkably with the treated doses (Figure 10a). The results reveal that ceftizoxime could cause more potent toxic activity than cefotaxime. The abnormal phenotypes observed in the groups treated with ceftizoxime (Figures 10b) are mainly as follows: The embryos had mild abnormality such as a slightly enlarged abdomen and a slightly swollen pericardial sac, deformed heart structures lacking blood cells, blood island congestion, underdeveloped brain region and eyes, and thickened yolk extension at the posterior end, which were similar to those of cefotaxime.

Cefmenoxime is a combined compound, whose C-7 subsitituent is the same as cefotaxime, and the C-3 substituent is the same as cefoperazone. Theoretically, it should have two toxic functional groups, and the toxic actions should be close to cefotaxime and cefoperazone. The conformations of the C-7

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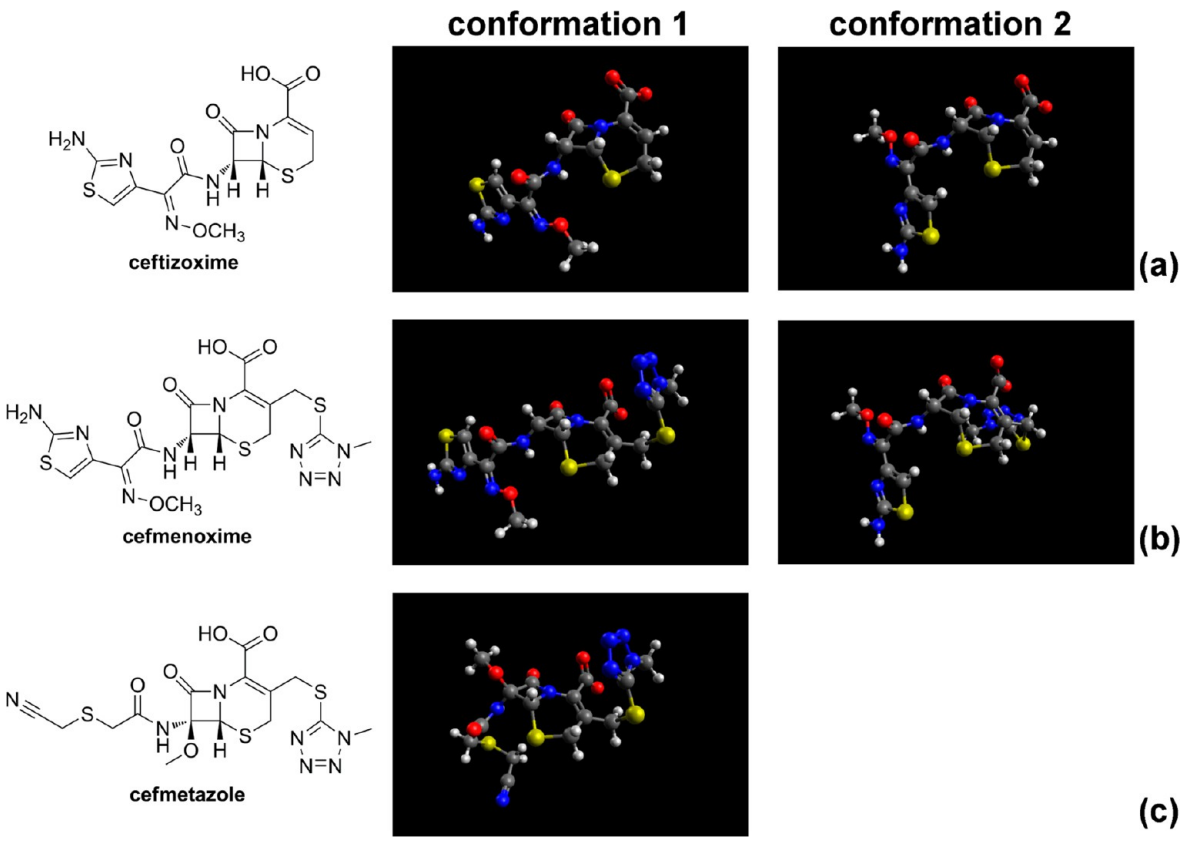

Figure 9. Structure of cephalosporins with different C-7 and C-3 substituents and their most stable conformations. (a) Ceftizoxime, (b) cefmenoxime, and (c) cefmetazole. Conformation 1 and conformation 2 mean the two most stable conformations of cephalosporins with 2-amino-5-thiazolyl residues at the C-7 position.

substituent are just like cefotaxime, and the side chain at the C-3 position is more likely to adopt a folded conformation (Figure 9b). The substituent at the C-7 position should have a greater chance of exhibiting toxic activity than the C-3 substituent. So that like cefoperazone, the toxicity of cefmenoxime was driven by the C-7 side chain at lower concentrations, and both functional groups exhibited toxic activity at higher concentrations in zebrafish embryo testing. The zebrafish model was used to confirm the following suggestion (Figure 10c): The ED_{50} of cefmenoxime on teratogenicity was at about 24 mmol/L, and the LD50 was about 49 mmol/L during the first three days post-fertilization. The abnormal phenotypes of the treated groups (Figure 10c) are mainly as follows: embryos treated with lower dosage of cefmenoxime presented mild deformation such as a slightly enlarged abdomen and swollen pericardial sac, blood pool congestion, and yolk invagination, which were similar to what cefotaxime did. When the concentration reached close to 40 mmol/L, the second abnormal phenotype was observed such as shortened body length, weak heartbeats with small and abnormal heart structures, and an apparent enlarged opaque abdomen, a part of which was similar to what the MTT, the C-3 side chain of cefoperazone, did.

Cefmetazole is a very special compound, the C-7 substituent is unique, but the C-3 substituent is MTT, the same as cefoperazone. The cyan group at the C-7 position of cefmetazole is a well-known toxic group. Theoretical calculation indicated that both the C-7 and C-3 substituent adopted the folded conformations but that the cyan group was directed outward. The substituent at the C-7 position should have a greater chance of exhibiting toxic activity than the substituent at the C-3 position. Therefore, the cyan group should be the main toxic fuctional group and cause different abnormal phenotypes in the zebrafish model (Figure 9c). The presumption was validated by zebrafish embryonic experiments (Figure 10). The ED_{50} of cefmetazole on teratogenicity appeared at about 9 mmol/L and the LD_{50} at 14.5 mmol/L. The abnormal phenotypes of the cefmetazole treated groups are mainly as follows (Figure 10d). The embryos exhibited mildly enlarged abdomens and swollen pericardial sacs, reduced body clarity, and shortened body lengths. After hatching, the larvae were inactive; more severely, some of them rotated but did not move straight ahead when they were touched. At a high concentration of 14 mmol/L, the body axis became short, bent, and almost colorless; the abdomen and yolk extension easily went through diabrosis; most of the embryos died at 2 dpf when the concentration was up to 20 mmol/L. A part of the abnormal phenotype is similar to that of the MTT group. In brief, the theoretical suggestion was supported by the experiment result.

Our study suggests that both the C-7 and C-3 substituents of cephalosporins are toxic functional groups. However, the structure−bioactivity relationship of the cephalosporin side chains revealed that structural modifications at C-7 and C-3 can improve antibacterial profiles of cephalosporins[5,6] and affect the interaction between drugs and β-lactamases.[7,8] It means that the C-7 and C-3 substituents of cephalosporins are not only the toxic functional groups but also the pharmacological functional groups. As the efficacy targets of cephalosporins are on bacteria, which are prokaryocyte, but the toxicity targets are on the eukaryocytes of mammalians, how to balance the efficacy and the toxicity becomes a challenge when modifying the substituents of

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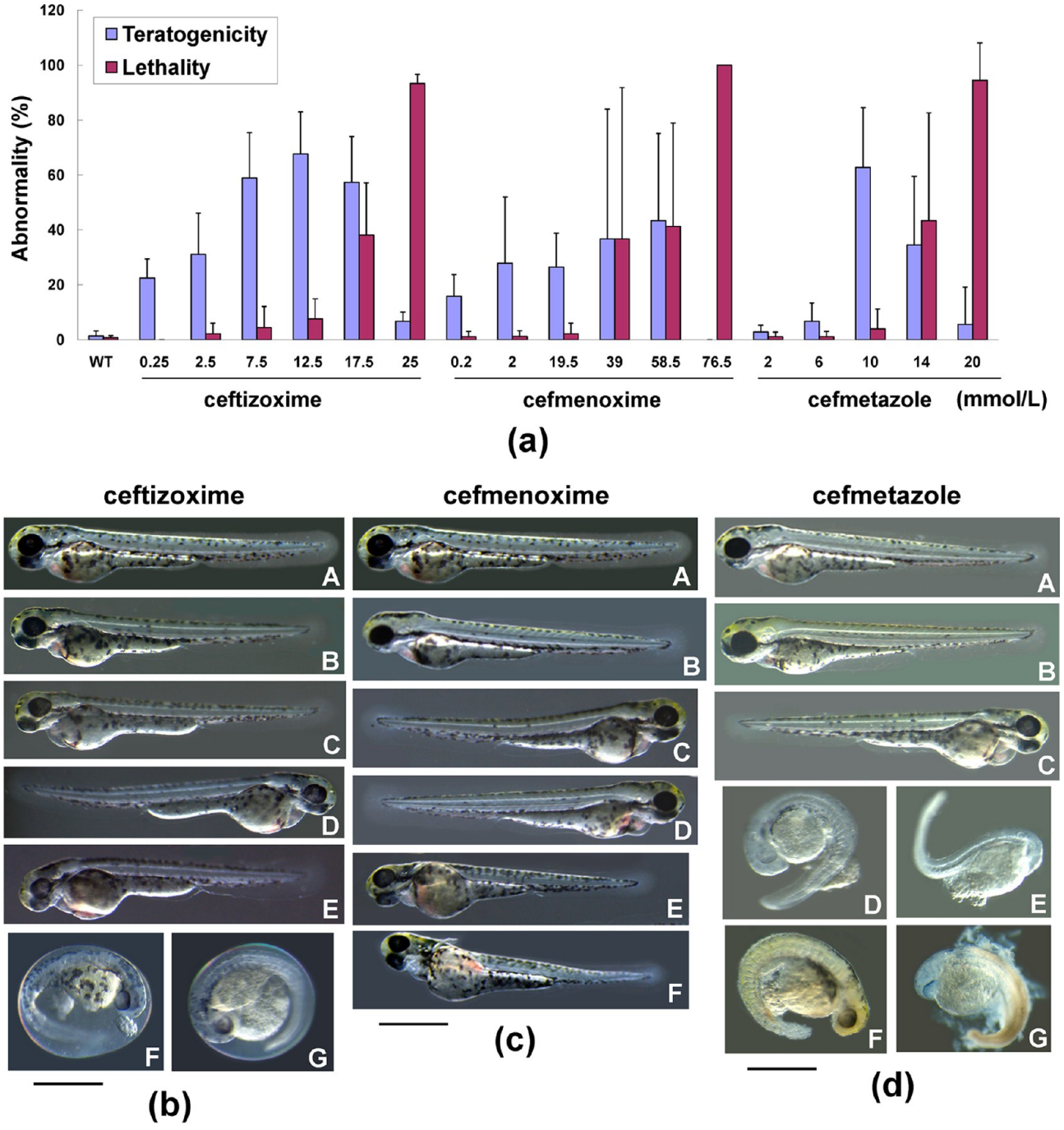

Figure 10. Toxicity of ceftizoxime, cefmenoxime, and cefmetazole in zebrafish embryo testing. (a) Comparison of the toxicity results of ceftizoxime, cefmenoxime, and cefmetazole on zebrafish embryonic development. The data are 3 dpf statistics. WT means wild type, which here represents untreated normal controls. (b) Abnormal phenotypes of zebrafish embryos caused by ceftizoxime treatment. In b, A, WT, an untreated control; B–G, abnormal phenotypes caused by ceftizoxime at concentrations of 0.25 mmol/L (B), 2.5 and 7.5 mmol/L (C), 12.5 mmol/L (D), 17.5 mmol/L (E), and 25 mmol/L (F, G). The embryos in A–E were photographed at 72 hpf and in F and G at 48 hpf. (c) Abnormal phenotypes of zebrafish embryos caused by cefmenoxime treatment. In c, A, WT, an untreated control; B–F, cefmenoxime treatment at concentrations of 2 mmol/L (B), 19.5 mmol/L (C, D), 39 mmol/L (E), and 58.5 mmol/L (F). The embryos in A–F were photographed at 72 hpf. (d) Abnormal phenotypes of zebrafish embryos caused by cefmetazole treatment. In d, A, WT, an untreated control; B–G, cefmetazole treatment at concentrations of 2 mmol/L (B), 10 mmol/L (C), 14 mmol/L (D,E), and 20 mmol/L (F,G). The embryos in A–G were photographed at 72 hpf. The scale bars represent 740 μm in b–d.

cephalosporins in new drug design. The fifth generation of cephalosporins, including ceftaroline and ceftobiprole, all bear 5-amino-1,2,4-thiadiazol residues at the C-7 position, and these residues are derivatives of the 2-amino-5-thiazolyl ring of cefotaxime. The oxime moieties are all in the *syn*/(*Z*) configuration, so the *anti*/(*E*)-isomers still exist. We suppose the (*E*)-isomers are more hydrophobic and toxic than (*Z*)-isomers based on our conclusion; therefore, more attention should be paid to these (*E*)-isomers for safety concerns.

Although there are no serious adverse effect reports of cephalosporins on teratogenesis in clinical use, Czeizel et al. also reported that treatment with the studied cephalosporins during

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pregnancy does not seem to present a detectable teratogenic risk to the fetus using pair analysis of cases.[38] However, as drug safety is substantially more difficult to demonstrate than its efficacy, and very little attention has been paid to sex differences and even less attention to the impact of pregnancy, gaps in knowledge in treating pregnant women exist.[39] Alternative strategies need to be developed to characterize the safety information of drugs. The concept of toxic functional groups may help us understanding safety differences of cephalosporins.

4. CONCLUSIONS

Five kinds of cephalosporins and their isomers were used to investigate the toxic functional groups of cephalosporins. Zebrafish embryo toxicity tests showed that the level of toxicity of 7-ACA is far lower than that of either cefaclor or cefoperazone. One abnormal phenotype caused by cefoperazone is similar to the phenotype caused by cefaclor but different from the phenotypes caused by MTT at the C-3 position. These results suggest that the substituent at the C-7 position of cephalosporins is a toxic functional group and that it increases the toxic reaction of 7-ACA. The toxic properties of these cephalosporins with different C-3 structures were compared, and the compounds were shown to produce different abnormal phenotypes in zebrafish embryo toxicity tests. The abnormal phenotypes of ceftriaxone and cefepime at high concentration were similar to those caused by their C-3 substituent. These results suggest that the substituent at the C-3 position of cephalosporins is another toxic functional group. Theoretical calculations showed that TPSA values of stereoisomers are strongly correlated with the toxic effect. For *syn-*/*anti-* isomers of cephalosporins with the 2-amino-5-thiazolyl residue at the C-7 position, the molecular polarity of *anti*-isomers was found to decrease markedly because the formation of intramolecular hydrogen bonds and conformation change. This increased the membrane permeability of the *anti*-isomers and finally gave rise to more potent toxic activity. The calculation results indicated that molecular polarity may be crucial to the toxic effects of these compounds. All of the suggestions were further validated by using three different cephalosporins, ceftizoxime, cefmenoxime, and cefmetazole, with different C-7 and C-3 substituents in the zebrafish embryo toxicity model.

■ ASSOCIATED CONTENT

Supporting Information

Batch and content/purity information of the reference standards; atomic Cartesian coordinates (in Å) of all the minima conformers of cephalosporins and their stereoisomers; characteristic dihedral angle θ (<(O9′-C2′-C3′-N10′)) in cefotaxime, cefepime, ceftriaxone, and their (*E*)-isomers; absolute and relative energies of selected conformations of cephalosporins and stereoisomers; and the hydrogen bond length and angle in (*E*)-isomers of cefotaxime, cefepime, and ceftriaxone. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

7-ACA, 7-aminocephalosporanic acid; MTT, 1-methyl-1*H*-tetrazole-5-thiol; NMPD, *N*-methyl-2-pyrrolidone; GBMV, generalized Born with molecular volume; DFT, density functional theory; COSMO, conductor-like screening model; RI, resolution of the identity; TPSA, topological polar surface area

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