LAB/IN VITRO RESEARCH

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Assessment of Toxicity and Absorption of the Novel AA Derivative AA-Pme in SGC7901 Cancer Cells *In Vitro* and in Zebrafish *In Vivo*

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Asiatic acid (AA; 2α,3β,23-trihydroxyurs-12-ene-28-oic acid) is an active compound derived from *Centella asiatica,* a traditional medicinal plant used widely in many Asian countries, particularly for the treatment of cancer. However, the modified AA derivative N-(2α,3β,23-acetoxyurs-12-en-28-oyl)-l-proline methyl ester (AA-PMe) has shown markedly better anti-tumor activity than AA.

Material/Methods: We evaluated the toxicity of AA and AA-PMe on zebrafish morphology, mortality, and hatching rate and determined the effect on SGC7901 cancer cells by acute toxicity assay. AA-PMe absorption *in vitro* in SGC7901 cells and *in vivo* in zebrafish was determined by establishing a highly accurate and reproducible HPLC protocol.

Results: In zebrafish, the toxicity of AA-PMe was lower than AA, with an acute toxic dose of AA-PMe above 25 μM, compared to acute toxicity at doses above 10 μM for AA. However, chronic toxicity of AA-PMe began occurring at doses below 25 μM but became apparent for AA at doses below 10 μM. Although low doses of AA-PMe were tolerated acutely, it became chronically toxic during zebrafish development, resulting in morphological abnormalities, including peripheral and abdominal edema, hemorrhage, abnormal body shape, enlarged yolk sac, and reduced motility. At low concentrations, absorption of AA-PMe by cells and zebrafish embryos occurred in a dose-dependent manner, but this stabilized as the concentration increased.

Conclusions: This pharmacokinetic study outlines the cellular and organismal effects of AA-PMe and suggests a theoretical basis that may underlie its mechanism of action.

MeSH Keywords: **Centella • Stomach Neoplasms • Zebrafish**

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Background

Of the 250 000 known plant species on earth, specific therapeutic value has been demonstrated for approximately 5000 [1]. *Centella asiatica* is a traditional medicinal herb belonging to the Apiaceae family found in India, Sri Lanka, Madagascar, South Africa, Australia, China, and Japan [2]. Asiatic acid (AA), one of the pentacyclic triterpenoids derived from *Centella asiatica* (Umbelliferae), has been shown to exhibit anti-tumor, anti-inflammatory, and hepatoprotective properties and has also been studied as a treatment agent for depression and Alzheimer disease [3]. However, the efficacy of AA is relatively low and there have been many attempts to improve upon it. One such example, the highly potent AA derivative N-(2α, 3β, 23-acetoxyurs-12-en-28-oyl)-l-proline methyl ester (AA-PMe), was previously synthesized by our lab and studied for its anti-tumor and anti-angiogenic effects [4].

An increasing number of academic institutions and pharmaceutical companies are turning to the zebrafish (*Danio rero*) as a powerful *in vivo* model for drug discovery that will significantly speed the screening process of novel therapeutic candidates with important consequences for human health [5]. In contrast to traditional vertebrate models, zebrafish have rapid generation time, high fecundity, the convenience of external fertilization, and lower maintenance costs than mammalian models [6] but maintain an advantage over cell lines because they develop full organ and metabolic systems [7].

We have previously demonstrated the anti-tumor activity of AA-PMe in SGC7901 cells and in the zebrafish model, finding that AA-PMe induced apoptosis, inhibited proliferation and migration of human gastric cancer cells, and blocked blood vessel formation in both human umbilical vein endothelial cells (HUVECs) and zebrafish [8]. Here, we focus on the pharmacokinetic properties of AA-PMe in the gastric cancer cell line SGC-7901 and in zebrafish, and report decreased toxicity compared to its parent compound, AA.

Material and Methods

Chemicals and reagents

AA was isolated and purified from *C. asiatica* (L) Urban and the derivative AA-PMe was synthesized by our lab as previously described [4]. The structures of AA and AA-PMe are shown in Figure 1. Acetonitrile, methyl alcohol, and all HPLC-grade solvents were purchased from Sigma-Aldrich (USA); all other reagents were of analytical grade and obtained from Aladdin (Shanghai, China).

Cell culture, zebrafish husbandry, and embryo collection

SGC7901 gastric cancer cells were kept by our own lab and cultured in DMEM supplemented with 10% FBS (Gibco, USA).

Adult Tuebingen zebrafish were maintained in filtered, oxygenated tap water at a temperature of 28±0.1°C, pH 7±0.2, in a 14: 10 h light/dark cycle and fed live brine shrimp once daily and dry food twice a day [9]. All animal experiments in this investigation were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and approved by the Animal Care and Use Committee of Nanjing Normal University, China (Permit Number 2090658).

Zebrafish embryos were produced by pairwise mating in a fish hatching box with a 1: 2 (male: female) ratio and collected from spawning adults in tanks overnight. Embryos were collected within 1 h after fertilization, washed 3 times, and raised in an illumination incubator at 28°C in E3 embryo medium containing the antifungal solvent 0.01% methylene blue

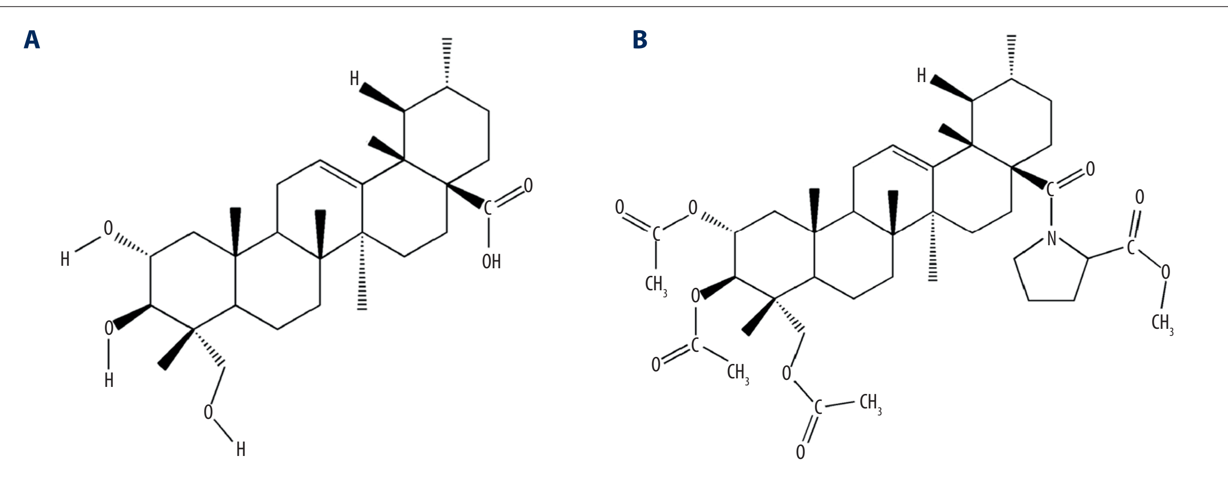

Figure 1. Structures of (**A**) AA and (**B**) AA-PMe.

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and salts (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) [10]. Embryos were observed on a daily basis for normal development, and tanks were monitored for water pollution and dead embryos. Media was replaced completely every 24 h [11]. Transgenic zebrafish and wild-type zebrafish were obtained from the Model Animal Research Center of Nanjing University and all zebrafish studies were approved by the Nanjing University of Technology.

Toxicity assay

Toxicity exposure

In a preliminary experiment, embryos were exposed to AA-PMe at various concentrations. Eight-hour post-fertilization (hpf) embryos treated with doses below 1 μM AA-PMe showed no signs of abnormality or decreased viability, but embryos exposed to concentrations over 50 μM were dead at 24 hpf, indicating 50 μM is the maximum tolerable concentration (MTC) [12]. For these experiments, concentrations decreasing geometrically from the MTC were used.

Embryos with >95% viability were chosen for this experiment and after microscopic examination, normally-developed 24-hpf embryos were randomly distributed into 24-well plates with 6 embryos per well. Embryos were then exposed to E3 media only (control group) or varying concentrations of AA-PMe in triplicate wells.

Acute toxicity assay

We used 72 hpf zebrafish embryos (or larvae, if hatched) to determine acute toxicity because by this point, morphogenesis and the development of functioning primary organ systems is complete. An inverted dissecting microscope was used to directly observe zebrafish in the 24-well plates, and the 50% lethal concentration (LC_{50}) was calculated based on the number of dead zebrafish at each concentration.

Acute toxicity was further determined based on daily observations of embryo malformation and hatching, the amount of spontaneous embryo tail lashing movement (motility) at 24 hpf, and the heart rate of the embryos or hatched larvae at 48, 72, and 96 hpf. Fish were not fed and dead fish were daily removed from the wells for the duration of the experiment.

Determination of AA-PMe absorption

Preparation of standard stock solution

AA-PMe was dissolved in dimethyl sulfoxide (DMSO) to produce a 2.9 mg/ml stock solution, filtered through a 0.22-μm PTFE membrane filter, and stored at 4°C.

Preparation of sample solutions for HPLC

Preparation of intracellular fluid for AA-PMe absorbance analysis

Culture medium and cells were collected following AA-PMe treatment with increasing concentrations at the indicated time points. Cells were washed 3 times with PBS, digested with trypsin, and collected after centrifugation. SGC7901 cells (4×10^6/ml) were re-suspended in PBS and repeatedly frozen and thawed 3 times at –80°C. The suspensions were centrifuged at 13 000 rpm for 10 min to remove debris, and only the supernatant containing the intracellular fluid was used for analysis. We added 1200 μl acetonitrile to 400 μl intracellular fluid, followed by vortex mixing for 2 min. Finally, the solution was centrifuged at 13 000 rpm for 10 min, filtered through a 0.22-μm PTFE membrane, and then directly injected into the HPLC [13].

Preparation of extracellular fluid for AA-PMe concentration analysis

After centrifugation to remove cells and debris, the culture medium supernatant was collected as extracellular fluid. We added 1200 μl acetonitrile into 400 μl of extracellular fluid, followed by vortex mixing for 2 min. The resulting solution was centrifuged at 13 000 rpm for 10 min, filtered through a 0.22-μm PTFE membrane, and then directly injected into the HPLC to determine the amount of extracellular AA-PMe.

Preparation of extra-zebrafish medium for AA-PMe concentration analysis

Zebrafish larvae were randomly divided into 6 groups (n=30) in 6-well plates at 120 hpf. Larvae were maintained in either ddH_2O as a control or in ddH_2O containing 10 μM AA-PMe for 1, 2, 4, 8, 10, 16, or 24 h; biological triplicates were performed for each treatment. The supernatants and larvae were collected separately (see below), and 2 ml of 100% methyl alcohol was added at 1, 2, 4, 8, 10, 16, or 24 h). Samples were incubated overnight at 4ºC, filtered through a 0.22-μm PTFE membrane, and then directly injected into the HPLC to determine the levels of unabsorbed AA-PMe [14].

Preparation of intra-zebrafish fluid for AA-PMe absorbance analysis

The separated larvae were blown dry with nitrogen [15], sonicated for 60 min until the tissue was powdered, and dissolved in 2 ml of 100% methyl alcohol. Samples were incubated overnight at 4°C, centrifuged at 4000 rpm for 10 min, filtered through a 0.22-μm PTFE membrane, and then directly injected into the HPLC.

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Table 1. LC_{50} (72hpf) of AA-PMe and AA on zebrafish.

Compound	Concentration, μM	LC_{50} (72hpf), μM
AA	1, 5, 10, 25, 50	4.54
AA-PMe	1, 5, 10, 25, 50	10.17

LC_{50} – 50% lethal concerntration.

Chromatographic condition

Samples were separated and analyzed using a Hypersil ODS column (4.6×250 mm, 5 μm) in a SHIMADZU SPD-15C HPLC system at 35°C using a 3: 1 acetonitrile: water mobile phase pumped at a flow rate of 1.0 ml/min. The sample injection volume was 20 μl and the detection wavelength was 215 nm.

Results

The 50% lethal concentration acute toxicity assay

The LC_{50} of AA and AA-PMe at 72 hpf were determined to be 4.54 μM and 10.17 μM in 72 hpf, respectively. Thus, the lethal sensitivity sequence of the 2 compounds to zebrafish is AA (4.54 μM) >AA-PMe (10.17 μM) (Table 1).

Teratogenic effects caused by AA-PMe in developing zebrafish embryos and larvae

The optimal time-point for toxicity experiments and morphological assessments in zebrafish is at 72 hpf: by this point, zebrafish larvae have hatched from the chorion with almost fully-formed primary organs and functional organ systems [16]. Edema or bleeding under or around the abdomen, curvature of the spine and tail, absence of a pectoral fin, changes in pigmentation, non-incubated and enlarged yolk sacs, and decreased motility are all evidence of abnormal development [17]. Using bright-field microscopy, we observed no effects on development at 1 μM AA or AA-PMe (Figure 2A). AA began to impair development at 5 μM, causing excess abdominal fluid, and at 10 μM, AA induced abdominal fluid build-up and bleeding. In contrast, AA-PMe had no effect on development at doses below 25 μM, at which point we observed accumulating abdominal fluid; abdominal fluid and bleeding were also detected at 50-μM doses. Thus, AA-PMe resulted in a higher ratio of normally vs abnormally developed fish compared with AA at most doses (Figure 2B).

Effects of AA-PMe on embryonic development

While chronic teratogenicity occurred in response to relatively low concentrations of AA, at 25 μM, AA became acutely lethal, with almost all zebrafish died by the second day (Figure 3A). At lower concentrations of AA, there was an increasing dose-dependent effect, but even at 10 μM there was more than 50% mortality by 2 days post-fertilization (dpf) and 5 μM caused more than 50% mortality by 6 dpf. Similar effects were only seen at higher doses of AA-PMe: treatment with 25 μM and 50 μM AA-PMe significantly increased mortality by 3 dpf, and by 4 dpf nearly 100% mortality occurred at either dose. However, doses below 5 μM AA-PMe had little to no effect on mortality: 1- and 5-μM doses were largely tolerated, even at 6 dpf (Figure 3B). At 10-μM doses, AA-PMe led to increased mortality at 6 dpf but after reaching 50%, the mortality rate remained steady. Comparing the lethal rates of AA and AA-PMe at different concentrations, we observed a far steeper mortality curve for AA compared with AA-PMe (Figure 3C).

Treatment with AA and AA-PMe similarly affected embryo hatching rates at increasing concentrations. At 25- and 50-μM doses, none of the embryos hatched, compared with a 100% hatching rate at 1 μM 96 hpf (Figure 3D). While 5- and 10-μM doses of AA appeared to promote hatching at 48 hpf, the toxic effects of AA were apparent at later time points, resulting in a negative correlation between dose and hatching rate. Similar to AA, AA-PMe concentrations below 25 μM promoted embryo hatching at 48 hpf. While 1 and 5 μM AA-PMe continued to promote hatching at 72 hpf, by this point the toxic effects of 10 and 25 μM doses became apparent (Figure 3E). With 10 μM AA-PMe, there was no further decrease at 96 hpf, but 25 μM dropped the hatching rate to the same level as 50 μM. At all time points, 50 μM AA-PMe strongly inhibited hatching. As with mortality, AA-PMe had less of a negative effect on hatching rates compared with AA at most concentrations (Figure 3F). Moreover, both compounds appeared to slightly promote hatching before their toxic effects became apparent.

HPLC chromatograms of AA-PMe in intracellular and extracellular fluid of SGC7901 cells

Drawing standard curves

To establish a standard curve, intracellular and extracellular fluid were collected from untreated SGC7901 cells, and AA-PMe was added to produce intracellular (0.0, 0.05, 0.203, 0.335, 0.450, 0.594, and 0.725 μg/ml) and extracellular (0.073, 1.16, 2.32, 3.48, 4.64, and 5.8 μg/ml) fluid standards. Standard curves were drawn from these controls, with the x-axis representing AA-PMe concentration and the y-axis the peak area. The best linear fit for intracellular fluid occurred between 0.058 and 0.725 μg/ml, $y=13749x+3223.9$ ($r=0.9997$), with a lower detection limit of 14.5 ng/ml (S/N=3). For extracellular fluid, the best fit occurred at 0.073–5.8 μg/ml AA-PMe, $y=13506x+3875.1$ ($r=0.9993$), with a lower detection limit of 18.1 ng/ml (S/N=3).

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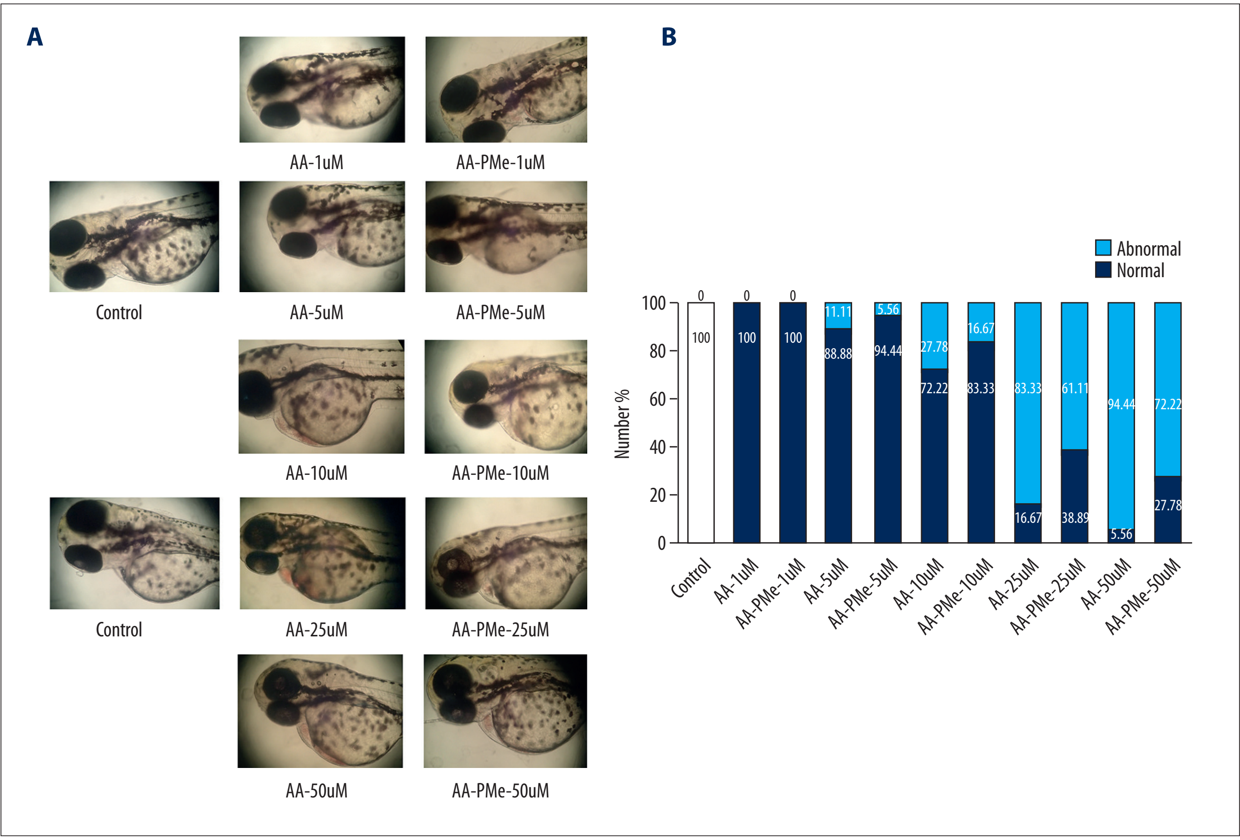

Figure 2. Morphological changes during early embryonic development in zebrafish caused by AA or AA-PMe treatment. (**A**) Embryos treated with different concentrations of AA or AA-PMe were imaged at 72 hpf. (**B**) The percentage of morphologically abnormal and normal zebrafish treated with different concentrations of AA or AA-PMe compared with controls (n=18 per group).

Specificity experiments

AA-PMe standard solution of intracellular/extracellular fluid and intracellular/extracellular fluid sample solution after drug treatment were added into corresponding blank intracellular/extracellular fluid, measured in terms of method. Figure 4 schematically displays the respective parameters of absorption. Since endogenous substances were nonintrusive for AA-PMe measurement, a good specificity was clearly shown.

Precision experiment

To ensure reproducibility, the intracellular (0.203, 0.450, and 0.725 μg/ml) and extracellular (1.16, 3.48, and 5.8 μg/ml) fluid standard measurements were repeated 5 times a day for 5 consecutive days to determine within-day and day-to-day precision. The within-day precision RSD of the intracellular standards were 1.47%, 1.79%, and 2.72%, and the day-to-day precision RSD were 2.98%, 3.26%, and 2.55%. For the extracellular standards, the within-day precision RSD were 0.78%, 1.38%, and 2.34%, while the day-to-day precision RSD were 2.17%, 2.39%, and 3.03%, indicating good precision.

Sample solution stability and repeatability experiment

Sample solution stability of 0.45 and 3.48 μg/ml intracellular and extracellular fluid after 0, 1, 2, 4, 8, 12, 24, and 48 h was similarly determined to ensure accurate measurements. For intracellular fluid, the RSD was 1.29%, while the RSD of extracellular fluid was 1.68%, indicating both solutions are highly stable within the test time. For the standards, these samples were run 5 times and the RSD were 1.34% for intracellular fluid and 1.48% for extracellular fluid.

Recovery ratio experiment

The sample recovery ratio was determined by adding 240, 200, or 160 μl of the 0.45 μg/ml (intracellular) and 3.48 μg/ml (extracellular) AA-PMe sample solutions into 9 tubes of 0.2 ml intracellular or extracellular fluid after drug treatment for 7 h. Following HPLC, the average recovery ratios of intracellular and extracellular fluid were determined to be 98.59% and 97.43%, respectively, which is a favorable recovery ratio.

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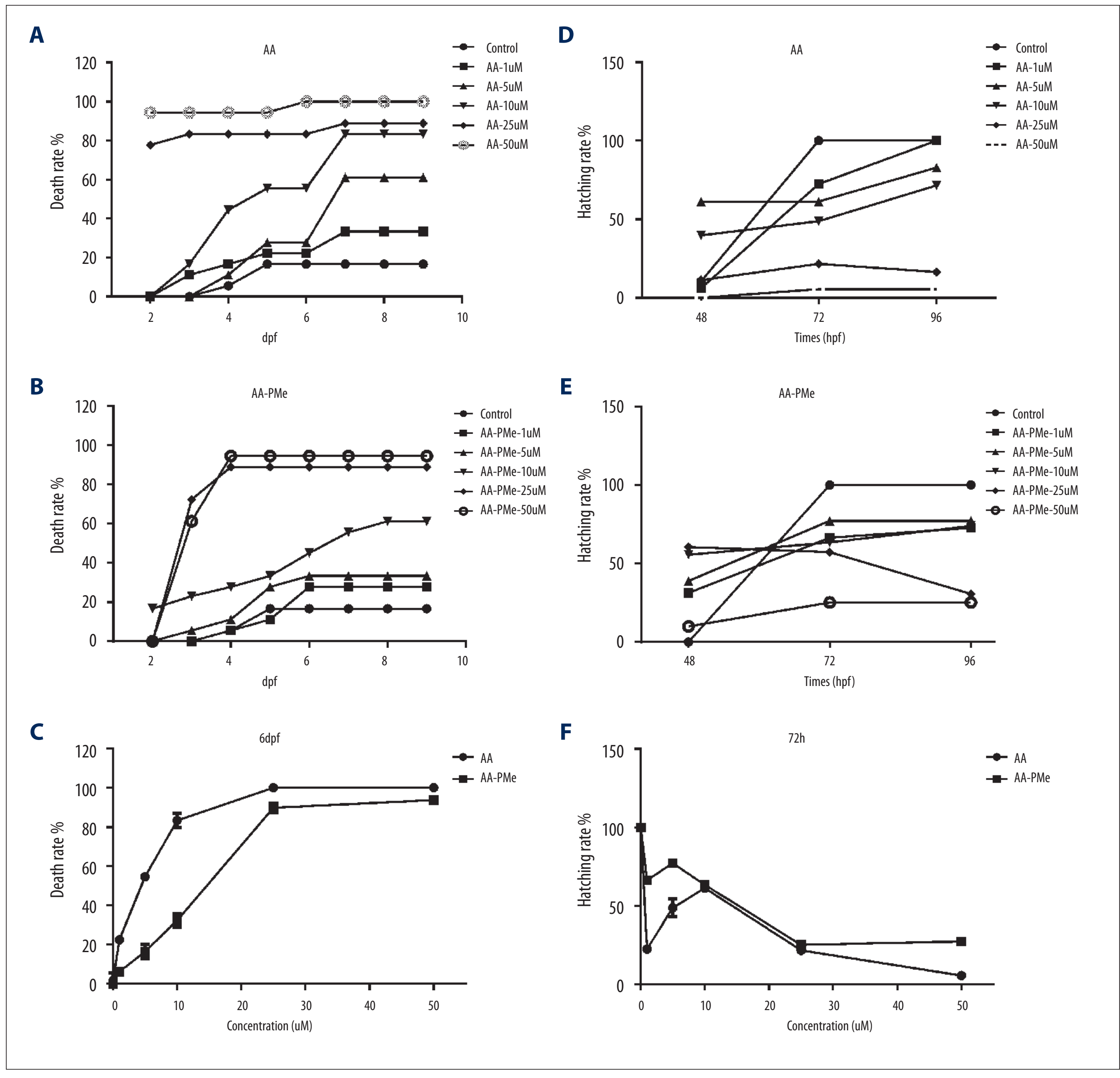

Figure 3. The effect of AA and AA-PMe exposure on embryo and larvae mortality and embryo hatching rate. Embryo and larvae mortality over time in response to various concentrations of (**A**) AA and (**B**) AA-PMe. (**C**) Comparison of larvae mortality at 6 dpf in response to increasing concentrations of AA and AA-PMe. Embryo hatching rates over time in response to various concentrations of (**D**) AA and (**E**) AA-PMe. (**F**) Comparison of embryo hatching rates at 72 hpf in response to increasing concentrations of AA and AA-PMe (n=18 per group).

Sample analysis

Logarithmic growth phase SGC7901 cells were treated with 0.725, 3.625, 7.25, 21.75, or 36.25 μg/ml AA-PMe for 1, 2, 4, 8, 10, 16, or 24 h and analyzed by HPLC (Tables 2, 3).

HPLC chromatograms of AA-PMe in intra-and extra-zebrafish fluid

Drawing standard curves

To establish a standard curve, intracellular and extracellular fluid were collected from untreated embryos and AA-PMe was added to produce intra-zebrafish (0.725, 1.450, 2.175, 2.900, 3.625, and 4.350 μg/ml) and extra-zebrafish (0.073, 1.16, 2.32, 3.48, 4.64, and 5.8 μg/ml) fluid standards. Standard curves

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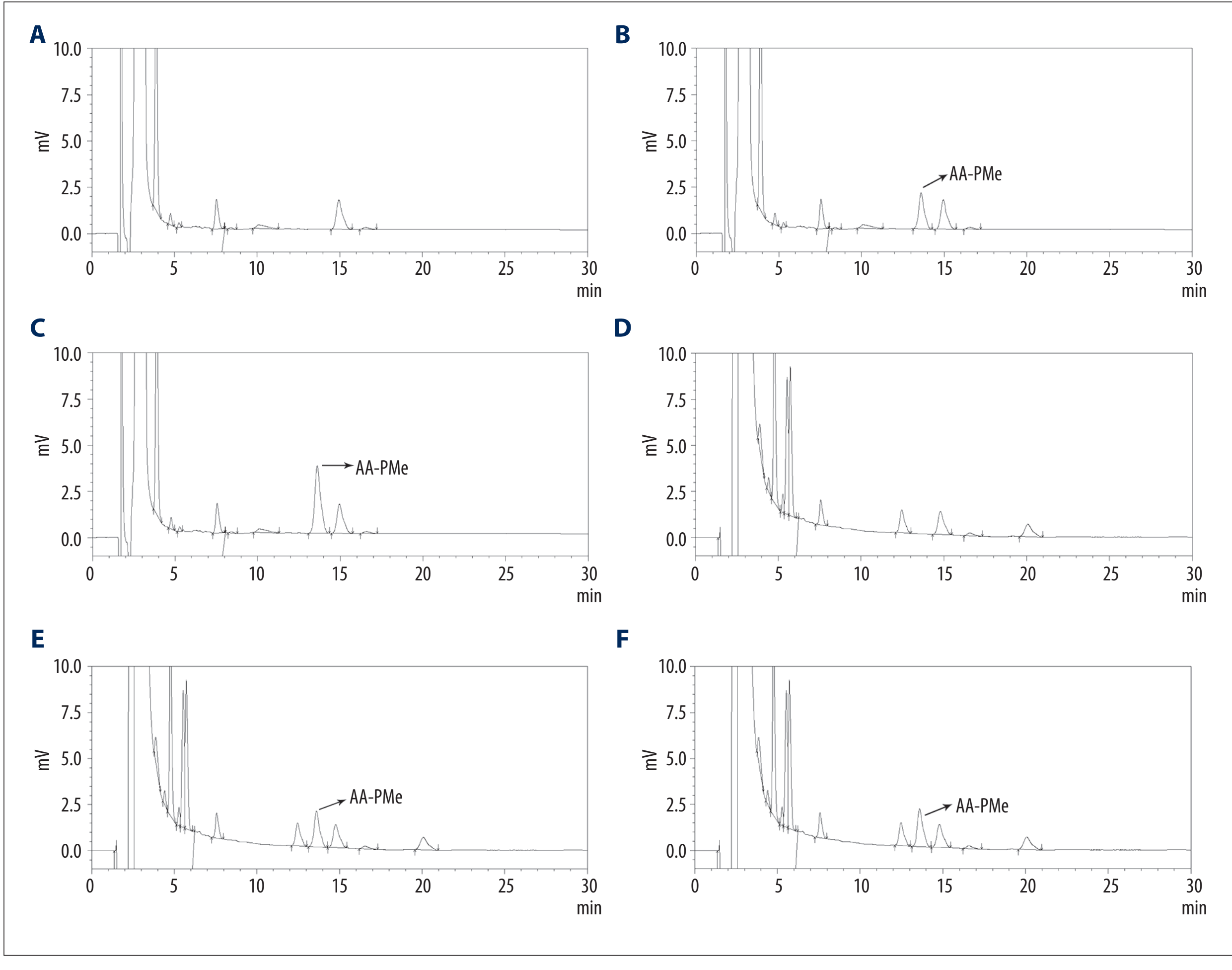

Figure 4. HPLC chromatograms of AA-PMe in intracellular and extracellular fluid of SGC7901 cells. (**A**) Blank intracellular solution, (**B**) intracellular solution standard, (**C**) AA-PMe test intracellular solution, (**D**) blank extracellular solution, (**E**) standard extracellular solution, (**F**) AA-PMe test extracellular solution.

were drawn from these controls, with the X-axis representing AA-PMe concentration and the Y-axis the peak area. Here, we found the best linear fit for intra-zebrafish fluid occurred at between 7.2505 μg/ml and 11.6005 μg/ml, y=9037.2x+2094.3 (r=0.9993), with a lower detection limit of 15.0 ng/ml (S/N=3). For extracellular fluid, the best fit occurred between 0.725 μg/ml to 4.350 μg/ml AA-PMe, y=9198.8x+1754.8 (r=0.9997), with a lower detection limit of 12.0 ng/ml (S/N=3).

Specificity experiments

AA-PMe standard solutions of intra- and extra-zebrafish fluid and drug-treated intra- and extra-zebrafish fluid samples were measured as described above in preparation of extra-zebrafish medium for AA-PMe concentration analysis (Figure 5). Untreated samples showed no peaks aligning with AA-PMe, indicating a high degree of specificity.

Precision experiment

To ensure precision, the intra-zebrafish (1.450, 2.175, and 4.350 μg/ml) and extra-zebrafish (8.120, 9.860, and 11.600 μg/ml) fluid standard measurements were repeated 5 times a day for 5 continuous days to determine within-day and day-to-day precision. The within-day precision RSD of the intra-zebrafish standards were 2.04%, 1.96%, and 0.74%, while the day-to-day precision RSD were 2.02%, 1.79%, and 2.74%. For the extra-zebrafish standard, the with-in-day precision RSD were 2.02%, 1.79%, and 2.74% while the day-to-day precision RSD were 2.62%, 2.36%, and 1.29%, suggesting good precision.

Sample solution stability and repeatability experiment

For the SGC7901 samples, solution stability of 2.175 and 9.860 μg/ml intra- and extra-zebrafish fluids, respectively, was determined after 0, 1, 2, 4, 8, 12, 24, and 48 h. For intra-zebrafish fluid, the RSD was 2.25%, while the RSD of extra-zebrafish fluid

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Table 2. AA-PMe content of SGC7901 intracellular fluid (μg/mL).

	0.725	3.625	7.25	21.75	36.25
1 h	0.0603	0.0703	0.1506	0.3461	0.2873
2 h	0.1038	0.1364	0.1602	0.4322	0.3443
4 h	0.1085	0.1946	0.2341	0.4727	0.4216
8 h	0.4674	0.4392	0.4569	0.6498	0.7157
10 h	0.0902	0.1967	0.2365	0.4564	0.3958
16 h	0.0674	0.1630	0.2204	0.4478	0.4770
24 h	0.0597	0.0639	0.1879	0.4563	0.4762

Table 3. AA-PMe content of SGC7901 extracellular fluid (μg/mL).

	0.725	3.625	7.25	21.75	36.25
1 h	0.1658	0.4472	1.5376	4.1318	5.5738
2 h	0.0731	0.3094	1.3142	3.3237	5.6810
4 h	0.0572	0.4607	1.1890	3.2949	5.7321
8 h	0.0559	0.5173	1.1454	3.2888	5.1403
10 h	0.0331	0.6385	1.3614	3.3990	5.7398
16 h	0.0270	0.3402	1.0763	3.3809	5.6615
24 h	0.1867	0.5695	1.1456	2.9725	5.1197

was 1.27%, indicating both solutions are highly stable within 48 h. For the standards, these samples were run 5 times and the RSD were 2.30% for intra-zebrafish fluid and 2.14% for extra-zebrafish.

Recovery ratio experiment

The sample recovery ratio was determined by adding 240, 200, or 160 μL of the 4.350-μg/ml (intra-zebrafish) and 8.120-μg/ml (extra-zebrafish) sample solutions into 9 tubes of 0.2 ml intra- or extra-zebrafish fluid after drug treatment for 10 h. Following HPLC, the average recovery ratios of intra- and extra-zebrafish fluid were determined to be 98.94% and 98.85%, which is a favorable recovery ratio.

Sample analysis

Zebrafish embryos were treated with 7.25 μg/ml AA-PMe for 1, 2, 4, 8, 10, 16, or 24 h. and analyzed as described above by HPLC (Tables 4, 5).

Discussion

The toxicity evaluation of AA-PMe in zebrafish

Here, we investigated the toxic effects of AA-PMe at various concentrations on the morphology, mortality, and hatching rates of zebrafish (Figures 2, 3). At higher doses, AA-PMe was acutely lethal, while at lower doses it resulted in chronic toxicity, demonstrated by abdominal fluid collection and bleeding, pericardial edema, yolk sac swelling and bleeding, and other characteristics. However, compared to AA, AA-PMe showed less toxicity overall. Curiously, both AA and AA-PMe appeared to promote increased embryo hatching at early time points, but over time and at higher doses, this effect disappeared and both compounds began to inhibit hatching. These differences in toxicity between AA and AA-PMe are likely due to differences in their structures, but the effects of modifications and their structure-function relationships need to be analyzed further.

The absorption of AA-PMe in cells and zebrafish

After investigating toxicity *in vivo,* we optimized HPLC conditions to establish a method for the determination of AA-PMe in cellular and zebrafish models with a high degree of accuracy and good reproducibility.

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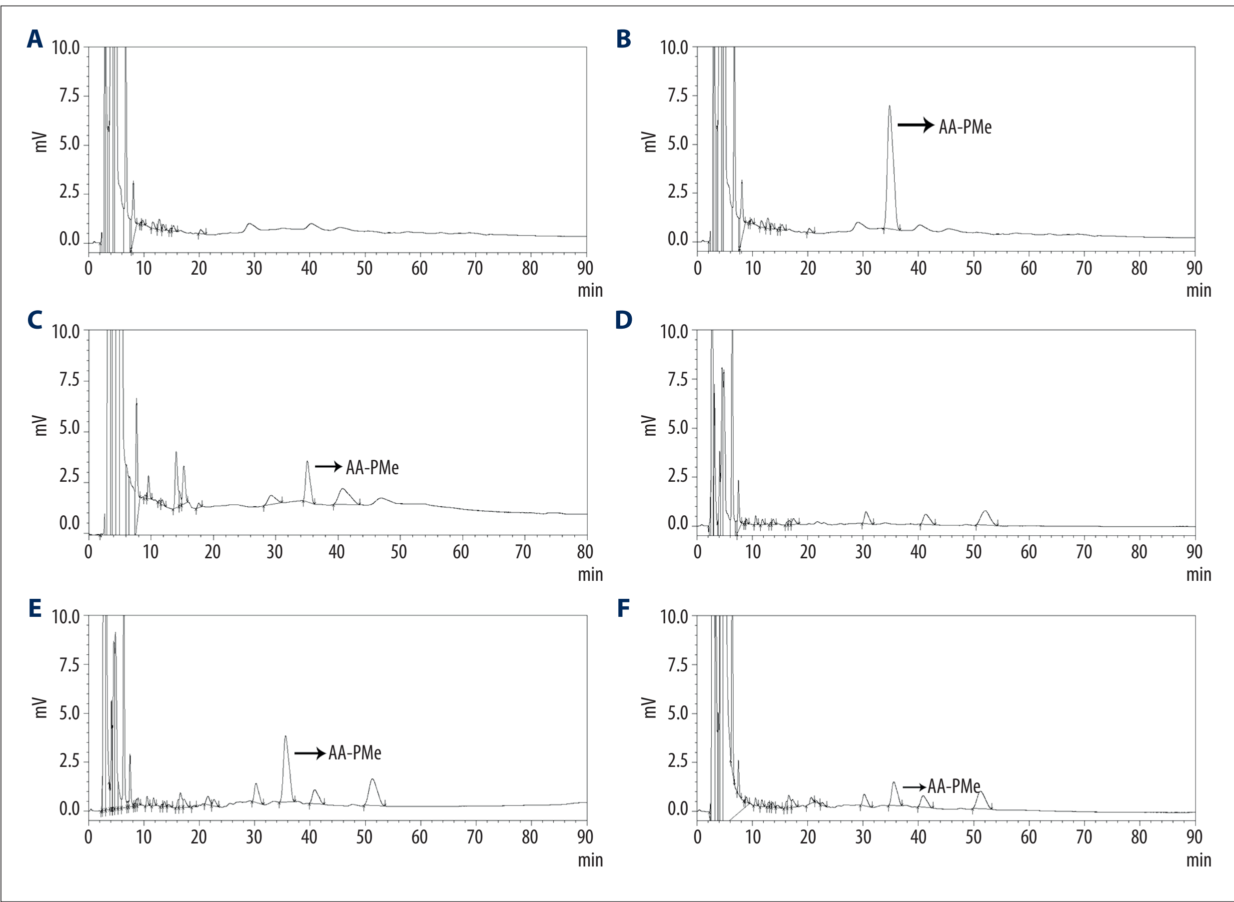

Figure 5. HPLC chromatograms of AA-PMe in intra- and extra-zebrafish fluid. Intra-zebrafish (**A**) blank intra-zebrafish solution, and (**B**) intra-zebrafish solution standard. (**C**) AA-PMe-treated intra-zebrafish solution. Extra-zebrafish (**D**) blank extra-zebrafish solution and (**E**) extra-zebrafish solution standard. (**F**) AA-PMe-treated extra-zebrafish solution.

Table 4. The content of AA-PMe intra-zebrafish (μg/mL).

	7.25
1 h	1.244
2 h	1.487
4 h	2.108
8 h	3.409
10 h	4.174
16 h	3.656
24 h	3.261

Table 5. The content of AA-PMe extra-zebrafish (μg/mL).

	7.25
1 h	11.28
2 h	9.95
4 h	9.77
8 h	8.399
10 h	7.659
16 h	8.594
24 h	8.677

In SGC7901 tumor cells we observed an increase in accumulation of intracellular AA-PMe over time that reached its maximum at 8 h before decreasing (Tables 2, 3). Intracellular drug accumulation also increased in a dose-dependent manner up to 0.44~0.72 μg/mL before plateauing, suggesting a saturation point for AA-PMe absorption by tumor cells. Above specific concentrations shown in Table 3, extracellular fluid levels of AA-PMe changed in a time-dependent manner. However, the total extracellular and intracellular drug levels did not necessarily equal the drug dosage, and this may be related to drug metabolism.

A similar pattern was observed in zebrafish, with intra-zebrafish AA-PMe increasing in a time-dependent manner until 10 h, when drug concentration peaked and remained at a steady state for 8 h before slowly declining (Tables 4, 5). Correspondingly, the

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concentration of extra-zebrafish AA-PMe gradually decreased to its lowest point at 10 h before steadily rising and reaching a steady state. Thus, zebrafish absorption and metabolism of AA-PMe appears to reach a balance after 10 h, stabilizing the intra- and extra-zebrafish fluid drug levels.

Overall, AA-PMe absorption by both cells and zebrafish appears to increase over time until equilibrium is achieved.

Conclusions

AA-PMe absorption by cells and zebrafish appears to increase over time until equilibrium is achieved. Our present study explored the cellular and organismal effects of AA-PMe and suggests a theoretical basis that may underlie its mechanism of action.

Conflict of interest

None.

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