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Screening of anti-cancer agent using zebrafish: Comparison with the MTT assay

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ABSTRACT

The MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay is a classical method for screening cytotoxic anti-cancer agents. Candidate drugs from the MTT assay need in vivo models to test their efficiency and to assess the absorption, distribution, metabolism, excretion, and toxicity of the drugs. An in vivo screening model could increase the rate of development of anti-cancer drugs. Here, we used zebrafish to screen a library of 502 natural compounds and compared the results with those from an MTT assay of the MCF7 breast cancer cell line. We identified 59 toxic compounds in the zebrafish screen, 21 of which were also identified by the MTT assay, and 28 of which were already known for their anti-cancer and apoptosis-inducing effects. These compounds induced apoptosis and activated the p53 pathway in zebrafish within 3 h treatment. Our results indicate that zebrafish is a simple, reliable and highly efficient in vivo tool for cancer drug screening, and could complement the MTT assay.

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1. Introduction

The traditional method for anti-cancer drug discovery, the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide) assay, is widely used to determine the cytotoxicity, proliferation and activation of potential medicinal agents based on the activity of mitochondrial dehydrogenase enzymes in cells [1,2]. The MTT cytotoxicity assay is widely used to screen for cytotoxic compounds during the development of chemotherapy or to screen for unwanted cytotoxic effects during preclinical studies [3]. The MTT assay usually produces a large number of candidates and requires the use of in vivo animal models to test the efficiency and assess the absorption, distribution, metabolism, excretion, and toxicity of these candidates [4]. Drug development may cease at any point because of low efficacy, toxicity or side effects, and less than 1% of candidates reach the market.

The use of an in vivo model at the screening stage could greatly decrease the costs and save time during anti-cancer drug development. Zebrafish is a small teleost fish species and has become an important model organism for the study of development, genetics, immunity, cancer and other diseases [5–10]. Due to the small size of the embryo, its rapid development and transparency, and the presence of similar molecules to mammals, zebrafish has emerged as a powerful in vivo platform for phenotype-based drug screens [11,12]. Zebrafish has been used not only to determine the bioactivity, toxicity, and off-target side effects of lead compounds, but also to validate novel drug targets, and to modify the structure of compounds [10,13–15]. A recent study demonstrated that zebrafish is an effective model not only for drug screening but also for drug optimization [11].

Previous studies have screened cell cycle inhibitors [16], angiogenesis inhibitors [17], apoptosis inducers [18] and cytotoxic agents [10] for novel cancer therapy using zebrafish. However, the potential for the use of zebrafish in anti-cancer drug screening has yet to be assessed, and no comparisons have been made with the MTT assay. In this study, we screened 615 compounds using zebrafish and the MCF7 breast cancer cell line and compared the differences between the two models.

2. Materials and methods

2.1. Zebrafish strains

AB and Tg (fli1:GFP) zebrafish were obtained from the Zebrafish International Resource Center (Eugene, Oregon, USA). Fish were housed in an automated fish housing system (ESEN, China) at 28 °C. Embryos were grown to 24 hpf, and then exposed to test compounds for 24 h. The embryos were observed under an Olympus SZX9 Zoom Ztereo Microscope (Japan).

2.2. Compounds

Five hundred and two compounds were assessed in the primary screen. The compounds were dissolved in DMSO and

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were diluted at a final concentration of 50 μM with Hanks for zebrafish or with DMEM for cell culture respectively.

2.3. Real-time RT-PCR

For the real-time RT-PCR assay, the embryos were treated with compounds for 3 h after which RNA was extracted. An RNeasy Mini kit (QIAgen) was used to isolate total RNA from ten treated embryos. After treatment with DNase, 2 μg RNA was reverse-transcribed using Moloney murine leukemia virus (MMLV) Reverse Transcriptase (Promega). The mRNA level was quantified using an Eppendorf Real-Time Cycler plus *β-actin* as a reference. The primer sequences were as follows:

p53:
5′-ATGTGGTGCCTGCCTCAGA-3′ (sense)
5′-CTTCGTCCTTCACCATCAGCTT-3′ (antisense)

β-actin:
5′-TGACAACGGCTCCGGTATG-3′ (sense)
5′-TTCTGTCCCATGCCAACCAT-3′ (antisense)

2.4. Analysis of apoptosis

Apoptotic cells were identified using the TMR-RED in situ cell death detection kit (Roche) as previously described [19]. Briefly, the embryos were fixed in 4% paraformaldehyde overnight and then washed three times with PBS for 5 min each at room temperature, followed by a 10 min incubation in acetone at −20 °C and two 5 min washes in PBS. Embryos were further permeabilized by incubating them in a freshly prepared solution containing 0.1% Triton X-100 and 0.1% sodium citrate in PBS for 15 min at room temperature followed by three 5 min rinses in PBS. They were then subjected to the TUNEL assay. The fluorescent signal was visualized and imaged using a Zeiss LSM510 microscope. Z-stack was superimposed using the extended focus feature of Zeiss LSM Image Examiner Version 3.2.0.115 software.

2.5. MTT assay

MCF7 breast cancer cells (2×10^3) were plated in each well of a 96-well plate and were allowed to adhere and spread for 24 h. The

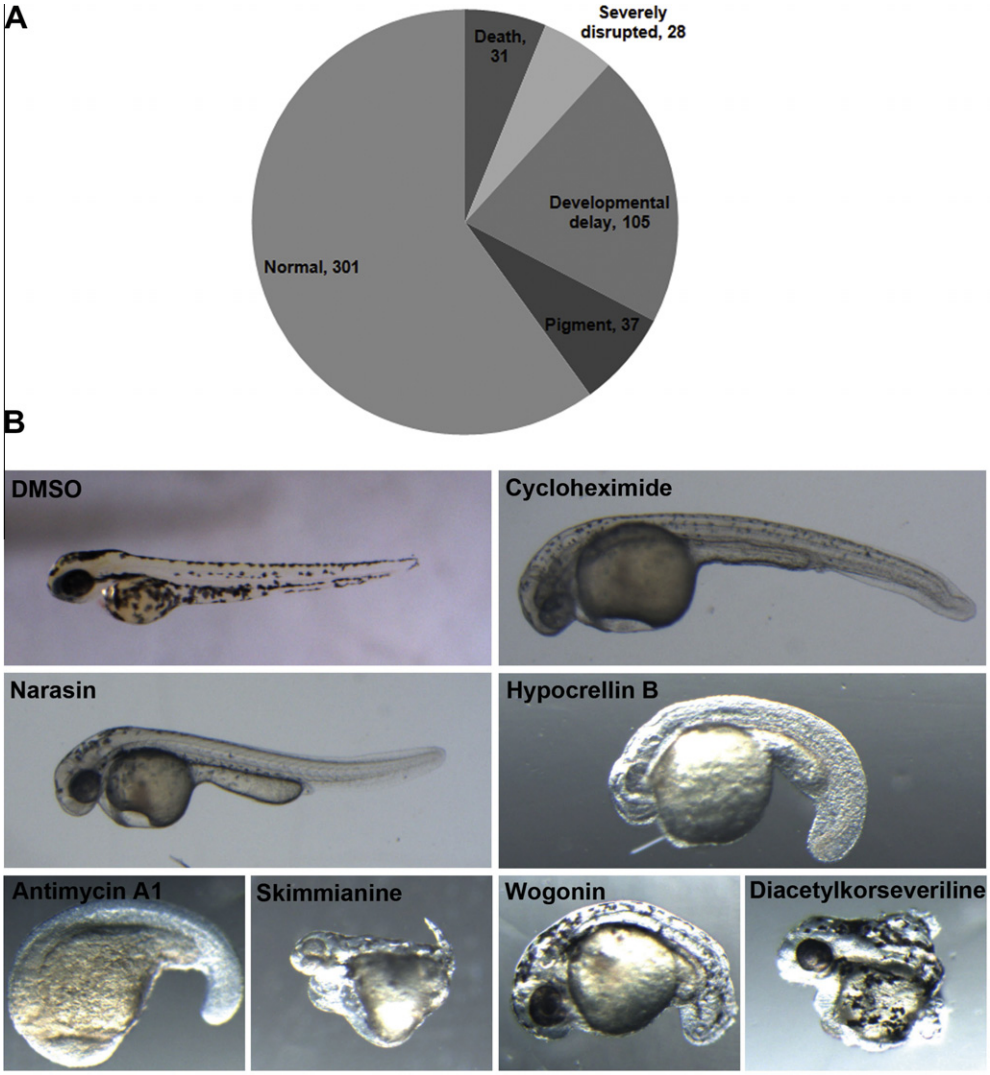

Fig. 1. Screening of toxic natural compounds using zebrafish embryos. (A) The compounds that affected zebrafish embryos are shown (201 of the 502 compounds screened). Of these, 31 and 28 compounds caused lethality and severely disrupted development respectively, 168 caused developmental delay or malformation, and 37 compounds reduced pigment levels. (B) Embryos showed normal (DMSO) or severely disrupted (cycloheximide, narasin, hypocrellin B and antimycin A1) development, and malformation (skimmianine, wogonin and diacetylkorseverilline). Embryos were treated with compounds at 50 μM for 24 h as indicated in the Section 2.

compounds were added to a final concentration of 50 μM, and the cells were cultured for 24 h at 37 °C. MTT solution (10 μl of 10 mg/ml, Roche) was added to each well, and the cultures were incubated for an additional 4 h. A further 100 μl of MTT solution was added and incubation continued overnight. The absorbance at 540 nm was determined in each well with a 96-well plate reader. The growth of the treated cells was compared with that of untreated cells.

3. Results

3.1. Screening potent anti-cancer compounds using zebrafish

Given that embryogenesis and carcinogenesis have similar molecular mechanisms [20,21], we used zebrafish embryos to screen the anti-cancer agents. A library of 502 natural compounds was tested for cytotoxic effects on the development of zebrafish embryos. At 24 h post-fertilization (hpf) the embryos were placed in 96-well plates (5 embryos per well), and incubated for 24 h with the compounds in Hanks at a concentration of 50 μM to assay toxicity. Any developmental abnormalities including those in the brain, blood, vasculature, heart, gut, trunk, tail and pigment were observed. Fig. 1A illustrates the effects of the compounds on the embryos and shows that 201 of the 502 compounds resulted in different phenotypes; some of these compounds also had multiple effects on phenotype. Of these, 31 caused toxicity leading to death, 28 severely disrupted development, 168 caused developmental delay or malformation, and 37 compounds reduced pigment development. Fig. 1B illustrates the severe developmental delay or malformation of zebrafish embryos caused by the active compounds.

3.2. Screening compounds by MTT assay in the MCF7 cell line

To analyze the efficiency of zebrafish screening for anti-cancer drugs, the compounds were tested by MTT assay, the traditional method for anti-cancer drug screening, using MCF7 breast cancer cells, and the results were compared with those from the zebrafish screen. The inhibitory efficiency of the compounds was assayed after 24 h of incubation of the cells in drug-containing medium at a concentration of 50 μM. Significant inhibition of growth was defined as 60% or less of control cell growth. Sixty-one of the 502 tested compounds reduced MCF7 growth by 60% when compared to the DMSO control.

Comparison of the zebrafish data with that from the MTT assay revealed an overlap between 21 of the toxic compounds (Fig. 2, Table 1), suggesting that both methods are effective for anti-cancer drug screening. In other candidate compounds identified in the zebrafish screen, the anti-cancer and apoptosis-inducing effects of 28 compounds were confirmed by the literature but not by the MTT assay (Table 1), and 10 were not identified by or had no toxic effect on MCF7 cells. These data indicate that the zebrafish screen and MTT assay are both effective in anti-cancer drug discovery, but that the zebrafish assay is more sensitive to some compounds.

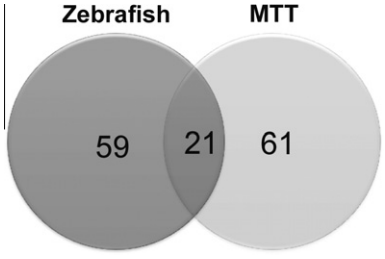

Fig. 2. Comparison of zebrafish screen with MTT screen. The zebrafish screen identified 59 toxic compounds, and the MTT screen identified 61. Of these, 28 compounds were toxic to both MCF7 cells and zebrafish.

Table 1
Zebrafish screening results and comparison to MTT assay.

Confirmed by	Zebrafish (+)	Confirmed by	MTT (+)
MTT	Brefeldin A	Zebrafish	Brefeldin A
	Bufalin		Bufalin
	Camptothecin		Camptothecin
	Capsaicin		Capsaicin
	Chaconine, α-		Chaconine, α-
	Cinobufagin		Cinobufagin
	Cycloheximide		Cycloheximide
	Cytochalasin E		Cyclopamine
	Echinomycin		Echinomycin
	Gliotoxin		Gliotoxin
	Harmine HCl		Harmine HCl
	Hypocrellin A		Hypocrellin A
	Ivermectin		Ivermectin
	Lapachone, β-		Lapachone, β-
	Menadione		Menadione
	Narasin		Narasin
	Plumbagin		Plumbagin
	Podophylotoxin		Podophylotoxin
	Rotenone		Rotenone
	Sanguinarine		Sanguinarine
	Vinpocetine		Vinpocetine
Reference	Amphotericin B	Reference	Actinomycin D
	Antimycin A1		Anisomycin
	Australine HCl		Berberine HCl
	Chelidonine, (+)-		Brucine n-oxide
	Daidzein		Cantharidin
	Deguelin		Catalpol
	Emodin		Cepharanthine
	Epibatidine, (±)-		Cerulenin
	Eudesmine		Chromomycin A3
	Evodiamine		Colchicine
	Forskolin		Curcumin
	Gossypol		Daunorubicin HCl
	Gramine		Dihydrotanshinone
	Honokiol		Gambogic acid
	Hypocrellin B		Gitoxigenin
	Indirubin		Harringtonine
	Mevastatin		Lycorine HCl
	Mycophenolic acid		Melatonin
	Myricetin		Mitomycin C
	Papaverine HCl		Nonactin
	Rifampicin		Ouabain (-)-
	Rottlerin		Parthenolide
	Scoulerine		Patulin
	Securinine		Puromycin
	Tschimganidin		Salinomycin
	Vincamine		Shikonin
	Wedelolactone		Streptonigrin
	Wortmannin		Taxol
Not confirmed	Deoxyphorbol 13-phenylacetate 20-acetate, 12-		Tetrandrine
	Ebelactone B		Tomatidine
	Nicotine, (-)-		Tubercidin
	Mezerein		Vinblastine sulfate
	Phorbol 12,13-dibutyrate		Vincristine sulfate
	Phorbol 12-myristate 13-acetate		Vinorelbine base
	Remerine HCl	Not confirmed	Deoxyshikonin
	Scopolamine N-oxide		
	HBr, (-)-		Emetine 2HCl
	Thapsigargin		Panaxatriol
	Tschimganine		Peruvoside
			Strophanthidin acetate
			Vineomycin A1

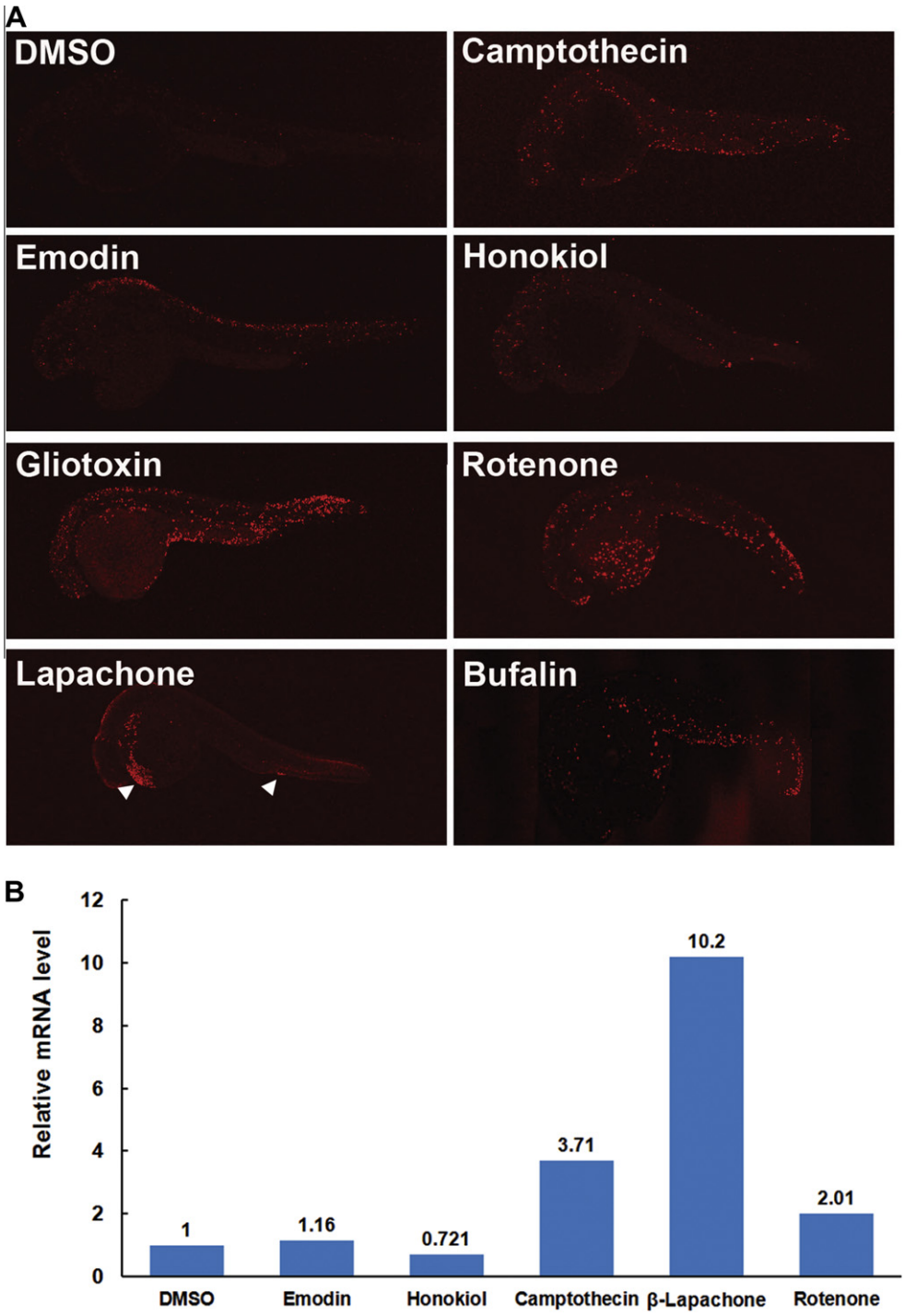

Fig. 3. The toxic compounds induced early apoptosis and p53 activation in zebrafish embryos. (A) TUNEL staining of the embryos treated with DMSO, camptothecin, emodin, honokiol, gliotoxin, rotenone, lapachone and bufalin. (B) P53 mRNA levels in the embryos treated with DMSO, emodin, honokiol, camptothecin, β-lapachone and rotenone. *β-Actin* expression was used as an internal control.

3.3. Anti-cancer agent induces apoptosis and activates p53 gene expression in zebrafish embryos at an early stage

Exposure to cytotoxic compounds can result in a variety of cell fates including cell necrosis, differentiation or programmed cell death (apoptosis). Previous reports have shown that camptothecin [22,23] induces tumor cell apoptosis via several pathways. We tested whether drugs could also induce apoptosis in zebrafish embryos using the TUNEL assay (Fig. 3A). In situ cell death detection showed that camptothecin, a Topo I inhibitor used in colon cancers, induced extensive apoptosis in zebrafish embryos after only 3 h. Other compounds such as emodin, honokiol, gliotoxin, bufalin and rotenone showed similar patterns of apoptosis to that observed in camptothecin-treated embryos. Several compounds showed specific apoptosis patterns; for example, β-lapachone [24] promoted apoptosis in blood stem cells (Fig. 3A), suggesting that the zebrafish in a useful tool for apoptosis analysis.

P53 is a key molecule in the apoptosis pathway. Most anti-cancer drugs induce apoptosis through the activation of p53 gene expression in the cancer cells. We investigated whether drugs that induce p53 mRNA in cancer cells could also significantly induce p53 gene expression in zebrafish embryos. Of the five compounds tested, camptothecin, β-lapachone and rotenone increased p53 mRNA levels within 3 h of incubation, indicating that zebrafish is an effective model for the rapid screening of anti-cancer agents.

4. Discussion

In the present study, we demonstrate a simple, quick and practical method for large-scale screening of cytotoxic agents for cancer therapy using zebrafish embryos. The main findings of this study are: (1) Most known cytotoxic anti-cancer drugs induced zebrafish embryo death within 24 h, showing similarity to the MTT assay; (2) some of these compounds induced apoptosis and activated the p53 pathway in zebrafish embryos within 3 h of treatment; (3) new toxic compounds that may be developed into anti-cancer drugs were identified in the zebrafish screen.

The zebrafish has previously been used to screen a cell cycle suppressor [16], and to identify and evaluate a novel and potent small molecule radiation sensitizer and an angiogenesis inhibitor using a chemical library [17]. The zebrafish has also been used to screen a small library of triazine compounds for inhibition of tubulin polymerization [25] and the inhibitor of wnt signaling in vivo [26] as a first step toward potential therapy of cancers. We found that 21 of the cytotoxic anti-cancer drugs that suppressed MCF7 breast cancer growth in vitro also induced zebrafish embryo death within 24 h of culture. This is consistent with the report by Murphey et al. that compounds that inhibit the G2/M phase of the cell cycle in vitro also inhibit the G2/M phase in zebrafish [13]. In our study, 28 compounds showed toxicity to zebrafish embryos but not to MCF7 cells, suggesting that the zebrafish screen is more sensitive to some compounds. This could be because: (1) the MTT assay may underestimate the anti-proliferative effect of a compound in the MCF7 cell line [12]; (2) the compounds could have different effects due to the difference between the in vivo and in vitro assay. The MTT assay can only be used to determine cytotoxicity, proliferation and activation in single cell lines, whereas it is possible to screen and observe drug efficiency in whole zebrafish. The combined use of two methods in anti-cancer drug screening may produce better results.

Apoptosis is an important mechanism in anti-cancer therapies, and can be used as an index in the analysis of cytotoxic drugs. Zebrafish has similar apoptotic molecules to humans, including p53, bcl2 and caspases, and these could be used to screen for apoptosis-inducing agents. The experimental results from zebrafish could be used as a reference before clinical trials. Interestingly, in the present study, most of the anti-cancer compounds tested also triggered apoptosis in zebrafish embryos within 3 h of treatment. Some cytotoxic compounds such as β-lapachone [24,27] induced apoptosis in blood stem cells in zebrafish specifically, but were not identified by screening with cell lines in vitro. Our data suggest that the zebrafish is an effective tool for whole mount screening of apoptosis-inducing drugs. In contrast to cell lines, the regulation of p53 in zebrafish is much more complicated because of the cross-talk between multiple organs. The p53 gene is activated in chemotherapy and radiotherapy and plays a key role in the induction of apoptosis in response to DNA damage in zebrafish [18]. Some DNA-damaging reagents such as camptothecin have been shown to increase p53 mRNA [28,29] and protein levels in zebrafish [12]. Activation of p53 leads to apoptosis mediated by stimulation of the intrinsic apoptosis pathway. Our results indicate that only some of the anti-cancer agents induce apoptosis in vivo via activation of p53 pathway signaling.

In conclusion, we found that cytotoxic anti-cancer agents that inhibit cell growth are also toxic to zebrafish embryos. Compounds that induced apoptosis and activated the p53 pathway in the cell line also acted on zebrafish in a similar manner. For some compounds, the zebrafish screen was more sensitive than the MTT assay. Our results indicate that the zebrafish is a highly efficient in vivo tool for cancer drug screening and may be used as a complement to the MTT assay.

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