COMPARATIVE TOXICITY EVALUATION OF TARGETED ANTICANCER THERAPEUTICS IN EMBRYONIC ZEBRAFISH AND SEA URCHIN MODELS

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Cancer drug resistance and poor selectivity towards cancer cells demand the constant search for new therapeutics. PI3K-Akt-mTOR and RAS-MAPK-ERK signaling pathways are key mechanisms involved in cell survival, proliferation, differentiation, and metabolism and their deregulation in cancer can promote development of therapy resistance. We investigated the effects of targeted inhibitors (wortmannin, GSK690693, AZD2014 and tipifarnib) towards these two pathways on early zebrafish and sea urchin development to assess their toxicity in normal, fast proliferating cells. PI3K inhibitor wortmannin and RAS inhibitor tipifarnib displayed highest toxicity while GSK690693, a pan-Akt kinase inhibitor, exhibited a less significant impact on embryo survival and development. Moreover, inhibition of the upstream part of the PI3K-Akt-mTOR pathway (wortmannin/GSK690693 co-treatment) produced a synergistic effect and impacted zebrafish embryo survival and development at much lower concentrations. Dual mTORC1/mTORC2 inhibitor AZD2014 showed no considerable effects on embryonic cells of zebrafish in concentrations substantially toxic in cancer cells. AZD2014 also caused the least prominent effects on sea urchin embryo development compared to other inhibitors. Significant toxicity of AZD2014 in human cancer cells, its capacity to sensitize resistant cancers, lower antiproliferative activity against human normal cell lines and fast proliferating embryonic cells could make this agent a promising candidate for anticancer therapy.

Keywords: PI3K-Akt-mTOR signaling pathway – RAS-MAPK-ERK signaling pathway – zebrafish (*Danio rerio*) – sea urchin (*Arbacia lixula*) – AZD2014

INTRODUCTION

One of the main obstacles for successful cancer treatment is the development of resistance to chemotherapy. PI3K-Akt-mTOR and RAS-MAPK-ERK signaling pathways are among the main cellular mechanisms involved in the regulation of cell survival, proliferation, differentiation, and metabolism [24, 26]. Deregulation of these pathways may result in resistance to therapy which is common for numerous cancer types [23, 24]. The role of PI3K-Akt-mTOR and RAS-MAPK-ERK pathway inhibition in chemosensitization of resistant cancers has been extensively studied and vari-

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ous inhibitors of these pathways were investigated as potential agents to overcome the problem of multidrug resistance [1, 27, 29]. Additional problem in cancer therapy is its nondiscriminatory nature between normal and cancer cells. Many agents do not spare normal cells and may have substantial cytotoxic effects on normal tissue. Some cell types in bone marrow or gastrointestinal lining have a rapid turnover, and can be more susceptible to drug toxicity. For instance, the toxic effect in bone marrow cells can lead to development of anemia and neutropenia, while toxicity in the gastrointestinal tract can result in colitis [5].

Zebrafish represents an excellent model to study the toxicity of different substances [12]. As an *in vivo* system for toxicology, zebrafish has many advantages including rapid and *ex utero* development, transparent and small embryos in early stages, high fecundity enabling high-throughput screening and cost effectiveness [4]. Zebrafish genome has approximately 70% of human genes and about 82% of potential human disease related genes have at least one zebrafish orthologue [16]. Additionally, the studies of known toxic substances on zebrafish revealed 63–100% of predictability which makes zebrafish a good model to study toxic effects [11, 14, 17].

The sea urchin embryo has long been used as a model for developmental studies and drug toxicity testing. Multiple factors such as inexpensive growth procedure, simple artificial spawning and fertilization, rapid synchronous development, optical transparency of embryos and well understood embryogenesis, make this organism suitable for conducting a variety of biological tests. Other advantages that make sea urchin embryo particularly convenient for toxicity studies include high permeability of eggs and embryos to chemicals, rapid cell divisions, observable markers of viability and biochemical similarity to vertebrates and mammals [28]. As a result, various antiproliferative agents have been tested for their effects on the sea urchin embryo development [20, 30].

The aim of this study was to assess the effect of different signaling pathway inhibitors in vertebrate and invertebrate embryonic models. For this reason, we followed early zebrafish (*Danio rerio*) and sea urchin (*Arbacia lixula*) development in order to assess inhibitors' toxicity on normal, fast proliferating cells. We have investigated four inhibitors of key components in PI3K-Akt-mTOR and RAS-MAPK-ERK pathways: wortmannin – specific inhibitor of PI3K; GSK690693 – ATP-competitive Akt kinase inhibitor, AZD2014 – dual mTORC1 and mTORC2 inhibitor and tipifarnib – RAS inhibitor.

MATERIALS AND METHODS

Drugs

Wortmannin, GSK690693, AZD2014, and tipifarnib were kindly provided by Selleckchem (Houston, TX, USA). All drugs were diluted in DMSO and 10 mM aliquots were stored at –20 °C. Before treatment, all drugs were freshly diluted in water.

Zebrafish husbandry

Tübingen wild type zebrafish (*Danio rerio*) were housed at 28 ± 1 °C on a 12:12 light–dark photoperiod, according to good laboratory practice. Adults and embryos were maintained in water containing 5.5 mg KCl, 294 mg $CaCl_2 \cdot 2H_2O$, 123 mg $MgSO_4 \cdot 7H_2O$ and 63 mg $NaHCO_3$ per L. Zebrafish were spawned every seven days. Spawn was discarded if more than 10% of eggs were unfertilized.

Zebrafish treatments

Experiments were performed in 24-well plates. Twelve embryos were placed in each well in 750 µl of the appropriate solution. Three different concentrations of each inhibitor were tested per each experiment (wortmannin: 0.5 µM, 1 µM, 2.5 µM; GSK690693: 0.5 µM, 1 µM, 5 µM; AZD2014: 0.1 µM, 0.5 µM, 1 µM, tipifarnib: 1 µM, 5 µM, 10 µM). Two different combinations of inhibitors were also tested: 1 µM wortmannin+1 µM GSK690693 and 1 µM GSK690693+1 µM AZD2014. Treatments started 2, 6 or 24 hours post fertilization (hpf) for single treatments and 6 hpf for combination treatments. Treatments for all concentrations were repeated three times using embryos obtained from three independent spawns. All experiments contained untreated and 0.01% dimethyl sulfoxide (DMSO) treated groups, as controls.

Developmental toxicity screen

The embryos were observed under the stereomicroscope PXS- IV (Optica Co. Ltd, Thailand) at 40x magnification and imaged with camera CKX41 (Olympus, Japan). Embryo survival was observed at 24, 48 and 72 hpf. Embryos that were coagulated or where no heartbeat could be detected were considered dead. Embryo hatching rate was observed at 48 and 72 hpf, as hatching normally occurs between 48 and 72 hpf. Developmental malformations (head malformation, eye malformation, eye pigmentation, otoliths malformation, chorda malformation, tail malformation, scoliosis, yolk deformation/edema, heart malformation, pericardial edema, tail circulation, growth retardation, body pigmentation) were observed at 72 hpf after hatching.

Handling of sea urchin gametes

Adult *Arbacia lixula* sea urchins were collected by scuba in the Adriatic Sea and were immediately transported (maximum 1 h transportation time) to the Institute for Marine Biology (Kotor, Montenegro). They were subsequently kept in circulating and filtered artificial sea water (ASW) (27.6 g/l NaCl, 0.67 g/l KCl, 1.36 g/l $CaCl_2 \times 2H_2O$, 6.29 g/l $MgSO_4 \times 7H_2O$, 4.66 g/l $MgCl_2 \times 6H_2O$, 0.18 g/l $NaHCO_3$, pH 7.5) at

21 °C. Eggs or sperm were obtained by injecting the animals with 1 ml of 0.55 M KCl, and fertilization of the eggs was carried out using the sperm of one male with eggs acquired from 2 to 4 females.

Sea urchin embryo treatments

Approximately 100 fertilized sea urchin eggs were placed in 1 ml of ASW per well in 6-well plates. Two different concentrations were assessed for each inhibitor: AZD2014 (0.25 and 0.5 μM), GSK690693 (2.5 and 5 μM) and tipifarnib (5 and 10 μM). Treatment was performed after visible assembly of the sea urchin fertilization membrane, which occurs approximately 20 min after sperm fusion with the egg. After 24 h, control and treated embryos were examined under Leica DMI4000 B microscope (Leica Microsystems GmbH, Germany) at 5× magnification. A minimum of 30 embryos were observed for each condition. The results are presented as percentage of nonviable and viable embryos in different developmental stages (undeveloped embryos, swimming blastula and late gastrula) in control and treated groups.

Statistical analysis

All data are shown as mean±SEM. Differences between control and single treatments were assessed using one-way analysis of variance (ANOVA) followed by Dunette's test. In combined treatments, the results were analyzed with t-test. The minimum significant level was set at $p \leq 0.05$. Statistical analyses were performed using GraphPad Prism 5 or 6.

RESULTS

Wortmannin causes decreased embryo survival and morphological malformations

There were no significant differences between untreated embryos and embryos incubated in 0.01% DMSO. Untreated embryos did not show mortality or developmental malformations at any time of observation. Hatching rate of untreated embryos was around 44% at 48 hpf and 100% at 72 hpf.

Wortmannin induced dose-dependent decreased survival rate in embryos treated 2 and 6 hpf when compared to the control group at 72 hpf. However, only 2.5 μM treatment applied at 2 hpf produced a statistically significant result (58%, Fig. 1A). Wortmannin also induced a dose- dependent hatching rate decrease in embryos treated 2 and 6 hpf when compared to the control group at 48 hpf (Fig. 1B). Still, only 2.5 μM treatment applied at 2 hpf caused statistically significant result by completely blocking the hatching.

Table 1
Embryo malformations induced by wortmannin

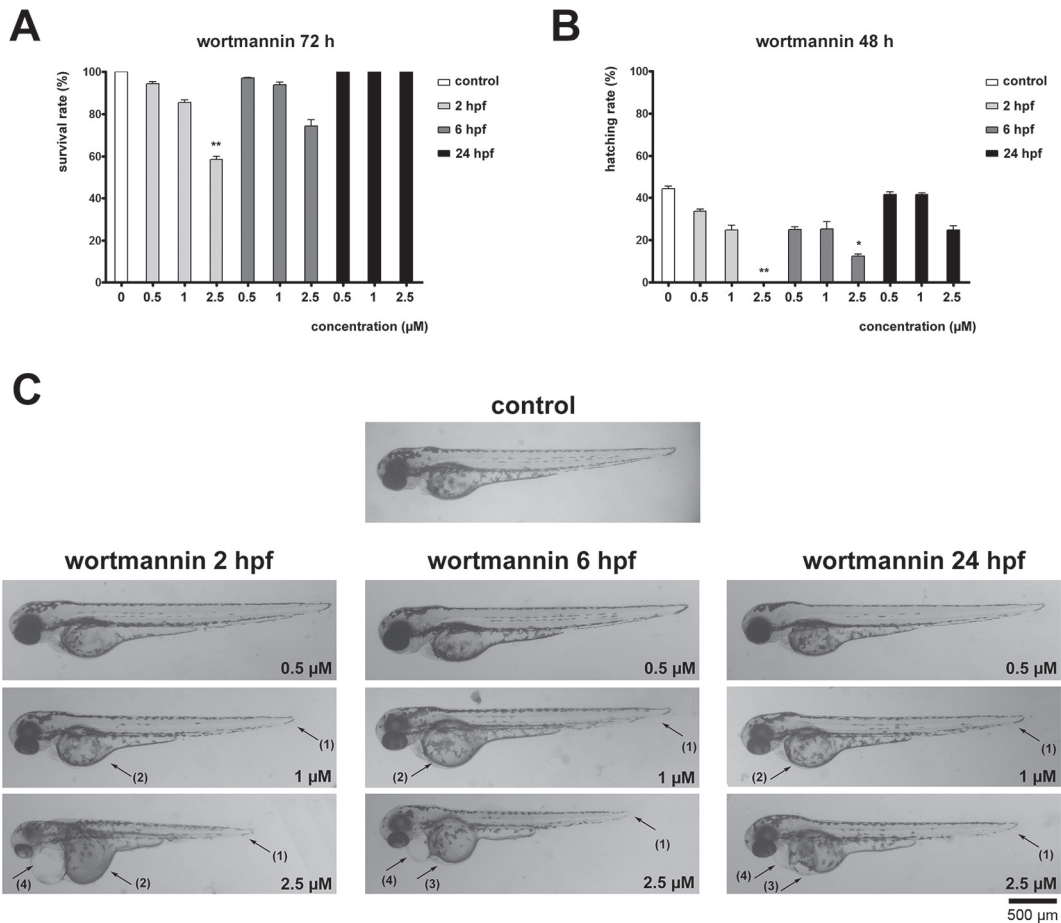| Malformations	2 hpf			6 hpf			24 hpf		
	0.5 μM	1 μM	2.5 μM	0.5 μM	1 μM	2.5 μM	0.5 μM	1 μM	2.5 μM
Decreased body length	np	100%	100%	np	100%	100%	np	100%	100%
Voluminous yolk sac	np	100%	np	np	100%	np	np	100%	np
Yolk sac edema	np	np	100%	np	np	100%	np	np	100%
Pericardial edema	np	np	100%	np	np	100%	np	52%	100%
Scoliosis	np	np	22%	np	np	np	np	np	np

*np – not present.

Fig. 1. Wortmannin affects survival and development in zebrafish embryos. A) Survival rate of 72 hpf zebrafish embryos treated with wortmannin at 2, 6 or 24 hpf. B) Hatching rate of 48 hpf zebrafish embryos treated with wortmannin at 2, 6 or 24 hpf. C) Representative images of morphological malformations in 72 hpf zebrafish embryos treated with wortmannin at 2, 6 or 24 hpf

Wortmannin treatment induced multiple malformations in zebrafish embryos. The observed effects were more prominent in earlier treatment starting points and were concentration dependent (Table 1, Fig. 1C). Treatment with 1 µM wortmannin decreased body length and produced voluminous yolk sac in comparison to the control group. Furthermore, embryos treated with 1 µM wortmannin 24 hpf had pericardial edema. Treatment with 2.5 µM wortmannin caused shorter body length compared to controls and 0.5 µM and 1 µM treatments, as well as yolk sac edema and pericardial edema. Additionally, embryos treated with 2.5 µM wortmannin 2 hpf showed scoliosis (Table 1, Fig. 1C).

GSK690693 and AZD2014 do not influence zebrafish embryo survival and development

The zebrafish embryos were also treated at different time points with increasing concentrations of GSK690693. GSK690693 did not affect survival or embryonic development, but decreased hatching of the embryos observed at 48 hpf in comparison to
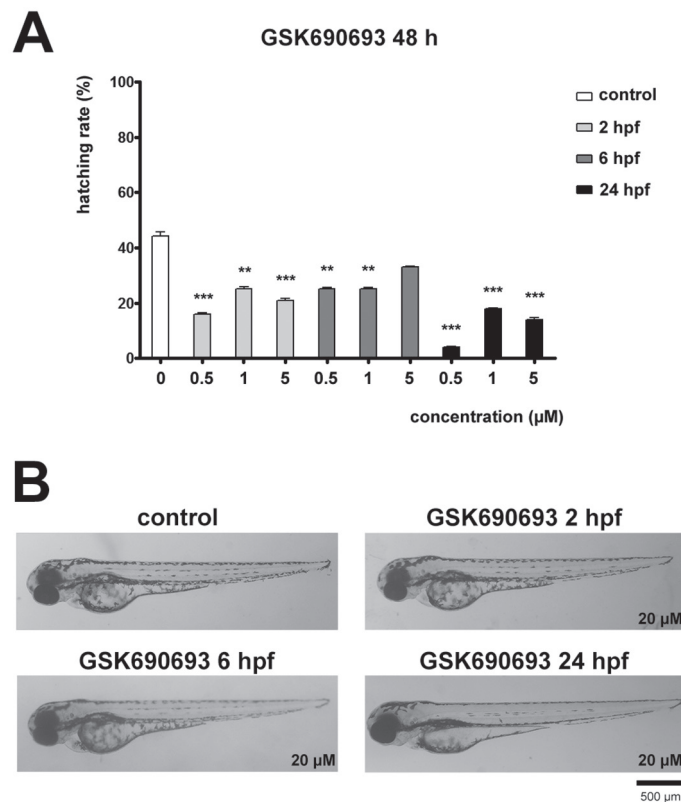

Fig. 2. GSK690693 affects hatching but not development in zebrafish embryos. A) Hatching rate of 48 hpf zebrafish embryos treated with GSK690693 at 2, 6 or 24 hpf. B) Representative images of 72 hpf zebrafish embryos treated with GSK690693 at 2, 6 or 24 hpf

control groups. All concentrations of GSK690693 decreased hatching rate of embryos at all treatment starting time points (Fig. 2A). No malformations during the development were observed in any of the GSK690693 treatments (Fig. 2B). Treatment with increasing concentrations of AZD2014 caused dose-dependent decrease in hatching rate at all treatment starting time points (Fig. 3A). No differences in survival of AZD2014 treated embryos compared to controls, or any malformations during the development were observed (Fig. 3B).
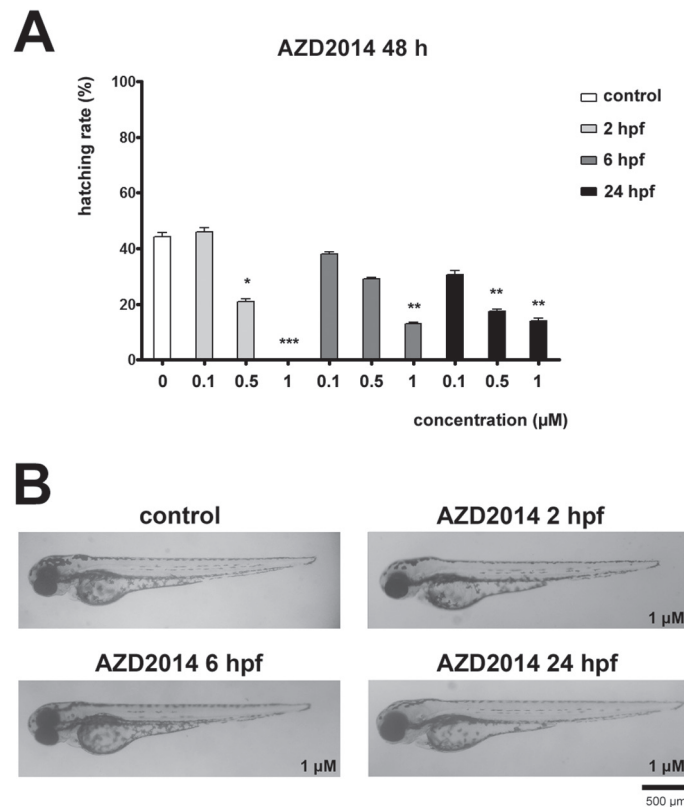

Fig. 3. AZD2014 affects hatching but not development in zebrafish embryos. A) Hatching rate of 48 hpf zebrafish embryos treated with AZD2014 at 2, 6 or 24 hpf. B) Representative images of 72 hpf zebrafish embryos treated with AZD2014 at 2, 6 or 24 hpf

Co-treatments with PI3K-Akt-mTOR pathway inhibitors cause decrease in zebrafish embryo survival and multiple malformations

Subsequently, the embryos were exposed to combined treatment with wortmannin and GSK690693 or GSK690693 and AZD2014. Combined treatment with 1 μM wortmannin and 1 μM GSK690693 significantly decreased embryo survival rate
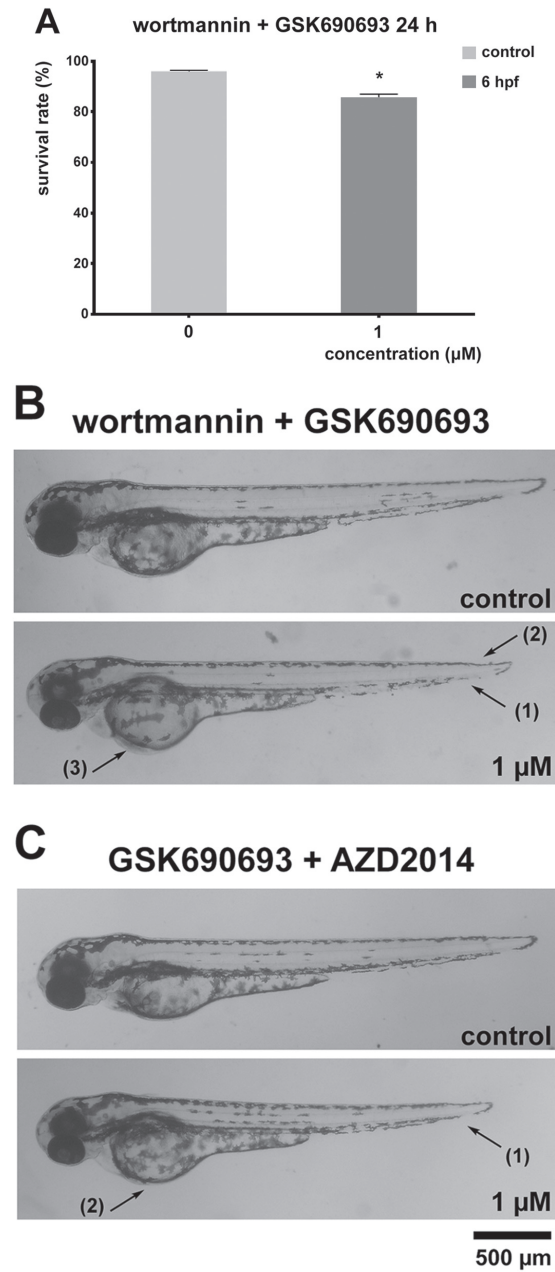
Fig. 4. PI3K-Akt-mTOR pathway inhibitors co-treatments affect survival and development in zebrafish embryos. A) Survival rate of 24 hpf zebrafish embryos treated with wortmannin and GSK690693 combination at 6 hpf. B) Representative images of morphological malformations in 72 hpf zebrafish embryos treated with wortmannin and GSK690693 combination at 6 hpf. C) Representative images of morphological malformations in 72 hpf zebrafish embryos treated with GSK690693 and AZD2014 combination at 6 hpf

(86%) observed at 24 hpf (Fig. 4A). Additionally, all embryos had decreased body length, while 79% of embryos had hemagglutination and 70% had malformation of the tail (Fig. 4B).

Combined treatment with 1 µM GSK690693 and 1 µM AZD2014 inhibitors did not alter survival or hatching rate, however decreased body length of all embryos was observed. Additionally, this combined treatment caused voluminous yolk sac in 65% of treated embryos (Fig. 4C).

Tipifarnib causes multiple morphological malformations in zebrafish embryos

Tipifarnib treatment did not affect the embryo survival or hatching rate, however, multiple malformations of the embryos were observed all treatment starting time points (Fig. 5). The most prominent malformation was decrease in body length (Table 2). Yolk sac edema and pericardial edema were also observed.

Table 2
Embryo malformations induced by tipifarnib

Malformations	2 hpf			6 hpf			24 hpf		
	1 µM	5 µM	10 µM	1 µM	5 µM	10 µM	1 µM	5 µM	10 µM
Decreased body length	np	100%	100%	np	100%	100%	np	np	100%
Voluminous yolk sac	np	65%	100%	np	np	100%	np	np	82%
Pericardial edema	np	np	27%	np	np	36%	np	np	np

*np – not present.

The effects of PI3K-Akt-mTOR and RAS-MAPK-ERK pathway inhibitors on early sea urchin development

Fertilized sea urchin eggs were treated with 0.25 µM or 0.5 µM AZD2014 20 min after fertilization and the effect on the embryo survival and development was observed after 24 h (Fig. 6). Treatment with 0.25 µM produced 8% late gastrula embryos while 52% of embryos were dead after 24 h. The mobility of live embryos was reduced compared to the untreated group. After treatment with 0.5 µM AZD2014 2% of embryos reached the late gastrula stage, 3% of embryos were arrested as deformed stationary blastulas (compared to 23% swimming blastulas in the control group) and 74% of embryos became nonviable.

After 24 h treatment with 2.5 µM GSK690693 there were no late gastrulas and 4% embryos were arrested at the swimming blastula stage which retained mobility despite their altered morphology (Fig. 6). However, the majority of embryos remained undeveloped (57% in treated group compared to 15% in control) and 39% of embryos were dead. Treatment with 5 µM GSK690693 produced 63% undeveloped
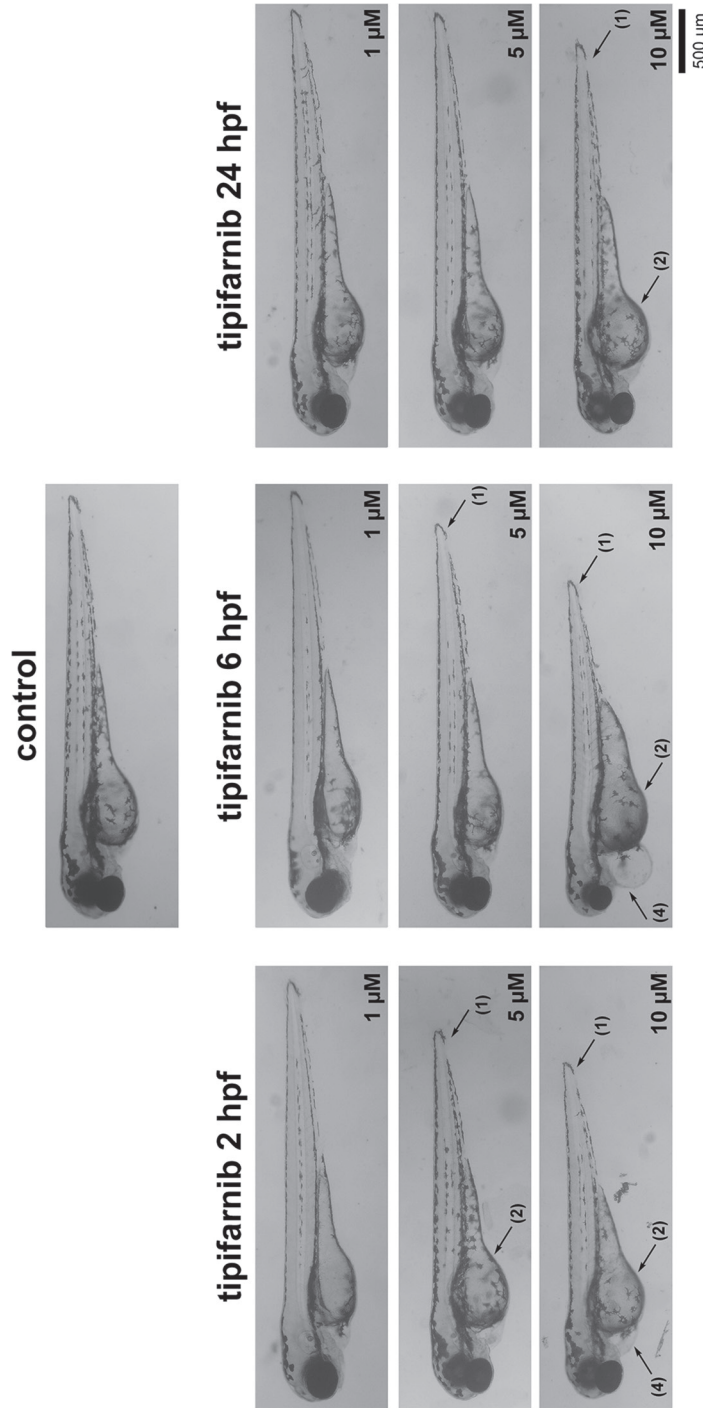
Fig. 5. Tipifarnib affects development in zebrafish embryos. Representative images of morphological malformations in 72 hpf zebrafish embryos treated with tipifarnib at 2, 6 or 24 hpf
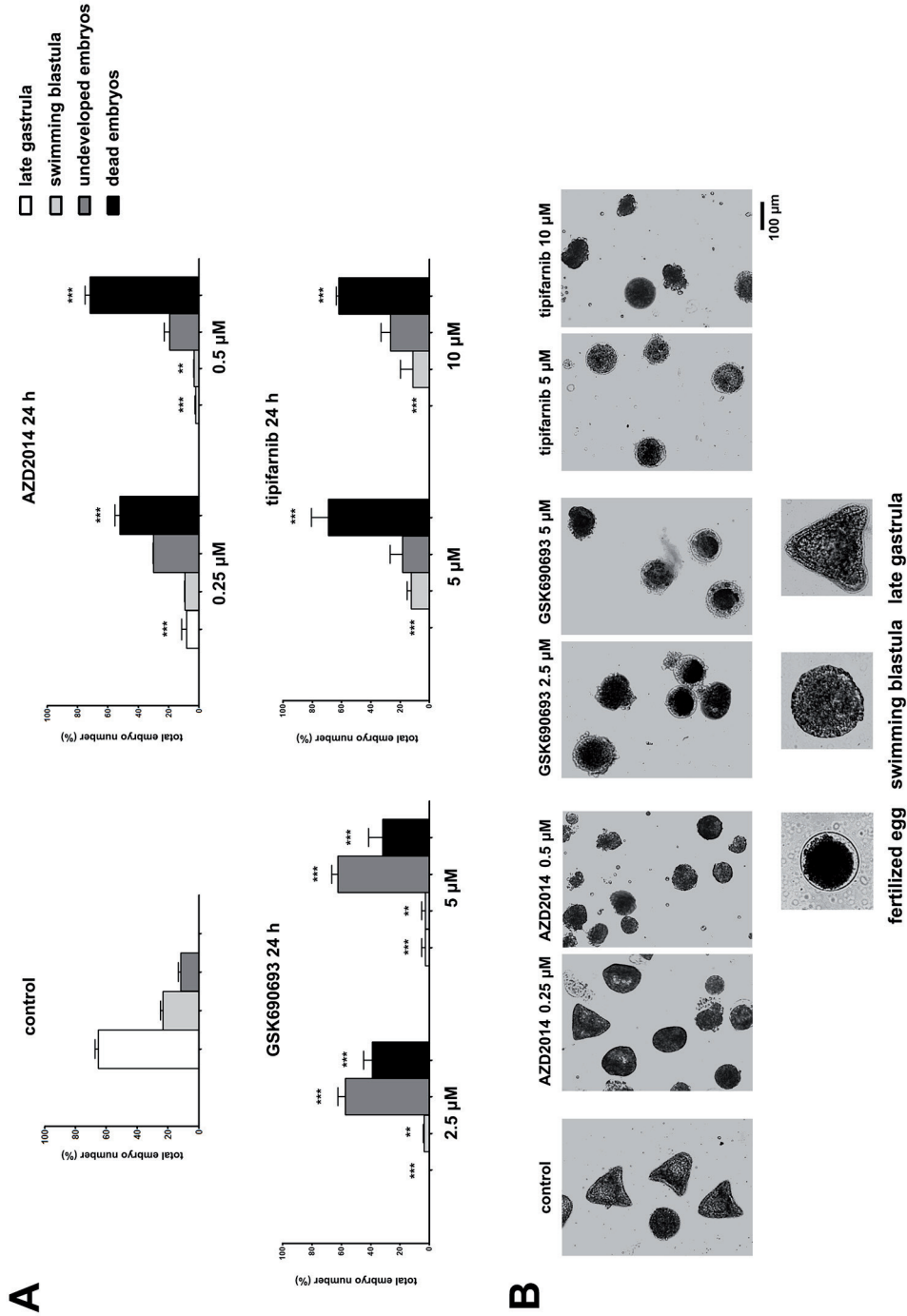
Fig. 6. The effects of PI3K-Akt-mTOR and RAS-MAPK-ERK pathway inhibitors on early sea urchin development. A) Survival and development of sea urchin embryos treated with GSK690693, AZD2014, or tipifarnib 20 min after fertilization. B) Representative images of developmental delay in sea urchin embryos after 24 h treatment with GSK690693, AZD2014, or tipifarnib

embryos, 3% of swimming blastulas with significantly reduced mobility and 32% of dead embryos. The morphology of undeveloped embryos was significantly altered.

After the fertilization membrane became visible, sea urchin embryos were also treated with 5 µM or 10 µM tipifarnib (Fig. 6). The impact on embryo viability and developmental delay was observed after 24 h. Treatment with 5 µM tipifarnib arrested the embryos at various stages of development. Compared to control where 62% of embryos reached the late gastrula, none of the embryos treated with 5 µM tipifarnib were observed at this developmental stage. Furthermore, the treatment had a high impact on viability since 69% of embryos became nonviable after 24 h while no dead embryos were found in the control group. Application of higher concentration of tipifarnib (10 µM) produced similar results.

DISCUSSION

One of the currently most investigated problems in cancer treatment is the development of non-toxic therapies to normal cells,mean while retaining the ability to successfully inhibit tumor growth. Novel adjuvant therapies are being developed to increase the effectiveness of existing drugs.

This study was addressed to the time- and dose-dependent responses of zebrafish and dose-dependent responses of sea urchin embryos on chemical inhibitors of PI3K-Akt-mTOR and RAS-MAPK-ERK signaling pathways components. The toxic effects of these inhibitors alone or in combinations were investigated *in vivo* in zebrafish and sea urchin embryos that served as models for fast proliferating, but not transformed cells. To the best of our knowledge, this is the first report investigating the effects of GSK690693, AZD2014 and tipifarnib on zebrafish and sea urchin embryo models.

We have selected several treatment time points for zebrafish embryos: blastula (2 hpf), gastrula (6 hpf) and pharyngula (24 hpf) stages, as numerous differences in speed and pattern of cell proliferation during these developmental stages have previously been reported [25]. The most prominent effects on the embryo survival and development were present in treatments started at the earliest time point in all studied inhibitors. Inhibitor concentrations selected for treatments were previously shown to have a significant effect on proliferation, and trigger autophagy and apoptosis in normal and cancer cell lines [10]. It should be taken into account that, compared to zebrafish embryos, sea urchin embryos were found to be generally more sensitive to a number of chemicals and drugs [6], which is in line with our observations.

Wortmannin, a PI3K inhibitor, has been previously shown to exhibit high toxicity during fertilization and development of sea urchin [9]. It has been reported that wortmannin treatment disrupts the mitotic spindle formation and subsequently the first cell division of the fertilized egg. Similarly, in our model wortmannin treatment resulted only in nonviable disintegrated embryos after 24 hours and was therefore excluded from our sea urchin experiments. In zebrafish embryos, treatment with 2.5 µM wortmannin at the blastula stage decreased embryo survival and hatching rate.

Additionally, several morphological malformations have also been observed. It has been previously shown that wortmannin exhibited high cardiotoxicity in zebrafish embryos although independently of PI3K inhibition [32]. It has also been reported that wortmannin affected cancer cell growth at micromolar concentrations ($IC_{50} = 30$ µM) and improved the activity of doxorubicin and cisplatin in thyroid carcinoma cell lines [27]. Our observation, that wortmannin exhibits substantial effects on zebrafish survival and morphology in much lower concentrations compared to human cancer cell lines (2.5 µM), does not favor this inhibitor as an anticancer agent as it affects normal as well as cancer cells.

GSK690693 is a small molecule that acts as ATP-competitive inhibitor of Akt1, Akt2 and Akt3. This agent affected sea urchin fertilization and development, but its effects on zebrafish development were negligible. It did not impact zebrafish embryo survival, but caused delayed hatching that could not be attributed to either the time of treatment or applied concentration. The process of hatching is complex and influenced by many physical and chemical factors. In zebrafish, hatching glands produce proteolytic enzyme that weakens chorion and facilitates hatching [8]. Also, oxygenation of water and other physic-chemical water properties could affect hatching [15]. Presence of pollutants in water has been shown to influence hatching rate [31]. As the differences between treated animals and controls in hatching rate were not present after 72 hpf (all the surviving embryos were hatched in all groups), the detailed analysis of hatching was not performed. GSK690693 has been found to be active *in vitro* against many cell types, inhibiting their growth in micro molar range (IC_{50} values from 8 to 25 µM) [7, 21, 27]. However, *in vivo* studies on xenograft models, GSK690693 exhibited effects not very promising to further promote it as anticancer agent [2, 7].

AZD2014 is an ATP-competitive mTOR kinase inhibitor, affecting both mTORC1 and mTORC2, currently in phase II clinical trials [13]. In our experimental setup, AZD2014 treatment did not affect embryo survival, but resulted in time- and dose-dependent delayed hatching at 48 hpf, compared to controls, but this difference disappeared after 72 hpf. AZD2014 treatment leads to body length shortening in some embryos only at highest, 1µM, concentration.

None of the AZD2014 treated embryos showed any other morphological malformations. Although sea urchin embryo viability was low and substantial developmental delay was observed in all treatments, only treatment with AZD2014 managed to produce a noteworthy number of embryos that reached the swimming blastula and late gastrula stage. It has been shown that AZD2014 sensitized oral squamous cancer cells and glioblastoma stem-like cells to treatment with ionizing radiation [19, 33]. *In vitro*, AZD2014 showed highest growth arrest potential in thyroid and colorectal cancer cells, acting in nanomolar range (IC_{50} values from 0.15 to 0.21 µM), compared to the other inhibitors used in this study [18, 27]. Additionally, it was demonstrated that it most effectively lowered IC_{50} values for classic chemotherapeutics doxorubicin and paclitaxel [27]. AZD2014 was also shown to inhibit proliferation and induce apoptosis in liver cancer cells [22]. In our previous work, we have found that AZD2014 induced autophagy in concentrations that had no effect on development,

and prevented cisplatin-induced apoptosis in zebrafish embryos. Autophagy modulation with AZD2014 triggered the cellular response to anticancer drugs more effectively in NCI-H460 (human non-small cell lung carcinoma) and zebrafish embryonic models compared to human normal HaCaT cells suggesting that these modulators are selective towards rapidly proliferating cells [10]. Taken together with these findings, our results further promote AZD2014 as a promising anticancer agent. Of all the inhibitors tested, AZD2014 had the least influence on the normal embryonic development.

Wortmannin and GSK690693 combination treatment significantly decreased zebrafish embryo survival after 72 h at even lower wortmannin concentration implying a synergistic action of the two PI3K/Akt/mTOR inhibitors. Combined treatment with inhibitors of downstream part of the PI3K/Akt/mTOR pathway, GSK690693 and AZD2014, did not significantly affect the survival of the treated embryos. Wortmannin + GSK690693 and GSK690693 + AZD2014 co-treatments produced morphological malformations not present in single treatments (hemagglutination and yolk sac malformation) which further implies the synergistic action between these inhibitors.

Tipifrnib is a Ras pathway inhibitor approved by USFDA for the treatment of elderly acute leukemia, and is in phase II clinical trials for several other types of cancer [3]. In our study, treatment with tipifarnib affected zebrafish embryo morphology as well as survival and development of sea urchin embryos, displaying higher toxicity on normal embryonic cells, compared to AZD2014 and GSK690693. Tipifarnib has been shown to effectively arrests cell growth in micro molar concentrations (IC$_{50}$ values ranging from 2 to 8 μM) [27]. Meanwhile it was very toxic to sea urchin embryos and caused zebrafish embryo malformations, we have shown that tipifarnib has prominent effect on normal proliferating cells.

CONCLUSIONS

In the search for new anticancer drugs, the main focus is placed on finding compounds with substantial capacity to inhibit the cancer cell growth, particularly in resistant cancers, while exhibiting negligible effects on normal cells during the course of chemotherapy. We found that AZD2014 had no major effects on fast proliferating normal cells of zebrafish embryos in concentrations that induced substantial toxicity in cancer cells. Treatment with AZD2014 also caused the least prominent effects on sea urchin embryo development compared to other investigated inhibitors which target signaling pathways involved in regulating cell survival and proliferation.

Although further investigations are needed, the significant toxicity of AZD2014 in human cancer cells, its capacity to sensitize resistant cancers, lower antiproliferative activity against human normal cell lines as well as fast proliferating embryonic cells could make this agent a promising candidate for anticancer therapy.

ACKNOWLEDGMENTS

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