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Adrenocortical carcinoma xenograft in zebrafish embryos as model to study in vivo cytotoxicity of abiraterone acetate

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Reviewer 1: The authors present a technical resource describing their work employing zebrafish embryos as xenograft model to study the in vivo cytotoxicity of abiraterone acetate. Overall, the paper appears quite interesting and provides a good outlook on the use of relatively cheap in vivo models such as zebrafish in the preclinical testing of drugs. The use of H295R cells can be regarded as a proof of principle and one would be curious to see how primary tumor cells will behave in such a xerograph model.

Accordingly to the Reviewer suggestion, results obtained with a primary cell culture established from a cortisol-secreting ACC patient, namely ACC29, have been added to the revised Ms. Results confirmed the cytotoxic effect of AbiAC on cortisol-secreting ACC cells (revised Fig. 5). Exposure of ACC29 xenografted embryos to 1 µM AbiAc significantly reduced the xenograft area: 56,987 ± 2,83 μM^2 in solvent-treated (control) embryos vs 39,776 ± 4,51 μM^2 in ABiAC-treated embryos (* $p < 0.05$). Furthermore, to strengthen our results, as internal negative control, we xenografted the non secreting, AbiAc insensitive SW13 cell line (*J Clin Endocrinol Metab*. 2016;101(12):4594-602), that belong to adrenal gland, although its origin is still under investigation (*Mol Cell Endocrinol. 2012;58-65*). Tumor area of SW13 xenograft in zebrafish embryos was not modified after 1 µM AbiAc exposure up to 3 days (revised Fig. 6).

Due to the addition of these results, the Ms has been extensively revised throughout each part, included the title that has been changed as: *AdrenoCortical Carcinoma xenograft in zebrafish embryos as model to study the in vivo cytotoxicity of abiraterone acetate.*

The paper can benefit from some revisions.

1) It would be useful to have a figure or supplementary figure on the dose finding/ toxicity.

A figure showing the AbiAc dose toxicity in zebrafish embryos has been added as supplemental data (https://figshare.com/s/0447ced4fc9703178db5).

2) It would be good to include schematic on the AbiAc treatment protocol in the paper. This would make it easier to understand the treatment.

The scheme of AbiAc treatment has been added to the revised Ms (revised Fig. 1).

3) The authors have established abiraterone concentrations at 24 and 48 hpf. The experiments exploring the effects in injected H295R cells have been conducted at a later time point (5 dpf). It would be useful to get some information on abiraterone concentrations at 5 dpf and also if this affects interrenal cortisol production due to blocking Cyp17a2.

Fig. 2 has been completed with Table 2, reporting the time-course of abiraterone concentrations up to 120 hpf (5 dpf).

The effect of 1 µM AbiAc on 5dpf embryo production of cortisol and progesterone has been measured and results are reported in Table 3.

4) Line 46: Please introduce Δ4A in the text.

The correction has been made

5) Lines 96, 164: All androgen synthesis is blocked not only in the adrenal. Please amend the statement.

We corrected the wrong sentence at line 97. At line 164, only the hydrolysis from AbiAc to Abi was mentioned.

6) Lines 165 and 166; Please specific which method had been used and stick with the abbreviation.

We corrected the method definition, eliminating high-performance and sticking to the acronym. Furthermore, we add the full form of methods throughout the paragraph.

7) **Line 239: States "were manually dechorionated at 24 hpf and divided", whereas the materials and methods described 48 hours. What time points have been used?**

We apologize for the mistake, the correct time point is 48hpf, as indicated now both in Methods and in the Results section: "*.* AB zebrafish embryos at 48 hpf were manually dechorionated, anesthetized with tricaine and…" (lines 327-329 revised Ms).

8) Line 272: Please clarify the technical limits to readers not familiar working with zebrafish larvae.

This point has been clarified by adding the sentence: at this stage of development microinjection is not recommended due to tissue fragility (lines 316-318 revised Ms)

9) **Lines 320-322: Other groups have measured cortisol synthesis and precursors in zebrafish using LC-MSMS. However, to assess an impact on endogenous cortisol production by abiraterone by far more embryos/ larvae need to be used (50-100 plus). These experiments can be easily conducted and will be very informative.**

We thank the Reviewer for his/her criticism. As above indicated, the effect of 1 µM AbiAc on embryo production of cortisol has been measured and results are reported (Table 3, lines 321-325 revised Ms): "AbiAc at 1 µM concentration induced as well a modification of AB zebrafish embryo cortisol level. Results are reported in Table 3. After 3 days exposure, the irreversible binding of AbiAc to CYP17A1 induced a significant reduction of cortisol production in AbiAc-treated embryos. Accordingly, as expected, progesterone become measurable in treated embryos, while it did not reach detection in solvent-treated embryos."

Hormone determinations were performed as described in Methods and analyzed by LC-MS/MS as described

10) Figure 4: The figure legend does not guide through the figure in the order of presented data. The labelling is hard to differentiate T3-AbiAc, T3+AbiAc; please make this a clearer.

The different significance of labelling has now been explained in the revised figure legend.

Reviewer 2: In this study, the authors present results on a zebrafish model used to assess cytotoxicity of abiraterone acetate (AbiAc), a CYP17A1 inhibitor, on growth of H295R ACC cell xenografts. After showing that the drug is metabolized in zebrafish embryos, the authors show data suggesting that it has an inhibitory effect on xenograft development.

Major concerns:

1)The authors should sequence the PCR product obtained to make sure that it has really been amplified from the zebrafish 3beta-HSD transcript.

The gene expression evaluation was done in zebrafish embryos without xenograft to ensure that only zebrafish mRNA could be amplified

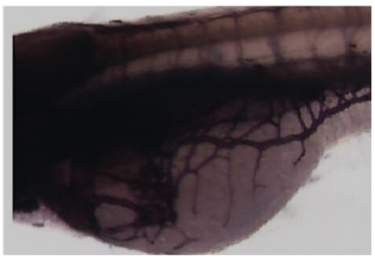
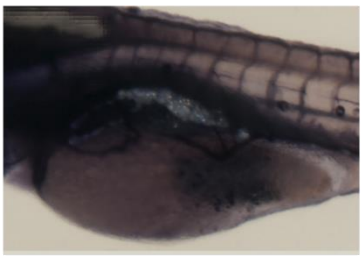
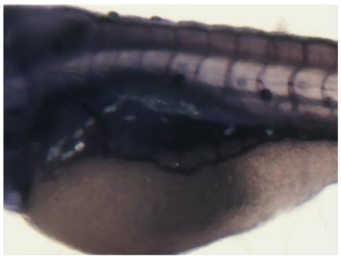
2) The reported AbiAc effects on ACC cell growth might be due to a non-specific toxic effect of the drug. In fact, as the authors report, a slightly higher concentration of the drug compared to the one used in xenografts experiment had important effects on embryo viability and development. An essential control needed is to measure the effect of AbiAc on the growth of xenografts from a human cell line not expressing CYP17A1, in order to show that the effects of the drug on ACC cells are specific.

Accordingly to the Reviewer observation, to strenghten our results, as internal negative control, we xenografted the non steroidogenic, CYP17A1 negative SW13 cell line *(J Clin Endocrinol Metab. 2016;101(12):4594-602*), that belong to adrenal gland, although its origin is still underi investigation (*Mol Cell Endocrinol. 2012;58-65*). Tumor area of SW13 xenograft in zebrafish embryos was not modified after 1 µM AbiAc exposure up to 3 days (revised Fig. 6).
Furthermore, results obtained with a primary cell culture established from a cortisol-secreting ACC patient, namely ACC29, have been added to the revised Ms. Results confirmed the cytotoxic effect of AbiAC on cortisol-secreting ACC cells (revised Fig. 5). Briefly, exposure of ACC29 xenografted embryos to 1 µM AbiAc significanty reduced the xenograft area: 56,987 ± 2,83 μM^2 in solvent-treated AB zebrafish embryos vs 39,776 ± 4,51 μM^2 in ABiAC-treated AB zebrafish embryos (* $p < 0.05$).
Due to this modification, the Ms has been extensively revised throughout each part, included the title that has been changed in: *AdrenoCortical Carcinoma xenograft in zebrafish embryos as model to study the in vivo cytotoxicity of abiraterone acetate*.

3) The manuscript is quite poor of data: the authors could take advantage of the zebrafish model by showing the effect of the drug on xenograft histology (tumor structure, vascularization) and expression of differentiation (steroidogenic enzymes) and proliferation markers (Ki67).

As the reviewer could see, the Ms has been extensively revised, adding new results, all of which strengthen the aim of this work, that is to reproduce in zebrafish embryos the cytotoxic effect mediated by AbiAc, reducing the ACC xenograft area in another *in vivo* animal model other than the athymic mice. Indeed, we measured the effect of the CYP17A1 inhibition induced by AbiAc on cortisol and progesterone levels to evaluate the AbiAc activity on zembrafish embryos.

We are taking into deep consideration the Reviewer indications, but we consider that they are outside the main aim of this work, that was submitted to Endocrinology as "Technical Resource" article type.
Indeed, we are setting up the experiments (included those requested by the Reviewer), in order to study and identify parameters that could become biomarkers of drug response to ACC in this model. Indeed, we performed tumor vascularization experiments, but the NCI-H295R cells did not induce vascularization in this model (see below).

Positive control	T3-AbiAc	T3+AbiAc
		

Positive control: Embryos injected with FGF2-T-MAE (3F2T) cells.

T3-ABiAc:NCI-H295R cells at 3 days post injection.

T3+AbiAc: NCI-H295R cells treated with AbiAc at 3 days post injection.

Furthermore, we did the Ki67 immunofluorescence experiments, but we need to better set up the conditions as we could count Ki67 positive cells in tumor xenograft in solvent-treated embryos, while, we can still count Ki67 positive cells in AbiAc-treated embryos: they were significantly reduced from 58,22 ± 12,33 to 8,84 ±3,2 (n=10 solvent-treated and n=9 AbiAc-treated embryos). However, embryos presented a diffuse immunofluorescent background that we have not been able to eliminate up to now. A hypothesis is that AbiAc could modify the lipid homeostasis (Adv Exp Med Biol. 2018;1112:293-307), thus influencing the unspecific secondary antibody binding. However, we are working out on these experiments.

Adrenocortical carcinoma xenograft in zebrafish embryos as model to study the *in vivo* cytotoxicity of abiraterone acetate

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Short title: ACC cell xenograft in zebrafish embryos

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ABSTRACT

Purpose: Abiraterone acetate (AbiAc) inhibits tumor growth when administered to immunodeficient mice engrafted with *in vitro* cell model of human adrenocortical carcinoma (ACC). Here, we developed and validated a zebrafish model engrafted with cortisol-secreting ACC cells to study the effects of AbiAc on tumor growth.

Methods: The experimental conditions for AbiAc absorption in AB zebrafish embryos as embryos number, AbiAc concentrations and absorption time-curve by LC-MS/MS were set up. The AbiAc effect on steroid production in AB zebrafish embryos was as well measured. ACC cells (NCI-H295R cell line, the primary cells ACC29 and the negative control cells SW13) were treated with the Dili fluorescent dye and about 240 cells/4 nl were injected in the subperidermal space of the yolk sac of AB zebrafish embryos (n=80±10). Cell area was measured with Noldus DanioScopeTM software.

Results: AbiAc absorption in AB zebrafish embryos was stage-dependent. Abiraterone (Abi) concentration decreased while its main metabolite, namely Δ4A, increased. Accordingly, we demonstrated that zebrafish expressed the enzyme 3β-hydroxysteroid dehydrogenase mRNA, that converts Abi in Δ4A. Furthermore, ABiAc reduced zebrafish embryos cortisol production and increased progesterone. Three days after cell injection (T3), the cortisol-secreting ACC cell area in solvent-treated embryos was significantly higher compared to 1 μM AbiAC-treated embryos, while no AbiAc effect was observed in SW13, that lacks the Abi target enzyme CYP17A1.

Conclusions: Zebrafish embryo xenografted with ACC tumor cells could be a useful, fast and reproducible experimental model to preclinically test the activity of new drugs potentially active in human ACC.

INTRODUCTION

AdrenoCortical Cancer (ACC) is a rare tumor with an estimated incidence between 0.7 and 2.0 per million population per year (1). Surgery is the only potentially curative treatment modality. Systemic therapies have a limited efficacy and the prognosis of locally advanced or metastatic ACC patients is often dismal. Mitotane is the only drug approved to treat ACC both in adjuvant setting and metastatic disease (2, 3); the drug pharmacokinetics and safety profile, however, limit its efficacy (4). Mitotane can be administered either alone or in association with etoposide, doxorubicin and cisplatin (EDP-M) (5, 6). The overall 5-year survival rate of metastatic ACC patients submitted to EDP-M is about 15%. In this scenario, the introduction of new potentially effective drugs, or the demonstration of efficacy in ACC of already available drugs is of paramount importance. Evaluating new targets and drugs using established cell lines is limited by the inexact correlation between responsiveness observed in cell lines versus that elicited in the patient. Tumor cell xenografts in athymic mice, generated from fresh tumor specimens, recapitulate the diversity of malignancies and have been the most used *in vivo* model over the last 50 years (7). This model, however, is time-consuming and expensive, and these are important limitations in the early phase of drug screening, when a large number of drugs needs to be evaluated for their potential anti-tumor activity.

It is therefore mandatory, especially in the case of rare diseases like ACC, to identify and validate reliable and faster experimental preclinical *in vivo* models that can offer a robust demonstration of anti-tumor efficacy of new drugs.

The zebrafish (*Danio rerio*) animal model has been developed in the past years, and became a widely used experimental vertebrate model in many fields of scientific research. In particular, zebrafish embryos represent a valuable tool to study human diseases, including cancer (8, 9), and to develop in vivo toxicological and pharmacological screenings (10-16).

Several zebrafish xenograft models of different tumor cells have been already validated for pre-clinical anti-cancer drug screening (17-20). Indeed, very low amount of cancer cells and drugs are needed to conduct the experiments; in addition only few days are necessary to obtain results in zebrafish embryos/larvae, as opposed to the several weeks required in mice models. No immunosuppressant treatment is necessary since lymphocytes mature after around 7 days post fertilization (21) and the transparency of zebrafish embryos and larvae allow the easy

observation of engrafted tumor cells. Taken together, these observations allow to affirm that the zebrafish xenograft models developed up to now are fast, simple and reproducible (17-20).

The aim of this study is to provide the first evidence that the zebrafish embryo model is a useful tool to evaluate *in vivo* cytotoxicity of drugs potentially efficacious on ACC. To do so, we validated in zebrafish embryo the results already obtained in immunodeficient mice xenografted with the NCI-H295R cells treated with abiraterone acetate (AbiAc) (22). AbiAc is an irreversible inhibitor of 17alpha-hydroxylase/17,20-lyase (CYP17A1), a key enzyme for steroid hormone synthesis (23). Since AbiAc inhibits androgen synthesis throughout the body, it is effective in the management metastatic prostate cancer (24, 25). Besides reducing androgen levels, the drug rapidly impairs cortisol synthesis (26) and appears potentially effective in the management of Cushing's syndrome often associated with ACC. Our group demonstrated in preclinical models that AbiAc is not only able to inhibit cortisol secretion but also exerts a cytotoxic activity in the ACC cell line and in ACC primary cell cultures (22), due to the drug induced increase of progesterone levels (22, 27). AbiAc inhibited tumor growth when administered daily for 16 days in NCI-H295R cells xenografted immunodeficient mice, thus confirming the *in vitro* findings (22). In the present study, we were able to reproduce in the zebrafish embryos the *in vivo* cytotoxic effect observed by AbiAc in the murine model and demonstrated that the zebrafish embryos xenografted with ACC cells are a valuable *in vivo* preclinical model to screen drugs potentially efficacious in ACC patients.

MATERIALS AND METHODS

Chemicals

AbiAc was purchased from Selleckchem (DBA, Segrate, MI, Italy) and resuspended in 100 % ethanol (stock solution: 25 mM). When required, AbiAc was directly added to the fish water at different concentration (0.5, 1, 2.5 μM), accordingly to the experimental conditions. Control embryos were treated with solvent alone.

Reagent-grade methanol (MeOH) LC-MS CHROMASOLV® and formic acid (98%) were purchased from Sigma Italia (Milan, Italy). Ultra-pure water was prepared using a Millipore Milli-Q purification system (Millipore Corporation, Billerica, MA, USA). Abi standard, Abi deuterated standard (SI) were purchased from Selleckchem; cortisol (1mg/ml in methanol), progesterone, Δ4A standard was purchased from Sigma Aldrich (Sigma Italia, Milano, Italy). Abi was resuspended in 100 % dimethylformamide; progesterone, Abi deuterated and Δ4A were resuspended in 100 % dimethylsulfoxide. Drugs were subsequently diluted in methanol.

Cell culture and labeling

The human cell lines NCI-H295R and SW13 were obtained from the American Type Culture Collection (ATCC) and cultured as suggested. The NCI-H295R cell line was established from a secreting human ACC and represented the most widely used experimental cell model to study ACC in vitro (28). The human SW-13 cell line has been established from a small cell carcinoma in the adrenal cortex. These cells do not produce steroids and their exact histopathologic characteristics are still under investigation (28). The human ACC primary cells, namely ACC29, were derived from a female patient underwent surgery for ACC (29) and established as previously described (22, 27). Cells were characterized as of adrenal origin, measuring the Steroidogenic Factor 1 gene expression (30,31) by q-RT-PCR and measuring cortisol production, as described in Fiorentini et al (22). The local Ethical Committee approved the project and written informed consent was obtained from the patient. ACC culture conditions were as indicated for NCI-H295R cells. Conditioned media from ACC29 were obtained as described in Fiorentini et al. (22). The doubling-time was calculated according to ATCC indication with the following formula: $DT=T \ln 2/\ln(Xe/Xb)$, where T is the incubation time in any units, Xb is the cell number at the beginning of the incubation time, Xe is the cell number at the end of the incubation time. The

cell viability was evaluated by trypan blue exclusion test. Briefly, cell suspensions containing 0.25 % trypan blue were dropped in a haemocytometer chamber and the viable cells were counted under a phase contrast microscope by two different operators. Cells ($3x10^6$ cells) were treated o/n with the vital red fluorescent dye CellTrackerTM CM-DiI (Thermo Fisher Scientific, Milano, Italy, final concentration 0.66 ng/ml), then detached with trypsin/EDTA, washed in PBS, resuspended in 50 µl of PBS and kept at 4 °C until use.

Measurement of cell viability

ACC29 cell viability was measured using the luminescence assay ATPliteTM (Perkin Elmer), that measures the ATP production of viable cells. Cells were plated at the density of 5×10^3 cells/well in 96 wells plate and treated with increasing concentrations of AbiAc (1-200 nM); untreated and AbiAc-treated cell viability was measured accordingly to the manufacturer instructions. Experiments were conducted at least three times, each point run in triplicate.

Fish maintenance and eggs collection

All zebrafish embryos were handled according to national and international guidelines, following protocols approved by the local Committee (OPBA protocol n. 211B5.24) and authorized by the Ministry of Health (authorization n. 393/2017-PR).

Healthy adult wild-type zebrafish (AB strain) were used for egg production. Fish were maintained under standard laboratory conditions as described (32), at 28 °C on a constant 14 h light/10 h dark cycle. Fishes were fed thrice a day with a combination of granular dry food and fresh artemia (Special Diet Services, SDS Diets). Nine months old male and female zebrafish were put in the breeding tank overnight in a 1:2 ratio. Immediately after spawning, fertilized eggs were harvested, washed and placed in 10 cm Petri dishes in fish water. The developing embryos were incubated at 28 °C and maintained in 0.003 % (w/v) 1-phenyl-2-thiourea (PTU, Sigma-Aldrich) to prevent pigmentation. Preliminary experiments were performed using different number of embryos at different stages of development in order to obtain the best experimental conditions.

AbiAc absorption quantification by liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Due to the rapid hydrolysis of AbiAc in abiraterone (Abi) (33), AbiAc absorption from embryos was evaluated by quantifying the concentration of Abi and its main metabolite Δ4A (34) by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

Ultra performance liquid chromatography (UPLC) was performed using a DionexTM UltiMateTM 3000 Thermo Fisher Scientific S.p.A (Milan, Italy) equipped with a LPG-3400SD quaternary analytical pump, a WPS-3000SL analytical autosampler, a TCC-3000SD thermostated column compartment. Chromatographic separation was performed using on XSELECT CSH C18 column (150 mm × 2.1 mm ID, particle size 3.5 µm) (Waters, Milano, Italy). Mobile phase (A) was water containing 0.1 % formic acid. Mobile phase (B) was methanol containing 0.1 % formic acid. An isocratic mobile phase was used with 70 % of (B) and a runtime of 15 min. The UPLC flow rate was 0.3 ml/min. The column temperature was 40 °C.

Collision-Induced Dissociation (CID)-MSn experiments were performed on an electrospray ionization mass spectrometer (LCQ Fleet Ion Trap MSn, Thermo Fisher Scientific). The positive ESI conditions were as follows. The source voltage was set at 4.5 kV and the sores current was set at 100 µA. The capillary voltage was set at 7 kV and the capillary temperature was 350 °C. The spray was stabilized with a nitrogen sheath gas (35 arb) and the auxiliary gas was set at 15 arb. The isolation width of precursor ions was 1 mass units. Ions were obtained in the range of m/z 300–400. For all SRM analyses the scan time was equal to 100 ms, the collision energy (CE) was fixed at 50 % and the isolation width of precursor ions was 2.5 mass units. Data were treated with the Xcalibur software (Version 4.0, Thermo Fisher Scientific). The calibration curves for the quantification of Abi and Δ4A were obtained both as described below.Twenty-five embryos for each batch (up to (120 hpf) were put at 4 °C. 100 µl of Internal Standard (IS) (300 nM) was added to each batch with 100 µl of Abi or Δ4A (dependent on the calibration curve) at different dilutions in order to obtain the final concentrations, respectively, 5-1000 nM Abi and 12.5-500 nM Δ4A. Samples were broken up with a pestle, homogenized using a pipette, vortexed for one minute, centrifuged at 15,000 rpm for 1 min at 4 °C, sonicated for 15 min, centrifuged at 15,000 × rpm for 10 min at 4 °C. The supernatant was transferred in a tube after filtration through a 0.2 µm PVDF filter and 5 µl were injected into the system LC-MS/MS. The SRM quantifier transitions were 350-->156 for Abi and 348-->156 for Δ4A.

188 Linearity was determined by least-squares regression (data not shown).

189 Each batch of 25 embryos (treated as above indicated) was put at 4 °C. 100 µl of SI (300 nM)
190 was added to each batch with 100 µl of MeOH. Samples were homogenized using a pipette,
191 vortexed for one minute, centrifuged at 15,000 rpm for 1 minutes at 4 °C, sonicated for 15
192 minutes, centrifuged at 15,000 × rpm for 10 minutes at 4 °C. The supernatant was transferred
193 in a tube after filtration through a 0.2 µm PVDF filter.

194 Five µl of each samples were analyzed by Ultra-performance liquid chromatography (UPLC)
195 interfaced with the electrospray ionization (ESI) tandem mass spectrometer (MSn).

196 **Cortisol and progesterone extraction and quantification by LC-MS/MS.** Cortisol and
197 progesterone extraction was performed as indicated in Fiorentini et al. (22). For ACC29
198 conditioned media, samples were the reconstituted in 20 µl of methanol and a volume of 10 µl
199 was directly injected into the LC-MS/MS system. For AB zebrafish embryos, batch of 100
200 solvent- or AbiAc-treated embryos each were prepared. Briefly, 70 µl of MeOH was added to
201 each batch. Samples were homogenized using a pipette, vortexed for 1 min, centrifuged at
202 14,000 rpm for 1 min at 4 °C, sonicated for 15 min, centrifuged at 15,000 × rpm for 10 min at
203 4 °C. The supernatant was transferred in a tube after filtration through a 0.2 µm PVDF filter.
204 Ten µl of each samples were analyzed.

205 UPLC was performed using a DionexTM UltiMateTM 3000 Thermo Fisher Scientific S.p.A (Milan,
206 Italy) equipped with a LPG-3400SD quaternary analytical pump, a WPS-3000SL analytical
207 autosampler, a TCC-3000SD thermostated column compartment. Chromatographic separation
208 was performed with XSELECT CSH C18 column (150 mm × 2.1 mm ID, particle size 3.5 µm)
209 (Waters, Milano, Italy). Mobile phase (A) was water containing 0.1 % formic acid. Mobile phase
210 (B) was methanol containing 0.1 % formic acid. A gradient mobile phase was used. The gradient
211 program was as follows: 58% B for 2 minutes; then from 58 to 100% B in 6 min, then 100% B
212 for 2 min, from 100 to 58% B in 2 min and re-equilibration to 58% B for 8 min. All analyses
213 were performed at 30 °C. CID-MSn experiments were performed on an electrospray ionization
214 mass spectrometer (LCQ Fleet Ion Trap MSn, Thermo Fisher Scientific). The positive ESI
215 conditions were as follows. The source voltage was set at 3.8 kV and the source current was set
216 at 100 µA. The capillary voltage was set at 11 V and the capillary temperature was 300 °C. The

spray was stabilized with a nitrogen sheath gas (35 arb) and the auxiliary gas was set at 15 arb. The isolation width of precursor ions was 1 mass units. Ions were obtained in the range of m/z 250–400. The calibration curves for the quantification of cortisol and progesterone were obtained diluting both reference standard as follow: 500 ng/ml; 250 ng/ml; 100 ng/ml; 50 ng/ml. Ten microliters of each standard dilution was analyzed by LC-MS/MS as previously described. Linearity was determined by least-squares regression (data not shown).

Tumor xenograft

AB zebrafish embryos at 48 h post fertilization (hpf) were dechorionated, anesthetized with 0.042 mg/ml tricaine (ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich) and microinjected with the labeled tumor cells into the subperidermal space of the yolk sac (8, 18). Microinjections were performed with the electronic microinjector FemtoJet coupled with the InjectMan N12 manipulator (Eppendorf Italia, Milano, Italy). Approximately 240 cells in a volume of 4 nl were injected into each embryo, which were then maintained in fish water plus PTU in a 32 °C incubator to allow tumor cell survival and growth. A picture of each injected embryo was acquired under a Leica MZ16F fluorescence stereomicroscope two hours post treatment (T0). AbiAc or solvent was directly added to the fish water. After three days (T3) pictures were taken at above described. A scheme of the AbiAc protocol is shown in Fig. 1. The tumor area of T0 and T3 AbiAc-treated and untreated groups was measured with Noldus DanioScopeTM software (Noldus Information Technology) and analyzed by GraphPad Prism software 6.01 version.

In silico analysis

The human 3β-Hydroxysteroid dehydrogenase (3β-Hsd) protein information collected in the UniProt database (35) were used to obtain the human 3β-HSD Ensemble gene entry (36). Ensemble full length protein sequence of human 3β-Hsd protein was used to search the zebrafish assembly on BLAST. The sequences of the zebrafish and human enzyme were aligned by using ClustalOmega online software (37, 38).

RNA extraction and real-time PCR

Total RNA was extracted from batch of 30 embryos at five different stage of development (24, 48, 72, 96 and 120 hpf) using the RNAeasy kit (Qiagen Italia, Milano, Italy). RNA was quantified by mySPEC microvolume spectrophotometer (VWR). One µg of each sample was transcribed into

cDNA using the M-MLV reverse transcriptase (Promega Italia, Milano, Italy). Relative gene expression of 3β-HSD was evaluated by quantitative RT-PCR with the ViiA7 Real Time PCR System (Applied Biosystems, Milano, Italy), using the iQ™SYBR Green Supermix method (Bio-Rad, Segrate (Mi), Italy), according to manufacturer's instructions.

The zebrafish full length 3β-HSD transcript was employed to design zebrafish (ZF) specific primers for PCR by using the Primer3web software version 4.1.0 (http://primer3.ut.ee/) (39). The ZF-3β-HSD oligonucleotide sequences of were: F: 5'-CTTTCAACGCAGCGCTCTAC-3', R: 5'-TCTTCCAGCAACAGTCGGAC-3', while for the ZF β-actin (housekeeping gene) were: F 5'-AATCCCAAAGCCAACAGAGA-3', R 5'-TCACACCATCACCAGAGTCC-3'.

Reactions were conducted under the following conditions: 1 cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 62 °C for 1 min. Differences in the threshold cycle Ct values between the β-actin housekeeping gene and ZF-3β-HSD were then calculated as an indicator of the amount of mRNA expressed. Analysis was performed in triplicate for each sample, using different groups of embryos.

Statistical analysis

Statistical analyses were done using GraphPad Prism software 6.01 version. One-way ANOVA followed by Dunnett's test was performed to identify statistically significant differences among different groups of data, considering a p value <0.05 as the threshold for a significant difference.

RESULTS

Cytotoxic and antisecretive effect of AbiAc in ACC29 primary cells. ACC29 primary cells were exposed to increasing concentrations of AbiAc (1-200 nM) for 4 days and analyzed for cell viability. AbiAc exerted a concentration-dependent reduction of cell viability, that reached the plateau of 59,75 ± 2,3 % at 25 nM concentration and did not further increase. The calculated IC_{50} was 6 nM (95%CI 2,9-11,3 nM) (40). Conditioned media of ACC29 were then analyzed for cortisol and progesterone production. Results demonstrated that cortisol production of untreated ACC29 cells was reduced from 2,24 ± 0,29 ng/ml/10^6 cells to 1,55 ± 0,29 ng/ml/10^6 cells in 100 nM AbiAc-treated cells ($p<0.05$ vs untreated cells). As expected, progesterone was undetectable in untreated cells and raised up to 1,3 ng/ml/10^6 cells when exposed to AbiAc.

ACC cell viability and doubling time. The viability and the doubling time of ACC cells maintained at 32 °C were investigated, in order to evaluate whether or not these cells can be a suitable model for zebrafish embryo xenograft. Cell viability was evaluated by trypan blue exclusion test at both 37 °C and at 32 °C and results demonstrated that at the lower temperature, ACC cells are viable (not shown), although with a reduction of the cell proliferation rate (Table 1).

Determination of AbiAc treatment concentration. To set up the method, AB strain zebrafish embryos were treated with increasing concentrations of AbiAc, to determine the optimal drug concentration to be used during *in vivo* experiments. Embryos maintained in fish water plus PTU were manually dechorionated at 48hpf and divided into 5 groups (n=90±10 each). Three groups were treated with 0.5, 1 and 2.5 µM AbiAc, respectively, directly added to the fish water; in the fourth group the solvent alone (methanol) was added to the fish water plus PTU (control group), while the last group was left untreated. Embryos were kept at 32 °C. After 3 days of treatment, exposure of embryos the 2.5 µM AbiAc resulted in death or deformity (41). Embryos of the other two treated groups, as well as embryos of the solvent-treated and control groups developed normally and their phenotype was similar to that of untreated embryos (41). Based on these findings, the concentration of 1 µM AbiAc was chosen for subsequent experiments.

Determination of AbiAc measurement. To evaluate the optimal embryo number to determine absorbed drug concentration, 1 µM AbiAc-treated embryos at 24hpf were divided into groups of

10-15-20-25-30 embryos each and analyzed by LC-MS/MS. As indicated in Methods, AbiAc is rapidly hydrolyzed to Abi (30), indeed AbiAc could not be detected in embryos, while Abi was measurable. The LC-MS/MS analysis revealed that in samples obtained from groups of embryos from 10 to 20, Abi was not detectable, while samples obtained from 25 embryos gave measurable and reproducible results, as shown in Fig. 2a, where a time-curve of the absorbed Abi concentration was performed. AB zebrafish embryos maintained in fish water plus PTU were divided into 5 groups (n=120±10), each group was treated with 1 μM AbiAc, directly added to the fish water, and embryos were collected at different time-points, up to 24 hrs, for the quantification of Abi. In particular, experiments were conducted in both 24 hpf embryos and in 48 hpf embryos. The LC-MS/MS results reported in Fig. 2 showed that Abi absorption increased with the stage of embryo development: interestingly, in 48hpf embryos, the Abi concentration after 24hrs of incubation is about 179 ± 29.5 nM, that is very close to the highest Abi concentration used in *in vitro* experiments with NCI-H295R cells, that was 200 nM (22). The time course, at both development stages, displayed typical kinetic of time-dependent concentration –curve observed in humans (42), suggesting the capability of embryos to metabolize Abi. This hypothesis was supported by gene expression results demonstrating that zebrafish expressed the mRNA encoding the enzyme 3β-HSD; indeed, by q-RT-PCR, we demonstrated that the ΔCt was 4.8 ± 1.2 in 24 hpf embryos and 5.52 ± 0.75 in 48 hpf embryos. The 3β-HSD gene was transcribed in its protein, as demonstrated by the capability of 48 hpf embryos to metabolize Abi in its main metabolite Δ4A (Fig. 3a and Fig.3b). Interestingly, by in silico analysis we observed a high level of similarity (63 %) and identity (46 %) with the human counterpart (38). The experimental conditions were set up in both 24 hpf and 48 hpf embryos, thus, based on results obtained, we chose to perform cell xenograft in 48 hpf embryos, due to the Abi concentration time-course, to technical limits in injecting 24 hpf embryos (in particular, at this stage of development microinjection is not recommended due to tissue fragility) and according to the protocol of Nicoli et al. (8). Finally, the Abi metabolism time-course was conducted at different hpf of embryos, up to 120 (Table 2) and results indicated that the capability of AB zebrafish embryos to metabolize Abi increased with increasing hpf.

AbiAc at 1 µM concentration induced as well a modification of AB zebrafish embryo cortisol level. Results are reported in Table 3. After 3 days exposure, the irreversible binding of AbiAc to CYP17A1 induced a significant reduction of cortisol production in AbiAc-treated embryos. Accordingly, as expected, progesterone become measurable in treated embryos, while it did not reach detection in solvent-treated embryos.

ACC cell xenograft in AB zebrafish embryos.

NCI-H295R cell line. AB zebrafish embryos at 48 hpf were manually dechorionated, anesthetized with tricaine and microinjected with NCI-H295R labeled cells into the subepidermal space of the yolk sac (n=80±10). A picture of each injected embryo was acquired under a fluorescence stereomicroscope two hours post treatment (T0), then embryos were divided into two groups (n=40±5): one maintained in fish water/PTU plus solvent (solvent-treated), while the other was maintained in fish water/PTU plus 1µM AbiAc without medium change up to the 3 days treatment. Embryos were then incubated at 32°C. Three days after treatment (T3), pictures were taken as described and the injected cell area in solvent-treated and 1 µM AbiAc-treated embryos was measured and analyzed as described in Methods. The injected cell area at T3 in solvent-treated embryos was 38,390 ± 1,432 μM^2 compared to 24,891 ± 1,302 μM^2 in AbiAc-treated embryos ($p<0.01$, Fig. 4a). The tumor area in solvent-treated embryos displayed a 1.63 ± 0.07 fold increased at T3 compared to T0, while in AbiAc-treated embryos the tumor area was almost unchanged, with a value of 1.01 ± 0.03 fold increase at T3 compared to T0. A representative image is shown in Fig. 4b. Experiments were repeated changing zebrafish embryo medium every day after xenograft, thus adding fresh AbiAc every day. Results demonstrated that the injected cell areas measured was superimposable to that reported above (not shown).

ACC29 primary cell culture. The cytotoxic effect of 1 µM AbiAc was evaluated as well in a primary culture obtained from a patient diagnosed with a cortisol-secreting ACC. Results are shown in Fig. 5 and demonstrated that, although ACC29 cells seemed to be less sensitive to AbiAc both *in vitro* (see above) and *in vivo*, at T3, the injected ACC29 cell area was significantly reduced in 1 µM AbiAc-treated embryos compared to solvent-treated embryos. The area was indeed 56,987 ± 2,83 μM^2 in solvent-treated embryos vs 39,776 ± 4,516 μM^2 in ABiAC-treated embryos (* $p<0.05$).

350 *SW13 cell line*. The non steroidogenic, AbiAc-insensitive (22, 28) cell line SW13 was used as the
351 internal negative control. The tumor xenograft area of SW13 cells was not modified by exposure
352 to 1 µM AbiAc (Fig. 6), indeed the area of solvent-treated tumor was 52,842 ± 5,163 μM^2 while
353 the area of AbiAc-treated tumor was 49,665 ± 3,705 μM^2.

DISCUSSION

The zebrafish model, due to its distinctive characteristics, is nowadays progressively being applied to design useful *in vivo* experimental studies in a number of human diseases (43), including malignancies (reviewed in 44). In particular, zebrafish embryos provide a powerful tool to develop functional cancer models that can be used for drug discovery and development and drug toxicity. In a rare and severe disease such as ACC, the finding of reproducible and reliable *in vivo* preclinical models is an open challenge, especially in light of the number of variables that have to be taken into account when studying ACC xenograft in mouse model (45). The timing of drug administration, the drug metabolism and the solvent in which the drug is dissolved (that per se may affect the animal safety and the reproducibility of results) are among the most relevant issues (43). The few available cell lines and their genomic instability, the immune competence of the host and site of implantation are additional important drawbacks (45). Here, we validated a NCI-H295R cell xenograft in AB zebrafish embryos as an *in vivo* model of ACC. We were able indeed to confirm the *in vivo* cytotoxicity of AbiAc using an animal model which offers several advantages over other models, like mice (17-21). It should be underlined that AbiAc was directly added into the fish water and was significantly adsorbed by embryos, reaching concentrations able to exert the cytotoxic effect, thus simplifying the treatment procedure. Furthermore, the ACC xenograft in zebrafish was fast, as 3 days of AbiAc treatment were sufficient to demonstrate a significant difference in the NCI-H295R cell proliferation rate. The AbiAc effect was due to the direct binding on its target enzyme as the tumor area of xenografts with non steroidogenic (28), CYP17A1 negative (22) SW13 cells was not modified after 3 days exposure to 1 μM AbiAc, confirming the insensitivity of these cells to the cytotoxic effect of AbiAc treatment, as observed in *in vitro* experiments (22). Interestingly, the effect of AbiAc lasted up to 3 days without adding fresh drug, probably due to the irreversible binding of Abi to CYP17A1. The inhibition of NCI-H295R cell area growth in AB zebrafish embryos was about 60% after 3 days, which is even higher than that we observed in immune incompetent mice, where it reached the 34% inhibition about 60 days after the cell injection and 15 days after the end of 16 days treatment (22). Furthermore, another finding that give support for the use of zebrafish embryos as a useful model for *in vivo* animal studies on ACC is the expression in embryos of enzymes of

the steroidogenic pathways similar to what observed in more evolved animal models. Indeed, they express the 3β-HSD enzyme that converted Abi in its active metabolite Δ4A, that itself inhibits additional enzymes involved in steroidogenesis, including CYP17A1, 3β-HSD and the 5α reductase SRD5A (34, 46). The combined effect of Abi and its main metabolite on CYP17A1 induced a decrease of cortisol and, as expected, an increase of progesterone levels. In the personalized medicine era, the low number of cells needed and the lower length of the experiments make the zebrafish model potentially candidate to prepare ACC patient-derived xenografts in order to perform a real-time selection of the most appropriate cytotoxic drugs for each patient. On these bases, we reproduced results obtained with the NCI-H295R cell line in a patient-derived xenograft, obtained from primary cells established from a cortisol-secreting ACC, thus giving support to the possibility to develop this *in vivo* method to screen available therapeutic options for a cancer such as ACC, with a poor prognosis and a scarcity of therapeutic options. We believe that the validation of this animal model can offer a useful tool to perform a preclinical first-screening of a large number of drugs, and this is advantageous in a rare and aggressive disease such as ACC, in which the treatment strategies are limited. We are aware that this animal model cannot completely replace the others already in use, but we believe that our findings could offer a suitable and fast model to perform an initial selection of potentially effective drugs, also with regard to the identification of dose toxicity, and choose the most promising compounds to be used in more advanced preclinical phases. Lastly, this procedure could also reduce the number of animal models to finalize the research.

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REFERENCES

(1) Fassnacht M, Dekkers OM, Else T, Baudin E, Berruti A, de Krijger R, Haak HR, Mihai R, Assie G, Terzolo M. European Society of Endocrinology Clinical Practice Guidelines on the management of adrenocortical carcinoma in adults, in collaboration with the European Network for the Study of Adrenal Tumors. *Eur J Endocrinol.* 2018;179(4):G1-G46. doi: 10.1530/EJE-18-0608.

(2) Berruti A, Grisanti S, Pulzer A, Claps M, Daffara F, Loli P, Mannelli M, Boscaro M, Arvat E, Tiberio G, Hahner S, Zaggia B, Porpiglia F, Volante M, Fassnacht M, Terzolo M. Long-Term Outcomes of Adjuvant Mitotane Therapy in Patients With Radically Resected Adrenocortical Carcinoma. *J Clin Endocrinol Metab*. 2017;102(4):1358-1365. doi: 10.1210/jc.2016-2894

(3) Terzolo M, Daffara F, Ardito A, Zaggia B, Basile V, Ferrari L, Berruti A. Management of adrenal cancer: a 2013 update. *J Endocrinol Invest*. 2014;37(3):207-17. doi: 10.1007/s40618-013-0049-2

(4) Paragliola RM, Torino F, Papi G, Locantore P, Pontecorvi A, Corsello SM. Role of Mitotane in Adrenocortical Carcinoma - Review and State of the art. *Eur Endocrinol.* 2018;14(2):62-66. doi: 10.17925/EE.2018.14.2.62.

(5) Berruti A, Terzolo M, Sperone P, Pia A, Della Casa S, Gross DJ, Carnaghi C, Casali P, Porpiglia F, Mantero F, Reimondo G, Angeli A, Dogliotti L. Etoposide, doxorubicin and cisplatin plus mitotane in the treatment of advanced adrenocortical carcinoma: a large prospective phase II trial. *Endocr Relat Cancer.* 2005;12(3):657-66. doi: 10.1677/erc.1.01025.

(6) Fassnacht M, Terzolo M, Allolio B, Baudin E, Haak H, Berruti A, Welin S, Schade-Brittinger C, Lacroix A, Jarzab B, Sorbye H, Torpy DJ, Stepan V, Schteingart DE, Arlt W, Kroiss M, Leboulleux S, Sperone P, Sundin A, Hermsen I, Hahner S, Willenberg HS, Tabarin A, Quinkler M, de la Fouchardière C, Schlumberger M, Mantero F, Weismann D, Beuschlein F, Gelderblom H, Wilmink H, Sender M, Edgerly M, Kenn W, Fojo T, Müller HH, Skogseid B; FIRM-ACT Study Group. Combination chemotherapy in advanced adrenocortical carcinoma. *N Engl J Med.* 2012;366(23):2189–97. doi: 10.1056/NEJMoa1200966.

(7) Kelland LR. Of mice and men: values and liabilities of the athymic nude mouse model in anticancer drug development. *Eur J Cancer.* 2004;40(6):827-36. doi: 10.1016/j.ejca.2003.11.028.

(8) Nicoli S, Presta M. The zebrafish/tumor xenograft angiogenesis assay. *Nature Protoc.* 2007;2(11):2918-23. doi: 10.1038/nprot.2007.412.

(9) Mione MC and Trede NS. The zebrafish as a model for cancer. *Dis Model Mech*. 2010;3(9-10):517–523. doi: 10.1242/dmm.004747.

(10) Langheinrich U. Zebrafish: A new model on the pharmaceutical catwalk. *Bioessays* 2003;25:904-912. doi: 10.1002/bies.10326.

(11) Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov*. 2005;4:35-44. doi: 10.1038/nrd1606.

(12) Peterson RT, Macrae CA. Systematic approaches to toxicology in the zebrafish. *Annu Rev Pharmacol Toxicol.* 2012;52:433-453. doi: 10.1146/annurev-pharmtox-010611-134751.

(13) Gianoncelli A, Bonini SA, Bertuzzi M, Guarienti M, Vezzoli S, Kumar R, Delbarba A, Mastinu A, Sigala S, Spano P, Pani L, Pecorelli S, Memo M. An Integrated Approach for a Structural and Functional Evaluation of Biosimilars: Implications for Erythropoietin. *BioDrugs* 2015;29(4):285-300. doi: 10.1007/s40259-015-0136-3.

(14) Guarienti M, Giacopuzzi E, Gianoncelli A, Sigala S, Spano P, Pecorelli S, Pani L, Memo M. Computational and functional analysis of biopharmaceutical drugs in zebrafish: Erythropoietin as a test model. *Pharmacol Res.* 2015;102:12-21. doi: 10.1016/j.phrs.2015.09.004.

(15) Basnet RM, Guarienti M, Memo M. Zebrafish Embryo as an In Vivo Model for Behavioral and Pharmacological Characterization of Methylxanthine Drugs. *Int J Mol Sci.* 2017;18(3):E596. doi: 10.3390/ijms18030596.

(16) Gianoncelli A, Bertuzzi M, Guarienti M, Vezzoli S, Bonini SA, Mastinu A, Sigala S and Memo M Parallelism of chemico-structural properties between filgrastim originator and three of its biosimilar drugs. *J Chem*. In press

(17) Marques IJ, Weiss FU, Vlecken DH, Nitsche C, Bakkers J, Lagendijk AK, Partecke LI, Heidecke CD, Lerch MM, Bagowski CP. Metastatic behaviour of primary human tumours

in a zebrafish xenotransplantation model. *BMC Cancer*. 2009;9:128. doi: 10.1186/1471-2407-9-128.

(18) Jung DW, Oh ES, Park SH, Chang YT, Kim CH, Choi SY, Williams DR. A novel zebrafish human tumor xenograft model validated for anti-cancer drug screening. *Mol Biosyst.* 2012;8(7):1930-9. doi: 10.1039/c2mb05501e.

(19) Tonon F, Zennaro C, Dapas B, Carraro M, Mariotti M, Grassi G. Rapid and cost-effective xenograft hepatocellular carcinoma model in Zebrafish for drug testing. *Int J Pharm*. 2016;515(1-2):583-91. doi: 10.1016/j.ijpharm.2016.10.070.

(20) Bootorabi F, Manouchehri H, Changizi R, Barker H, Palazzo E, Saltari A, Parikka M, Pincelli C, Aspatwar A. Zebrafish as a Model Organism for the Development of Drugs for Skin Cancer. *Int J Mol Sci.* 2017;18(7). doi: 10.3390/ijms18071550.

(21) Chen AT, Zon LI. Zebrafish blood stem cells. *J Cell Biochem*. 2009;108(1):35-42. doi: 10.1002/jcb.22251.

(22) Fiorentini C, Fragni M, Perego P, Vezzoli S, Bonini SA, Tortoreto M, Galli D, Claps M, Tiberio GA, Terzolo M, Missale C, Memo M, Procopio G, Zaffaroni N, Berruti A, Sigala S. Antisecretive and Antitumor Activity of Abiraterone Acetate in Human Adrenocortical Cancer: A Preclinical Study. *J Clin Endocrinol Metab*. 2016;101(12):4594-602. doi: 10.1210/jc.2016-2414

(23) Attard G, Reid AH, Auchus RJ, Hughes BA, Cassidy AM, Thompson E, Oommen NB, Folkerd E, Dowsett M, Arlt W, de Bono JS. Clinical and biochemical consequences of CYP17A1 inhibition with abiraterone given with and without exogenous glucocorticoids in castrate men with advanced prostate cancer*. J Clin Endocrinol Metab*. 2012;97(2):507–16. doi: 10.1210/jc.2011-2189

(24) Ryan CJ, Smith MR, Fizazi K, Saad F, Mulders PF, Sternberg CN, Miller K, Logothetis CJ, Shore ND, Small EJ, Carles J, Flaig TW, Taplin ME, Higano CS, de Souza P, de Bono JS, Griffin TW, De Porre P, Yu MK, Park YC, Li J, Kheoh T, Naini V, Molina A, Rathkopf DE. Abiraterone acetate plus prednisone versus placebo plus prednisone in chemotherapy-naive men with metastatic castration-resistant prostate cancer (COU-AA-302): final

497 overall survival analysis of a randomised, double-blind, placebo-controlled phase 3 study.
498 *Lancet Oncol.* 2015;16(2):152– 60. doi: 10.1016/S1470-2045(14)71205-7.
499 (25) Fizazi K, Scher HI, Molina A, Logothetis CJ, Chi KN, Jones RJ, Staffurth JN, North S,
500 Vogelzang NJ, Saad F, Mainwaring P, Harland S, Goodman OB Jr., Sternberg CN, Li JH,
501 Kheoh T, Haqq CM, de Bono JS. Abiraterone acetate for treatment of metastatic
502 castration-resistant prostate cancer: final overall survival analysis of the COU-AA-301
503 randomised, double-blind, placebo-controlled phase 3 study. *Lancet Oncol*.
504 2012;13(10):983–92. doi: 10.1016/S1470-2045(12)70379-0.
505 (26) Claps M, Lazzari B, Grisanti S, Ferrari V, Terzolo M, Sigala S, Vezzoli S, Memo M,
506 Castellano M, Berruti A. Management of severe cushing syndrome induced by
507 adrenocortical carcinoma with abiraterone acetate: a case report. AACE *Clinical Case Rep*.
508 2016;2(4):e337-e341
509 (27) Fragni M, Fiorentini C, Rossini E, Fisogni S, Vezzoli S, Bonini SA, Dalmiglio C, Grisanti S,
510 Tiberio GAM, Claps M, Cosentini D, Salvi V, Bosisio D, Terzolo M, Missale C, Facchetti F,
511 Memo M, Berruti A, Sigala S. In vitro antitumor activity of progesterone in human
512 adrenocortical carcinoma. *Endocrine.* 2018; Epub ahead of print. doi: 10.1007/s12020-
513 018-1795-x
514 (28) Wang T, Rainey WE. Human adrenocortical carcinoma cell lines. *Mol Cell Endocrinol*.
515 2012;58-65. doi: 10.1016/j.mce.2011.08.041
516 (29) Gianoncelli A, Guarienti M, Fragni M, Bertuzzi M, Rossini E, Abate A, Basnet R M, Zizioli
517 D, Bono F, Terzolo M, Memo M, Berruti A, Sigala S. Supplemental data: Clinical and
518 immunohistochemical characteristics of ACC29 patient.
519 https://figshare.com/s/25f65cba83c6ca84f2f1.
520 (30) Sbiera S, Schmull S, Assie G, Voelker HU, Kraus L, Beyer M, Ragazzon B, Beuschlein F,
521 Willenberg HS, Hahner S, Saeger W, Bertherat J, Allolio B and Fassnacht M. High
522 diagnostic and prognostic value of steroidogenic factor-1 expression in adrenal tumors.
523 The Journal of clinical endocrinology and metabolism. 2010; 95:E161-171. doi:
524 10.1210/jc.2010-0653.

(31) Hantel C, Shapiro I, Poli G, Chiapponi C, Bidlingmaier M, Reincke M, Luconi M, Jung S., Beuschlein F. Targeting heterogeneity of adrenocortical carcinoma: Evaluation and extension of preclinical tumor models to improve clinical translation. Oncotarget. 2016 Nov 29;7(48):79292-79304. doi: 10.18632/oncotarget.12685.

(32) Westerfield M. A guide for the laboratory use of zebrafish (Danio rerio). The zebrafish book. 4th ed: University of Oregon Press Eugene; 2000.

(33) Stappaerts J, Geboers S, Snoeys J, Brouwers J, Tack J, Annaert P, Augustijns P. Rapid conversion of the ester prodrug abiraterone acetate results in intestinal supersaturation and enhanced absorption of abiraterone: in vitro, rat in situ and human in vivo studies. *Eur J Pharm Biopharm*. 2015;90:1-7. doi: 10.1016/j.ejpb.2015.01.001.

(34) Li Z, Bishop A, Alyamani M, Garcia JA, Dreicer R, Bunch D, Liu J, Upadhyay SK, Auchus RJ, Sharifi N. Conversion of abiraterone to Δ4A drives anti-tumour activity in prostate cancer. Nature 2015; 523: 347-351. doi:10.1038/nature14406

(35) The UniProt Consortium. UniProt: the universal protein knowledgebase. *Nucleic Acids Res*. 2017;45(D1):D158-D69.

(36) Zerbino DR, Achuthan P, Akanni W, Amode MR, Barrell D, Bhai J, et al. Ensembl 2018 *Nucleic Acids Res*. 2018;46(D1):D754-D761. doi: 10.1093/nar/gkx1098

(37) Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Söding J, Thompson JD, Higgins DG. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol.* 2011;7:539. doi: 10.1038/msb.2011.75

(38) Gianoncelli A, Guarienti M, Fragni M, Bertuzzi M, Rossini E, Abate A, Basnet R M, Zizioli D, Bono F, Terzolo M, Memo M, Berruti A, Sigala S. Supplemental data: ClustalOmega analysis. https://figshare.com/s/9288b1491c88d38d8db8.

(39) Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG.. Primer3--new capabilities and interfaces. *Nucleic Acids Res*. 2012;40(15):e115. doi: 10.1093/nar/gks596.

(40) Gianoncelli A, Guarienti M, Fragni M, Bertuzzi M, Rossini E, Abate A, Basnet R M, Zizioli D, Bono F, Terzolo M, Memo M, Berruti A, Sigala S. Supplemental data: Cytotoxic effect of AbiAc on ACC29 primary cell culture. https://figshare.com/s/5dc2d3564988c472c607.

(41) Gianoncelli A, Guarienti M, Fragni M, Bertuzzi M, Rossini E, Abate A, Basnet R M, Zizioli D, Bono F, Terzolo M, Memo M, Berruti A, Sigala S. Supplemental data: AbiAc toxicity in zebrafish embryos. https://figshare.com/s/0447ced4fc9703178db5.

(42) Abiraterone Acetate, Micromedex® 1.0 (Healthcare Series), (electronic version). Truven Health Analytics, Greenwood Village, Colorado, USA. Available at: http://www-micromedexsolutions-com.proxy.unibs.it [Accessed December 13, 2018].

(43) Cayuela ML, Claes KBM, Ferreira MG, Henriques CM, van Eeden F, Varga M, Vierstraete J, Mione MC. The Zebrafish as an Emerging Model to Study DNA Damage in Aging, Cancer and Other Diseases. Front Cell Dev Biol. 2019;6:178. doi: 10.3389/fcell.2018.00178

(44) Letrado P, de Miguel I, Lamberto I, Díez-Martínez R, Oyarzabal J. Zebrafish: Speeding Up the Cancer Drug Discovery Process. *Cancer Res*. 2018;78(21):6048-6058. doi: 10.1158/0008-5472.CAN-18-1029

(45) Hantel C, Beuschlein F. Xenograft models for adrenocortical carcinoma. *Mol Cell Endocrinol*. 2016 421:28-33. doi: 10.1016/j.mce.2015.05.031

(46) de Mello Martins AGG, Allegretta G, Unteregger G, Haupenthal J, Eberhard J, Hoffmann M, van der Zee JA, Junker K, Stöckle M, Müller R, Hartmann RW, Ohlmann CH. CYP17A1-independent production of the neurosteroid-derived 5α-pregnan-3β,6α-diol-20-one in androgen-responsive prostate cancer cell lines under serum starvation and inhibition by Abiraterone. *J Steroid Biochem Mol Biol*. 2017; 174:183-191. doi: 10.1016/j.jsbmb.2017.09.006.

FIGURE CAPTIONS

Figure 1. Scheme of AbiAC treatment in AB zebrafish embryos xenografted with ACC cells

Figure 2: The LC-MS/MS results of the quantification of Abi treated with 1 μM AbiAc, directly added to the fish water, starting from different timepoints: 24 (a) and 48hpf (b). Batch of 25 embryos for each group were collected after 1.5 h, 3 h, 6 h and 24 h of treatment, for the quantification of Abi.

Figure 3: a) The chromatogram relating to the extraction of a sample of 25 embryos treated at 48 hours with AbiAc. From the chromatogram, the presence of a peak relative to the Abi and other minor peaks related to its metabolites has been found. The main metabolite Δ4A has been identified. b) The time course of Abi (blue) absorption in 48hpf embryos in comparison with the time course of Δ4A (orange).

Figure 4: AbiAc exposure of AB zebrafish embryos reduced the tumor xenograft area in NCI-H295R cell line. The tumor area of T0 and T3 AbiAc-treated and solvent-treated groups was measured with Noldus DanioScopeTM software (Noldus Information Technology) and analyzed by GraphPad Prism software 6.01 version. The increase of tumor area in solvent-treated vs AbiAc-treated embryos was statistically significant (p value <0.01) after one-way ANOVA followed by Dunnett's test analysis (Fig. 4a). A representative image is shown in Fig. 4b.
T0: time point at injection (control embryos 48hpf); T3-AbiAc: time point 3 days later in fish water with solvent alone (untreated embryos 120hpf); T3+AbiAc: time point 3 days later in fish water with 1 μM AbiAc (treated embryos 120hpf).

Figure 5. AbiAc exposure of AB zebrafish embryos reduced the patient-derived tumor xenograft ACC29. For details, see Figure 4 legend.

Figure 6. The CYP17A1 negative, AbiAc insensitive SW13 cells xenografted in AB zebrafish embryos were not responsive to ABiAc exposure. For details, see Figure 4 legend.

Table 1. Temperature-induced modifications of cell Doubling Time (DT) of ACC cells

Cell line / primary colture	**DT at 37°C**	**DT at 32°C**
NCI-H295R	52 h	90 h
SW-13	21 h	26 h
ACC29	50 h	74 h

The DT was calculated according to ATCC indication with the following formula: DT=T ln2/ln(Xe/Xb), where T is the incubation time in any units, Xb is the cell number at the beginning of the incubation time, Xe is the cell number at the end of the incubation time.

Table 2. Quantification of Abi absorption in zebrafish embryos exposed to 1 µM AbiAc, directly added to the fish water.

	[ABI] nM				
Embryos hpf	24	48	72	96	120
T1 = 1.5h	242,68±12,12	316,99±21,82	328,62±12,30	341,37±13,10	308,65±33,33
T2 = 3h	285,74±43,14	415,92±35,19	394,10±15,53	363,49±19,67	303,35±14,71
T3 = 6h	272,69±17,30	334,35±24,67	378,25±15,72	274,04±17,14	363,21±16,07
T4 = 24h	111,33±21,44	179,48±29,54	12,12±2,66	4,18±0,60	8,19±4,36

A time-course of Abi quantification was conducted on batches of 25 embryos each by LC-MS/MS, as described in Methods, starting from 24 hpf up to 120 hpf embryos.

Table 3. Effect of 1 µM ABiAc on AB zebrafish embryo cortisol and progesterone synthesis

	Solvent-treated AB zebrafish embryos (n=100)	AbiAc-treated AB zebrafish embryos (n=100)
Cortisol	9,35 ± 0.1 ng	4,25 ± 0.06 ng *
Progesterone	undetectable	3,53 ± 0.17 ng *

*p<0.001 vs solvent-treated AB zebrafish embryos

Hormone determinations were performed as described in Methods and analyzed by LC-MS/MS as described

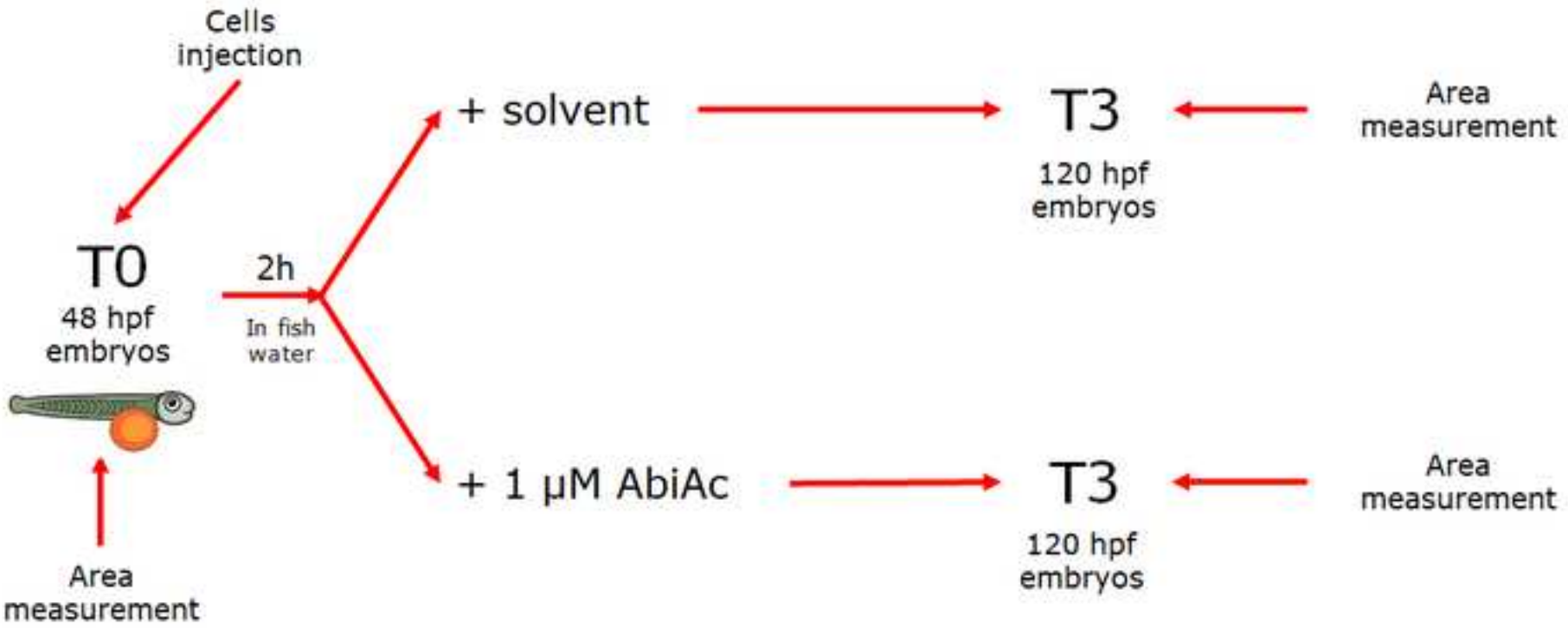


Figure 2

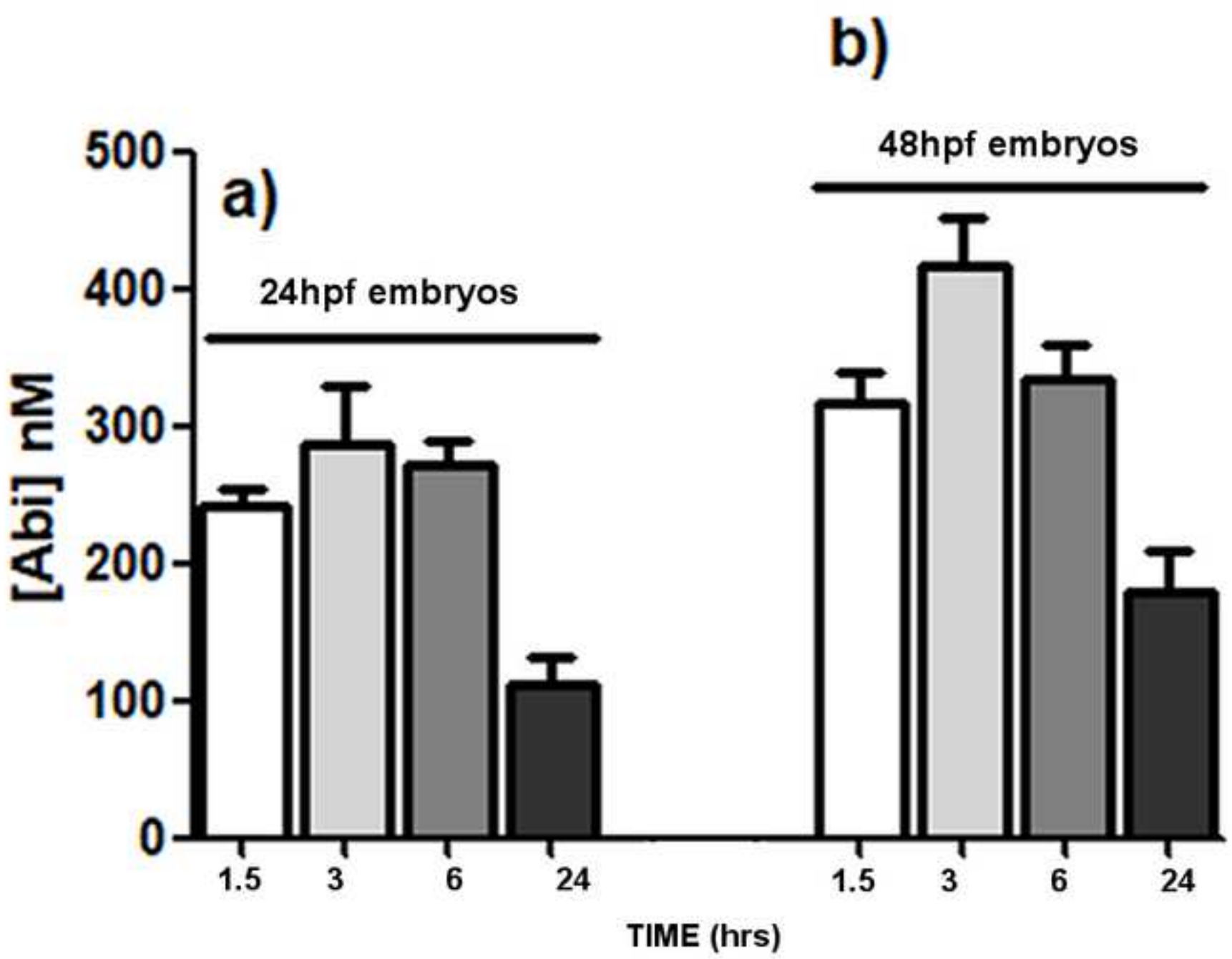
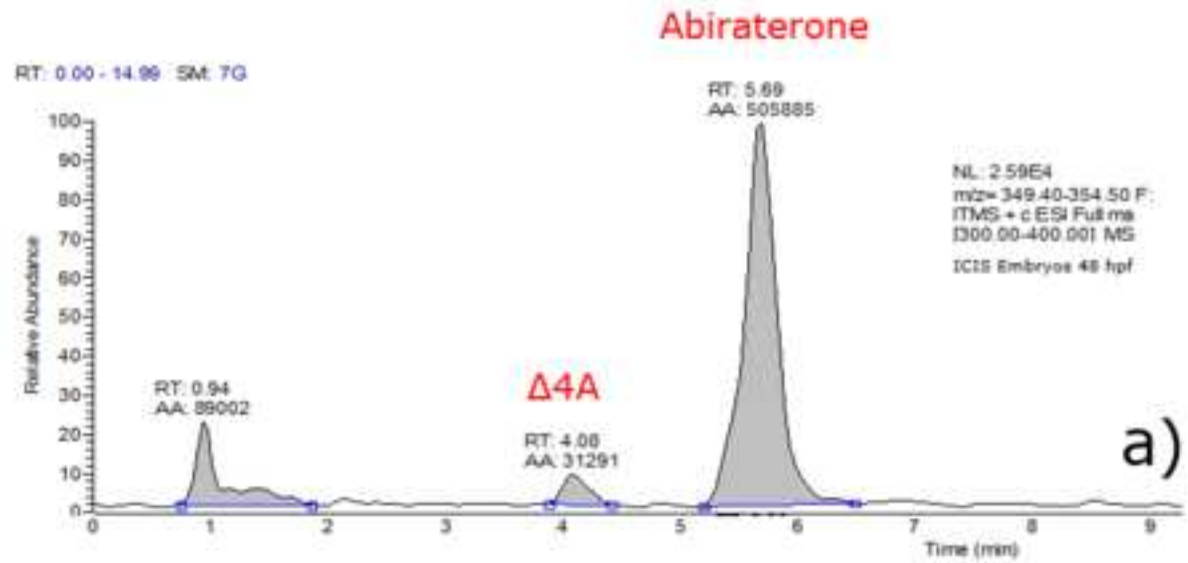


Figure 3



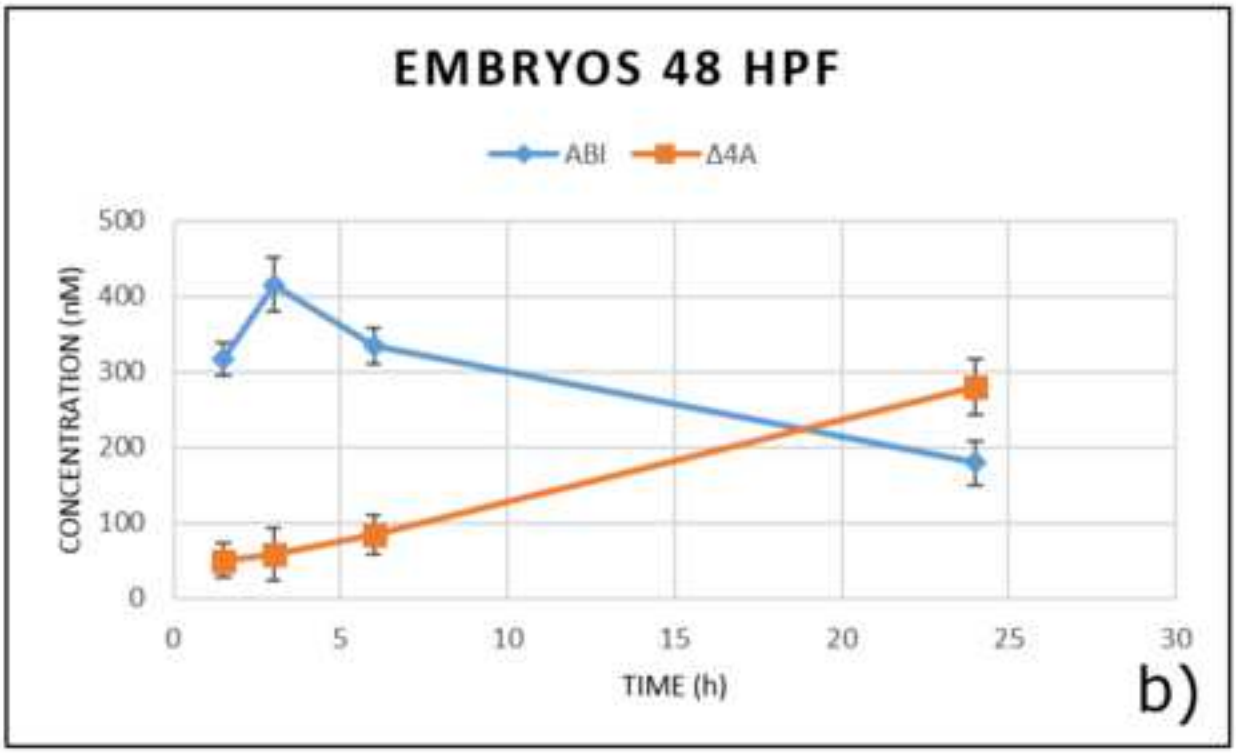


Figure 4

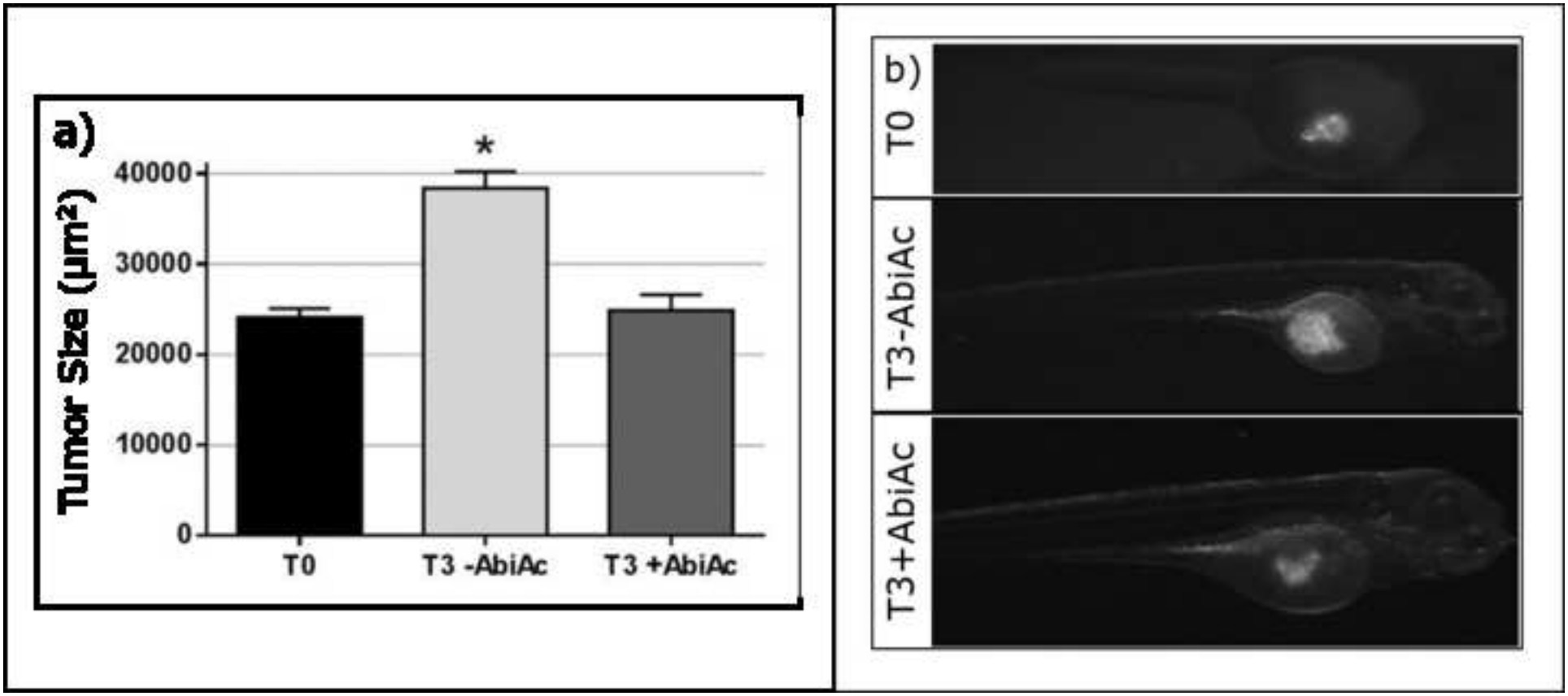


Figure 5

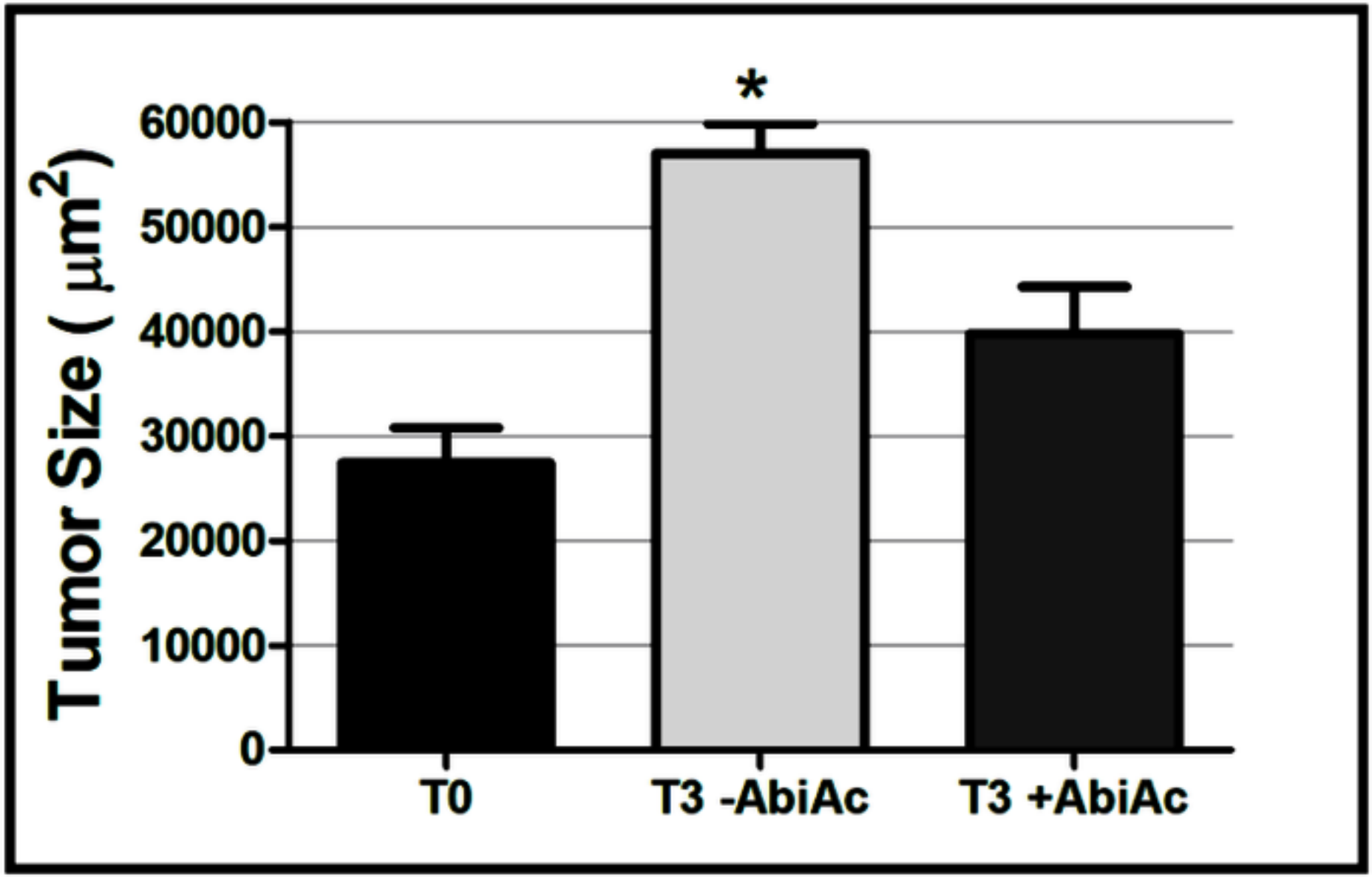


Figure 6