AUTOMATED TYROSINE KINASE INHIBITOR CARDIOTOXICITY ASSAY IN ZEBRAFISH

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Abstract

The zebrafish embryos have recently gained relevance in biomedical research thanks to some of its characteristics including embryo transparency, small size, ease of manipulation and possibility to evaluate different internal organs avoiding invasive methodologies. Combined with the possibility to adapt the model with an automatic device and the reduced cost associated to each assay, the model is an ideal killer experiment in early phases of drug discovery as well as a novel method to increase the selection arguments to reduce the candidates to enter into the Drug Development processes.

Cardiotoxicity is one of the most important reasons for drug attrition during the process of Drug Development. Evaluation of cardiotoxicity and especially HERG channel inhibition is described in regulatory guidelines, but limitations demands the development of new complementary assays that can also evaluate the heart function from a holistic point of view. Biobide has set up a novel in vivo automated platform that allows testing compounds in zebrafish embryos.

To evaluate and validate the quality of the analysis system, the model and the value of the information, we have used a panel of blind-coded Tyrosine kinase inhibitors that had been previously described in other in vitro and in vivo assays. The results indicate that our automated method provides with high informative and complementary data that can significantly improve the process of selection of new candidates with low or no cardiotoxicity.

Method

Embryos expressing a green fluorescent protein under a heart specific promoter were collected at 0 hours post fertilization (hpf) and raised with E3 media at 28.5C in an incubator. Once they reached 48 hpf, embryos were poured into an embryo sorter and sorted based on the expression of fluorescence and size of the embryo. Embryos were incubated with each compound at 3 different concentrations 5, 10, 50 µM and analyzed at 3 and 24 hours of incubation. All the processes were done automatically in the platform of Biobide (figure 1). Heart beat alterations were automatically analyzed with algorithms specially developed (figure 2) and a final comparison with other methods was done with data provided by AB Sciences.

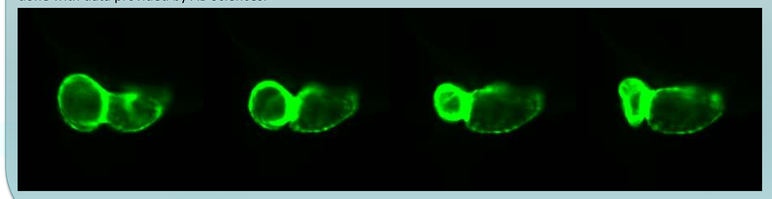

Figure 1. **Images obtained with the automated system.** Heart beat analysis is performed using videos of fluorescent transgenic hearts of 48 hpf zebrafish embryos.

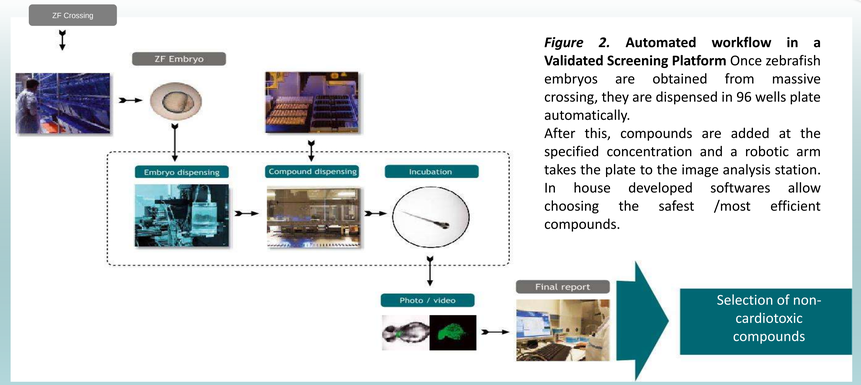

Figure 2. **Automated workflow in a Validated Screening Platform** Once zebrafish embryos are obtained from massive crossing, they are dispensed in 96 wells plate automatically.
After this, compounds are added at the specified concentration and a robotic arm takes the plate to the image analysis station. In house developed softwares allow choosing the safest /most efficient compounds.

Results

Description of the Tyrosine Kinases

Tested drug	Clinic		Incidence cardiac event in clinic
	General Description	*Cardiac effects*	
SORAFENIB	Is an antitumoral agent. Among the adverse effects of Sorafenib cardiovascular effects can occur, such as cardiomyopathy and hypertension to QT prolongation. Sorafenib also causes disthyroidism that aggravates cardiotoxicity. The registratory trials of Sorafenib reported cardiotoxicity in approximately 10% of the patients, but more recent observational studies of the general population show that cardiac events may ocur in as many as 40% of patients.	Hypertension, Hypothyroidism that aggravates cardiotoxicity, mytochondrial dysfunction, ischemia	17% Hypertension, Warning Full prescribing information
SUNITINIB	Sunitinib is a multitargeted TKI used to extend survival of patients with metastatic renal-cell carcinoma and gastrointestinal stromal tumours, but concerns have arisen about its cardiac safety, with reported rates of cardiotoxicity are as high as 33.8% .	QT prolongation, Cardiomyopathie and Hypertension, mytochondrial dysfunction, ischemia	18% patients, 24% Hypertension, WarningFull prescribing information
DASATINIB	Dasatinib is a Src family TKI used in patients with chronic myelogenous leukemia (CML). There is limited data on Dasatinib and cardiotoxicity. However, QT prolongation has been observed in Phase II trials of Dasatinib, and severe cardiac toxicity has occasionally been reported with Imatinib.	QT prolongation	Low to moderate
IMATINIB	Imatinib is a TKI used to treat certain types of cancer. The most common side effects include weight gain, reduced number of blood cells (neutropenia, thrombocytopenia, anemia), headache, edema, nausea, rash, and musculoskeletal pain. Severe cardiotoxicities are occasional.	-	No or low rate
NILOTINIB	Nilotinib is used in treatment of leukemia. Nilotinib prolongs the QT interval, and sudden deaths have been reported rarely.	QT prolongation	Warning FPI
PAZOPANIB	Pazopanib is a second-generation multitargeted TKI that has exhibited antiangiogenic and antitumor activity. Pazopanib has been shown to prolong the QTc interval (2% of the cases in clinical studies). The concurrent use of Pazopanib with other agents that prolong the QTc interval may result in potentially life-threatening cardiac arrhythmias, including Torsades de Pointes.	QT prolongation and Hypertension	37% Hypertension, Warning Full prescribing information
MASITINIB	Masitinib is a selective inhibitor of c-Kit and PDGFR registered in Europe (Masivet®) and the USA (Kinavet®) for the treatment of canine mast cell tumor, a common cutaneous cancer in dogs. Masitinib is being evaluated in nine phase III studies in human medicine, including pancreatic cancer, GIST, metastatic melanoma, multiple myeloma, mastocytosis, severe persistent asthma, rheumatoid arthritis, Alzheimer's disease, and multiple sclerosis	-	-
BOSUTINIB	Bosutinib functions as a dual inhibitor of Src and Abl kinases, with little if any inhibitory effect over PDGF-R and kit. It is used in the treatment of leukemia.	-	-
PONATINIB	Ponatinib is a multi-targeted TKI, being its primary target BCR-ABL and it is an experimental oral drug candidate for the treatment of chronic myeloid leukemia.	-	-

Figure 3. Description of the drugs tested in the zebrafish cardiotoxicity platform in Biobide, related to their cardiotoxic known effects in humans.

Cardiotoxicity classification is based on the use of the euclidian distance algorithm.

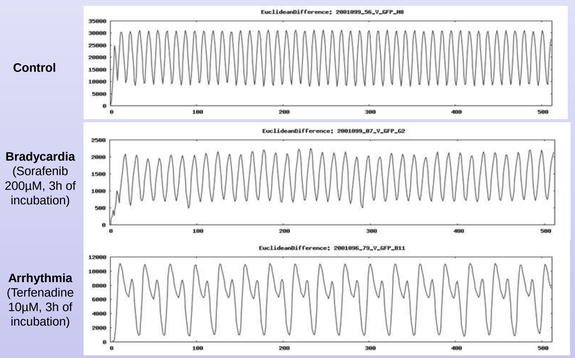

Figure 4. The videos were analyzed using the Cardio. V.3.0.0.1 software (Made in Biobide) which provides data about the cardiac rate, rhythmicity of the heart beat, and fibrillation/death embryos. The software is based on the application of the Euclidean Distance transformation. The analysis of the graph, permits the quantification of morphological and functional features of the cardiac activity.
The sensitivity of the transform Euclidean Distance was higher than the one obtained with other algorithms.

Tyrosine kinases potential cardiotoxicity was assessed in zebrafish embryos automatically in the platform at 3 and 24 hours post incubation

Drugs	Solubility	Concentration (µM)						Final conclusion
		3h treatment			24h treatment			
	Water µM	5	10	50	5	10	50	
SORAFENIB	8	Br	Br	Br	D	D	D	Bradycardia at 3h, acute tox at 24h
SUNITINIB	150	NE	NE	NE	NE	Br/A[1]	Br/A[1]	[1]Dysfunction Ventricle and Bradycardia
								zERG blocker
DASATINIB	200	NE	NE	NE	NE	NE	Br	Moderate Bradycardia
IMATINIB	200	NE	NE	NE	NE	NE	NE	No cardiotoxicity
NILOTINIB	2	NE	NE	Br	NE	NE	NE*	Moderate Bradycardia
PAZOPANIB	31	NE	NE	NE	NE	NE	NE	No cardiotoxicity
MASITINIB	100	NE	NE	NE	NE	NE	NE	No cardiotoxicity
BOSUTINIB	16	NE	NE	D	NE	NE	D	No clear cardiotoxicity but acute toxicity and heart failure.
PONATINIB	15	NE	NE	NE	D/A[1]	D/A[1]	D/A*	[1]Dysfunction Ventricle
								zERG blocker

Figure 5. Results of the tested drugs in the *in vivo* zebrafish automated platform in Biobide (toxicities in red)
No effect (NE); Bradycardia (BR); Arrhythmia (A) ; Death (D); * Precipitation observed

The zebrafish assay is a good tool to predict cardiotoxicity in humans and can complement in vitro assays.

Drugs	hERG# Inhibition (%)	hCM Prolif (IC50µM)	hROS prod°	Mito Respº (%) **	Incidence of cardiac event in clinic	Zebrafish
SORAFENIB	9	>10	-21	33	17% HTN Warning FPI	**Detected**
SUNITINIB	52 (0.27µM)*	9.5	331	93	18% patients 24% HTN Warning FPI	**Detected**
DASATINIB	23 (14µM)*	<1	167	134	Low to mod rate	**Detected**
IMATINIB	15	>10	15	160	No or Low rate	**Not detected**
NILOTINIB	40 (0.42µM)*	3	240	35	Warning FPI	**Detected**
PAZOPANIB	33	>10	39	ND	37% HTN Warning FPI	**Not detected**
MASITINIB	23	>10	11	100	-	**Not detected**
BOSUTINIB	96	>10	7	56	-	**Not classified because of dose**
PONATINIB	86	<1	ND	125	-	**Detected**

Figure 6. List of TKIs and potential cardiac activity in vitro. (cardiotoxicites in red)
% inhibition at 3µM Predictor hERG (Invitrogen) ;*Patch clamp IC_{50} µM; human cardiomyocites hCM ; hROS ROS production by treated cardiomyocytes ; ** % Respiration expressed as a ratio to a non toxic compound in isolated murin hearts

Conclusions

- This zebrafish test classifyed correctly 7 out of the 9 compounds that were tested.
- All compounds that were reported to be cardiotoxic in clinic were also positive in the zebrafish assay with the exception of Pazopanib that was not identified in any of the assays. The main cardiotoxic activity is based on hypertension, an endpoint that was not evaluated in the zebrafish assay and that could be implemented in future assays.
- Testing of compounds at 3 and 24 hours post incubation was informative and complementary. The incubation at 24 hours allowed the evaluation of compounds inducing alterations in metabolism or cardiomyocite survival such as with the Sunitinib.
- Testing compounds in zebrafish embryos can help establishing the therapeutical window when combining different concentrations and specific analysis with general toxicity analysis.
- Bosutinib induced embryo toxicity observed as heart failure and reported as embryo dead. No cardiotoxicity was reported at lower doses in accordance with the results observed in clinic. New analysis at concentrations between 10 and 50 µM would be required to finally classify this compound.
- The zebrafish cardiotoxic data is complementary to other already developed assays to evaluate cardiotoxicity.

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