Dimethyl-sulfoxide is a Suitable Solvent for Fluorescent Microscopy Detection of Medium and Strong Heat Shock Inductors Using Transgenic Zebrafish

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Heat shock inductors have potential as treatment for degenerative and protein misfolding diseases. Dimethyl-sulfoxide is widely used as a solvent in pharmacological screening tests and has been shown to have heat shock induction effects. Transgenic Tg (hsp70l:EGFP-HRAS_G12V)io3(AB) zebrafish larvae were exposed for 24 hours to dimethyl-sulfoxide in concentratios of 0.1-2%, and to moderate heat shock inductors pentoxifylline and tacrolimus. Positive controls were exposed to 35, 38 and 40°C for 20 min, and incubated for 24 h at 28°C. Heat shock response was measured by fluorescence microscopy and signal intensity quantification in FIJI. Dimethyl-sulfoxide caused a dose-dependant increase in fluorescent intensity, but significantly lower compared with exposure to 38 and 40°C. Pentoxifylline and tacrolimus induced a significantly higher increase in fluorescence compared with 0.5% dimethyl-sulfoxide. Thus, although dimethyl-sulfoxide has independent heat shock induction effects, concentrations of up to 0.5% are suitable for heat shock response screening tests.

Keywords: dimethyl-sulfoxide, heat shock response, zebrafish

The heat shock response (HSR) is a phylogenetically conserved adaptative reaction, consisting of the synthesis of heat shock proteins (Hsp), in response to alterations in tertiary protein structure caused by various stressors, including heat, oxidative stress, ethanol and heavy metals. Heat shock proteins are classified by molecular weight in several classes (small Hsp, Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100) [1, 2]. Hsp act as molecular chaperones, controlling misfolded protein sequestration, reconfiguration or degradation [3], and as anti-apoptotic factors [4].

HSR induction has been shown to have protective effects in numerous pathologies, including degenerative diseases [5], ischemia-reperfusion injury [6], systemic inflammatory response [7] and ionizing radiation exposure [8, 9]. This wide therapeutic potential has spurred interest in HSR modulation research from both the academic sector and pharmaceutical industry [10].

Zebrafish (Danio rerio) are small model organisms used in biomedical research in hundreds of laboratories worldwide, both academic and corporate [11], due to their small size, high prolificity, relatively high biological similarity with humans and very low maintenance costs [12].

Dimethyl-sulfoxide (DMSO) is widely used as a solvent for *in vitro* [13-15] and zebrafish based pharmacological screening tests, in concentrations up to 2% [16]. However, several studies have shown that starting with concentrations of 0.01%, DMSO causes HSR induction [17-19].

Thus, we verified if DMSO induction of the HSR can be detected by fluorescent microscopy image analysis and investigated if DMSO is a suitable solvent for the detection of HSR to known inductors, using tacrolimus [20] and pentoxifyllin [21] as representatives.

Experimental part

Animals

Transgenic Tg (hsp70l:EGFP-HRAS_G12V)io3(AB) zebrafish, with enhanced green fluorescent protein linked to Hsp70 promoter, were generated by Marina Mione, FIRC Institute of Molecular Oncology, Milan, and obtained from the European Zebrafish Resource Center of the Karlsruhe Institute of Technology (KIT). The breeding stock was maintained in standard conditions (salt enriched, pH buffered, reverse osmosis purified water, 28°C temperature, 14:10 h light-dark cycle).

Experiments were carried on lots of 72 h post fertilization larvae (n=8/lot).

Chemicals

DMSO was purchased from Lach-Ner (Nertovice, Czech Republic). Pentoxifylline and tacrolimus were obtained pre-dissolved in DMSO from Selleckchem Europe (Munich, Germany). Solutions were prepared fresh in the day of the experiment.

Heat shock induction

Zebrafish larvae lots were incubated at 28°C for 24 h in 2 mL well plates with DMSO/standard water in concentrations of 0.05, 0.1, 0.5, 1, 1.5, and 2%. A negative control lot was incubated in standard water. Positive controls were exposed for 20 min to 35, 38 and 40°C, by immersion in sealed Eppendorf tubes in a heated water bath, followed by incubation at 28°C for 24 h.

Subsequently, new lots of 40°C exposed (as described above) larvae were incubated in standard water and 0.5% DMSO, respectively. Additional lots were incubated in 50 μM pentoxifylline and tacrolimus, dissolved in 0.5% DMSO/standard water solutions. Negative controls were incubated in 0.5% DMSO/standard water.

Fluorescence microscopy image acquisition and analysis

After incubation, zebrafish larvae under lidocaine anesthesia (0.3 mg/mL) were examined with a Zeiss AxioImager M1 HBO100 epifluorescence microscope with a FITC filter set (excitation: 488/20, dichroic: 509, emission: 530/25 nm). Images were obtained with a Jenoptik

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All authors have equal contributions to the study and the publication.

ProgRes CCD camera (Jena, Germany), 1.8 s exposure time, and imported as unprocessed .TIFF files using Isis FISH Imaging System v5.1 (Metasystems GmbH, Germany) imaging software.

Quantification of fluorescence intensity was realized in the FIJI distribution of ImageJ, by measuring mean pixel gray value on the green channel of a region of interest limited ventrally by the dorsal aspect of the abdominal cavity, caudally by a perpendicular line crossing the anogenital pore, cranially by a line through the caudal limit of the yolk sack and dorsally by the dorsal edge of the larva (fig. 1).

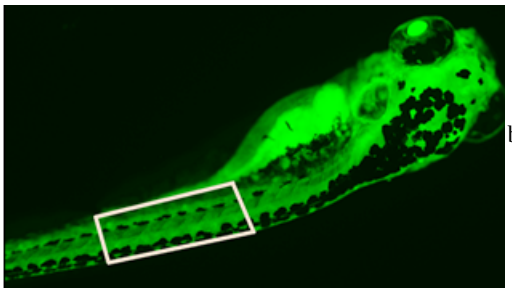

Fig. 1. Approximate borders of the region of interest

The region of interest was chosen to avoid measuring the physiologic HSR activation present in the yolk sack, gut and eye lens at 96 h post fertilisation. Background fluorescence was corrected by substracting the mean gray value of an equally sized empty region adjacent to the region of interest.

Data representation and statistic analysis

Data is presented as mean ± standard deviation, normalized to the mean value of negative control lots. Statistical significance was calculated with the Student t test in Microsoft Excel, with a p value of <0.05.

Results and discussions

DMSO has a mild HSR induction effect detectable by fluorescence microscopy

The water incubated negative control had a fluorescence intensity value of 100±36%. DMSO caused a dose dependent activation of the HSR. The lowest DMSO concentration that caused a significant (p=0.032) increase in fluorescence intensity, to 143±16%, was 0.1%. Incubation in 0.5% DMSO increased fluorescence intensity to 173±53%, significantly higher (p=0.035) compared with controls. Fluorescence intensity entered a plateau at 1%, 1.5 and 2% DMSO, with values of 229±69%, 240±22%, and 239±45%, respectively, with no significant difference between the three lots. Exposure to 35°C caused an increase of signal intensity to 194±98%, with no statistical significance due to high variability. Exposure to both 38 and 40°C caused a marked increase in fluorescence intensity (figs. 2 and 3), with values of 480±135% and

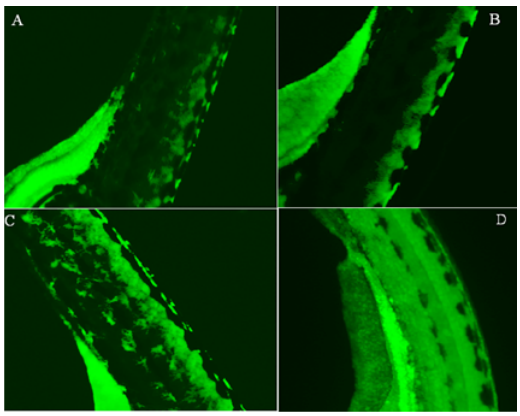

Fig. 2. Fluorescence microscopy images for negative controls (A), DMSO 0.5% (B), DMSO 2% (C), and 40°C exposed larvae (D)

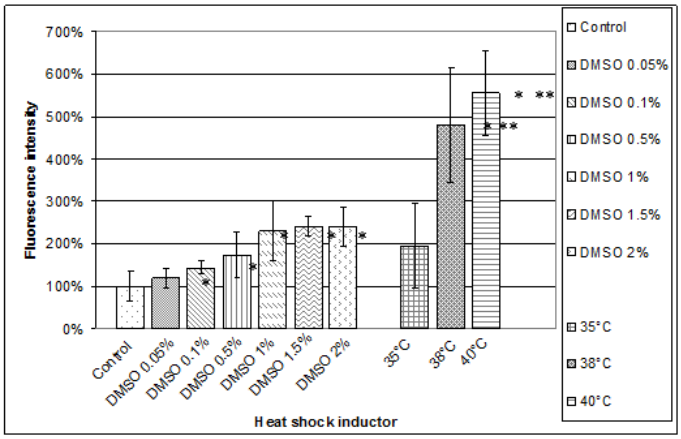

Fig. 3. Relative intensity of hsp70-EGFP expression, normalized to controls (naive larvae, incubated in water at 28°C). * p<0.05 compared with controls, ** p<0.05 compared with DMSO 2%.

554±99%, significantly higher than all other lots, but with no significant difference to each other (fig. 3).

0.5% DMSO and heat exposure have a synergistic HSR induction effect

In the second experiment, 0.5% DMSO negative controls had a fluorescence intensity value of 100±24%. Larvae exposed to 40°C and incubated in standard water had a fluorescence intensity of 345±60%, significantly higher compared with controls and significantly lower (p=0.0003) compared with the lot exposed to 40°C and incubated in 0.5% DMSO, which had a fluorescence intensity of 511±44% (fig. 4).

0.5% DMSO is suitable fo the detection of HSR induction by pentoxifylline and tacrolimus

Zebrafish larvae incubated in a 50µM pentoxifylline 0.5% DMSO solution had a fluorescence intensity of 145±42%, significantly higher (p=0.032) compared with

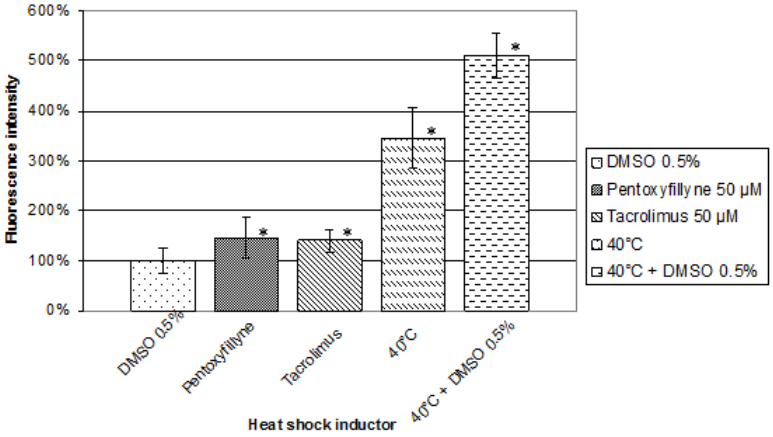

Fig. 4. Relative intensity of hsp70-EGFP expression, normalized to larvae incubated in 0.5% DMSO. * p<0.05 compared to controls

the 0.5% DMSO control. Incubation in a 50μM tacrolimus 0.5% DMSO solution resulted in a fluorescence intensity of 139±22%, significantly higher (p=0.004) compared with the 0.5% DMSO control (fig. 4).

DMSO is a polar aprotic solvent, widely used in zebrafish larvae based pharmacological screening assays. Several studies have used western blotting to detect significant increases in Hsp70 levels after DMSO exposure starting with concentrations of 0.01% [17-19].

Using computerized quantification of EGFP fluorescence in a specific region of interest of Hsp70I: EGFP_HRAS zebrafish larvae, we have detected a significant increase in Hsp70 expression after 24 h of exposure to DMSO in concentrations starting with 0.1%.

Differences in methodology include detection method (western blot versus fluorescence microscopy), zebrafish strains, DMSO exposure time (over 90 hours versus 24 hours), and sampling region (whole organism versus a paravertebral region of interest). Transgenic zebrafish strains using the Hsp70I promoter have been shown to present *eakiness*, an active baseline transcription in the absence of inductors [22], which can account for higher EGFP expression levels in negative controls. While zebrafish larvae are more sensitive to toxicants compared with early stage embryos [23], prolonged exposure starting with early stages has been shown to increase adaptative responses [24]. As the HSR initiates at 2-4 h after exposure [25] to the inductor and reaches its peak at 24 h [26], prolonged exposure to DMSO is not necessary in screening tests aiming to discover HSR inductors. Furthermore, DMSO has been shown to present organ specific toxicity [27, 28], which can manifest as an increase in Hsp when the entire organism is taken into account. Considering the HSR system is histologically ubiquitous [1, 22, 25], organ specific activation is relevant from a toxicological perspective, but less so when searching for general HSR inductors.

While high concentrations of DMSO (1-2%) have resulted in a significantly higher HSR compared with controls, exposure to 40°C, considered a strong inductor [25], elicited a robust HSR over twice as intense. After exposure to 35°C, a subliminal temperature for the induction of the HSR, there was no statistically significant difference to controls, consistent with examined literature [22, 25].

After exposure to a 40°C heat shock, incubation in 0.5% DMSO has resulted in a significantly higher fluorescent signal compared to incubation in standard water, suggesting a synergistic interaction between the two HSR inductors.

Incubation with moderate HSR inductors tacrolimus [20] or pentoxifylline [21] in concentrations of 50μM, resulted in a significantly higher HSR compared with 0.5% DMSO, showing that although 0.5% DMSO is a modest HSR inductor, its use as a solvent is suitable for the detection of moderate and strong HSR inductors.

Conclusions

HSR induction by DMSO is detectable in transgenic zebrafish by fluorescence microscopy signal intensity analysis of a paravertebral region of interest starting with concentrations of 0.1%. However, DMSO concentrations of up to 0.5% can be used in pharmacological screening assays for the detection of moderate and strong HSR inductors.

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Manuscript received: 6.07.2017