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Characterization of HSP90 and novel isoform-specific TRAP-1 inhibitors

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TABLE OF CONTENTS

INTRODUCTION

The heat shock response (HSR) is a conserved process that induces an increase of molecular chaperone expression such as the heat shock proteins (HSPs), in which heat shock factors (HSFs) play an essential role. HSR is mainly activated in response to cellular proteotoxic stress such as increase in temperature, oxidative stress, glucose depletion and the over-expression of misfolded proteins. However, these are not the only stressors that induce this complex mechanism, as small molecules such as HSP90 inhibitors, proteasome inhibitors, amino acid analogs, and ribosome biogenesis inhibitors also stimulate HSR (Kurop et al., 2021). When HSR is activated, different molecular chaperones and cell signaling pathways are expressed to prevent protein misfolding and aggregation, and enhance refolding of damaged proteins to promote cellular recovery from the stressful agent that caused damage.

HSPs are ATP-dependent molecular chaperones that stabilize and refold proteins or facilitate their degradation, minimizing the danger of aggregation in the protein-rich intracellular environment, and their expression is regulated by HSFs (Trepel et al., 2010). Upon stressful conditions, HSFs are hyperphosphorylated, oligomerized and translocated to the nucleus where they bind to heat shock elements (HSEs) as a trimer, upregulating and downregulating thousands of genes (Roos-Mattjus et al., 2021). The family of HSFs comprises six members in humans: HSF1, HSF2, HSF4, HSF5, HSFX and HSFY (Kmiecik et al., 2022). Among these transcription factors, HSF1 is considered to be the major activator of HSR and responsible for HSP expression under acute stress conditions, whereas HSF2 takes part in differentiation and development, particularly in the context of hemin-induced erythroid cell differentiation and spermatogenesis. Nevertheless, there is evidence suggesting that HSF1 and HSF2 can form heterotrimers, and therefore, that HSF2 could also be important in regulating molecular chaperone expression and induction of HSR (Smith et al., 2022). However, unlike HSF1, HSF2 has a limited role in promoting HSR, thus HSF1 knock out cells cannot activate HSR, meaning that HSF2 cannot compensate the loss of HSF1 (McMillan et al., 1998).

The ATP-dependent molecular chaperone, HSP90, is one of the most abundant and essential proteins in eukaryotic cells. Its full functional activity is gained when it attaches to co-chaperones and it dimerizes through its C terminal domain. In addition, this homodimer binds to a large repertoire of client proteins including kinases, phosphatases, growth factor receptors, and nuclear hormone receptors, stabilizing them (Pearl, 2016). Furthermore, HSP90 is involved in maintaining the mitochondrial membrane potential (Massimi et al., 2017). Each monomer contains three highly conserved domains: the amino-terminal domain (NTD), which mediates ATP binding; the middle domain (MD), important for ATP hydrolysis and binding to clients; and the carboxy-terminal domain (CTD), responsible for dimerization. During ATP deprivation, HSP90 adopts a V-shaped open conformation, whereas ATP presence enhances conformational changes leading to N-terminally closed state (Prodromou 2016).

In humans, HSP90 has two major cytoplasmic isoforms: HSP90α and HSP90β. The first is the main isoform and is induced by stress conditions, whereas the latter is a constitutively expressed isoform and more

abundant under physiological conditions (Biebl et al., 2019). As the isoforms have different amino acid sequences in certain areas, it is thought that they have isoform-specific functions and differential binding to client proteins. Moreover, humans have two more organelle-specific HSP90 isoforms, including GRP94 in the endoplasmic reticulum, and Tumor Necrosis Factor Receptor-Associated Protein-1 (TRAP-1) in the mitochondrial matrix (Sreedhar et al., 2004). This isoform is associated with mitochondrial integrity, oxidative cell death, organelle-compartmentalized protein folding, and transcriptional responses to proteotoxic stress (Altieri et al., 2012).

Initial preclinical observations in cancer suggested that HSP90 inhibitor therapy might respond favorably since HSP90 regulates oncogenesis by stabilizing the functional conformation of oncogenic signaling proteins (Massimi et al., 2017) which are essential for malignant transformation and progression (Smith et al., 2022). This way, several HSP90 inhibitors, targeting all isoforms, have been studied. Nevertheless, none of these have been approved for clinical use by FDA due to their side effects and toxicity (Pesonen et al., 2021). 17-AAG (17-allylamino-17- demethoxygeldanamycin, tanespimycin) is a "first-generation" N-terminal inhibitor of HSP90, which was synthetized using geldanamycin and radicicol as templates, the first isolated HSP90 inhibitors that bind to the N-terminal ATP binding pocket of HSP90 (McCollum et al., 2008). However, 17-AAG did not show clear clinical benefit, due to a level of unacceptable toxicity in clinical trials. For this reason, "second-generation" inhibitors were synthesized which showed greater efficacy and reduced toxicity than the first-generation (Li et al., 2021). Zelavespib (PU-H71) is a N-terminal purine-based inhibitor with potential antineoplastic activity which has been used in clinical trials against lymphoma and solid tumors (PubChem Compound Summary). Ganetespib (STA-9090) is a synthetic N-terminal small molecule inhibitor and it is under investigation for the treatment of several cancers, such as breast cancer (PubChem Compound Summary). HSP990 (NVP-HSP990) is a N-terminal inhibitor based on a 2-amino-4-methyl-7,8-dihydropyrido[4,3-d] pyrimidin-5(6H)-one scaffold (PubChem Compound Summary). These inhibitors act by inhibiting the activity of oncogenic proteins such as EGFR, N-Ras, Ki-Ras and c-Akt by resulting in their proteasome-mediated degradation (Massimini et al., 2017). However, most of these inhibitors induce HSR since HSP90 regulates the activity of HSF1, and this is counterproductive when treating cancer. Under physiological conditions, HSF1 monomers are kept inactive as they are attached to chaperones, such as HSP70 and HSP90. When the cell is exposed to proteotoxic stress or HSP90 inhibitors, HSF1 dissociate from the chaperone complex, undergo trimerization, is translocated to the nucleus and is activated to coordinate the expression of HSPs (Anckar et al., 2011).

OBJECTIVES

This thesis has two aims. First, to characterize non-isoform specific HSP90 inhibitors PU-H71, STA-9090 and HSP990 by analyzing U2OS (human osteosarcoma epithelial) cell survival with CCK8 assay and HSF1, HSF2, HSP70, β-tubulin and p53 expression, in a way to understand the inhibitory effect in U2OS cell viability and cytotoxicity, HSR induction and client protein stability. Related to this, we wanted to optimize CCK8 assay in U2OS for future applications.

Second, to characterize isoform-specific novel TRAP-1 inhibitors, which might be a promising alternative to minimize the toxicity of non-isoform-selective inhibitors. This will be done by analyzing U2OS wild-type (WT), HSF1 knockout (KO) and HSF2 KO cell viability and HSR induction in inhibitory conditions. Cells lacking HSF1 and HSF2 will allow us to understand these proteins' function and cytotoxic effect during their absence and to know if WT and KO cells are equally sensitive to TRAP-1 inhibitors. We hypothesized that TRAP-1inhibitors should not induce HSR as they do not target all HSP90 isoforms. At the same time, we hypothesized that KO cells would be more sensitive than WT cells to TRAP-1 inhibitors, being HSF1 KO cells the most sensitive ones, as seen in Bagatell et al., 2000 and Joutsen et al., 2020 and because HSF1 is the major regulator of the cytoprotective HSR.

MATERIALS AND METHODS

CELL CULTURE AND EXPERIMENTAL TREATMENTS

U2OS cells cultured in the following conditions: they were maintained at 37 °C in humidified 5% CO_2 atmosphere and grown in Dulbecco's modified eagle's medium (D6171, Sigma-Aldrich) which was complemented with 100 U/mL penicillin-100 µg/mL streptomycin mixture, 200 mM L-Glutamine and 10% Fetal Bovine Serum (F7524, Sigma-Aldrich). Same conditions were used for WT, HSF1 KO or HSF2 KO cells. Cell line was obtained from Valerie Mezqer lab, Université de Paris, CNRS, France. KO cells were generated with CRISPR-Cas9 (Joutsen et al., 2020; Pesonen et al., 2021).

Heat shock treatments were conducted on cell dishes wrapped in Parafilm and submerged in a water bath at 42 °C for 1 hour, and for the recovery phase, after the immersion, the Parafilm was taken out and cells were kept in the incubator at 37 °C for 3 hours. To inhibit HSP90, cells were treated with N-terminal inhibitors, 17-AAG, Zelavespib (PU-H71), Ganetespib (STA-9090) and HSP990 (NVP-HSP990), and to inhibit TRAP-1, four N-terminal inhibitors (C1, C2, C3 and C4) were used. The inhibitors were diluted in DMSO (Dimethyl sulfoxide) before treating the cells with the concentrations described in the figure legends. Negative controls were treated with DMSO. TRAP-1 inhibitors were obtained from professor Brian Blagg, University of Notre Dame, Kentucky, USA.

CELL SURVIVAL ASSAY

A CCK8 (Cell Counting Kit-8, Dojindo) assay was performed for the determination of the number of viable cells. The CCK8 assay is a sensitive colorimetric assay that uses WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble orange colored product formazan dye upon reduction by cellular dehydrogenases in the presence of an electron mediator (1-Methoxy PMS). The amount of produced formazan is directly proportional to the number of viable cells.

After optimization, cells were seeded and cultured at a density of 7000 U2OS cells/well in 150 µL of medium into 96-well microplates. Next day, the cells were treated with indicated concentrations of the

inhibitors, as described in the figure legends, by doing serial dilutions. Treatment time for HSP90 inhibitors was 24 h or 48 h, but it was later optimized for TRAP-1 inhibitors, where just 48 h was used. Each sample was in triplicate.

After inhibitor treatment, a mixture of 6 µL CCK8 reagent and 100 µL medium per well was added and then incubated for 2 h, 3 h or 4 h in dark, before measuring the absorbance at 450 nm using a microplate reader (Hidex). Outliers were removed and mean absorbance was measured for each treatment. Corrected absorbance was calculated by subtracting the blank absorbance. An empty well with only WST-8 was used as blank.

Relative cell survival was determined by dividing absorbance values of inhibitors treated cells by cells treated with DMSO. The mean relative cell survival percentages of the biological repeats were displayed in graphs (n= 1-3), as determined in each figure legend. This way, CCK8 was used for the determination of cell viability in cell proliferation.

IMAGE ACQUISITION

A density of 125 000 cells/well were plated in a 12-well-plate, and next day, incubated in the previously given conditions for 48 h with HSP90 inhibitors at specific concentrations, as mentioned in the figure legend. Before immunoblot analysis, digital images of these cell samples were taken in order to evaluate the cytotoxicity of cells and to evidence that cells were dying after treatment. Digital images were taken with 10X magnification objective in bright field inverted microscope (Leica) and scale bar was added with ImageJ Java image processing.

IMMUNOBLOT ANALYSIS

Cells were lysed in Laemmli buffer (30% glycerol, 3% SDS, 187.5 mM Tris-HCl pH 6.8, 0.015% bromophenol blue, 3% β-mercaptoethanol). Cells were scraped and suspended in appropriate amount of 3×Laemmli buffer after the washing with cold PBS (Phosphate-buffered saline), and boiled for 10 minutes.

Proteins were separated by resolving cell lysates on an 8% SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis), and then, they were transferred to a 0.45 µm pore size nitrocellulose membrane (Protran). Transfer success was evaluated using Ponceau S Solution (Sigma). After, the membranes were boiled for 10 minutes in Milli-Q water inside a folded piece of Whatman paper (Cytiva) and blocked with 5% skimmed milk powder in 1×PBS-0.3% Tween for 30 minutes.

The primary antibodies were diluted in PBS containing 0.5% BSA and 0.02% NaN_3. The membranes were incubated with the primary antibodies overnight at 4 °C, and then, the membranes were incubated at least 1 h at RT in the secondary antibodies conjugated to horseradish peroxidase and diluted in 1×PBS-0.3% Tween+0.5% skimmed milk powder (*Table 1*). Before the incubation with the primary antibodies and the secondary antibodies, membranes were washed 3 × 10 minutes with 1×PBS-0.3% Tween.

After antibody incubation steps, band intensity was detected via ECL (Enhanced chemiluminescence, PerkinElmer) solution 1:1, imaged with The Invitrogen iBright imaging systems and relative HSP70+β-tubulin, p53 and HSF2 levels were quantified with ImageJ (n= 2-4). The previously described washing steps were done before and after incubation with ECL. Relative proteins levels were calculated by dividing the protein signal with tubulin signal in each well, and then, by normalizing the ratios with DMSO. Samples treated with 17-AAG were used as positive control.

Table 1. Concentrations of primary and secondary antibodies used in Western Blot. Primary antibodies were diluted in 1×PBS + 0.5% BSA + 0.02% NaN_3. Secondary antibodies were conjugated to horseradish peroxidase and diluted in 1× PBS-0.3% Tween+0.5% skimmed milk powder.

Primary antibody	Concentration	Secondary antibody	Concentration
Anti-HSF1 (ADI-SPA-901, Enzo Life Sciences)	1:5000	Anti-rabbit-HRP (Promega)	1:20 000
Anti-HSF2 (HPA031455, Sigma-Aldrich®)	1:1000	Anti-rabbit-HRP (Promega)	1:5000
Anti-HSP70 (ADI-SPA-810, Enzo Life Sciences)	1:1000	Anti-mouse-HRP (GE Healthcare/Amersham)	1:10 000
Anti-p53 (DO-1, Invitrogen)	1:500	Anti-mouse-HRP (GE Healthcare/Amersham)	1:20 000
Anti-β-Tubulin (Clone AA2, T8328, Sigma-Aldrich®)	1:1000	Anti-mouse-HRP (GE Healthcare/Amersham)	1:10 000

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism 7 Software (GraphPad Software by Dotmatics, https://www.graphpad.com/). The data for relative HSP70 levels and relative p53 levels were analyzed using non-parametric one-way ANOVA and corrected with Dunn's multiple comparisons test, relative survival of U2OS-WT after treatment with HSP90 inhibitors was analyzed with parametric one-way ANOVA and corrected with Dunnett's multiple comparisons test and relative survival of U2OS-WT, HSF1 KO and HSF2 after treatment with TRAP-1 inhibitors with two-way ANOVA and corrected with Tukey's multiple comparisons test. The significance level was set to *P* values less than 0.05. Mean+SEM is shown in the figures.

RESULTS

HSP90 inhibitors reduce U2OS-WT cell viability and induce cytotoxicity

17-AAG has been thoroughly studied in cancer, and it is known that this HSP90 inhibitor is a potent inducer of apoptosis in some cell lines through the mitochondrial membrane depolarization (Massimini et al., 2017). To assess whether PU-H71, STA-9090 and HSP990 affects U2OS-WT cell viability at increasing concentrations, cell viability assay was performed with CCK8 reagent. As positive control for relative cell survival, we used 17-AAG.

Between all concentrations that were used in the experiment, the first CCK8 experiments were done with the highest ones (17-AAG: 1 µM, 2.5 µM, 5 µM; PU-H71: 1 µM, 5 µM; STA-9090: 0,5 µM, 1 µM; HSP990: 0.1 µM, 0.5 µM), and then, as high toxicity was observed, same experiment was performed with lower concentrations (17-AAG: 0.5 µM; PU-H71: 0.5 µM, 2 µM; STA-9090: 0,1 µM, 0.25 µM; HSP990: 0.05 µM, 0.075 µM). Absorbance was measured after 2 h (*data not shown*), and 3 h, with which statistical analysis was performed.

After 24 hours of treatment, there is no statistically significant decrease in cell survival, indeed, percentages of viable cells remain at around 80%. However, 17-AAG, PU-H71, STA-9090 and HSP990 treatments reduced cell survival after 48 hours of treatment with statistical significance. Cell viability decreases logarithmically (R^2>0.9) when concentration for each inhibitor increases (*Fig. 1*).

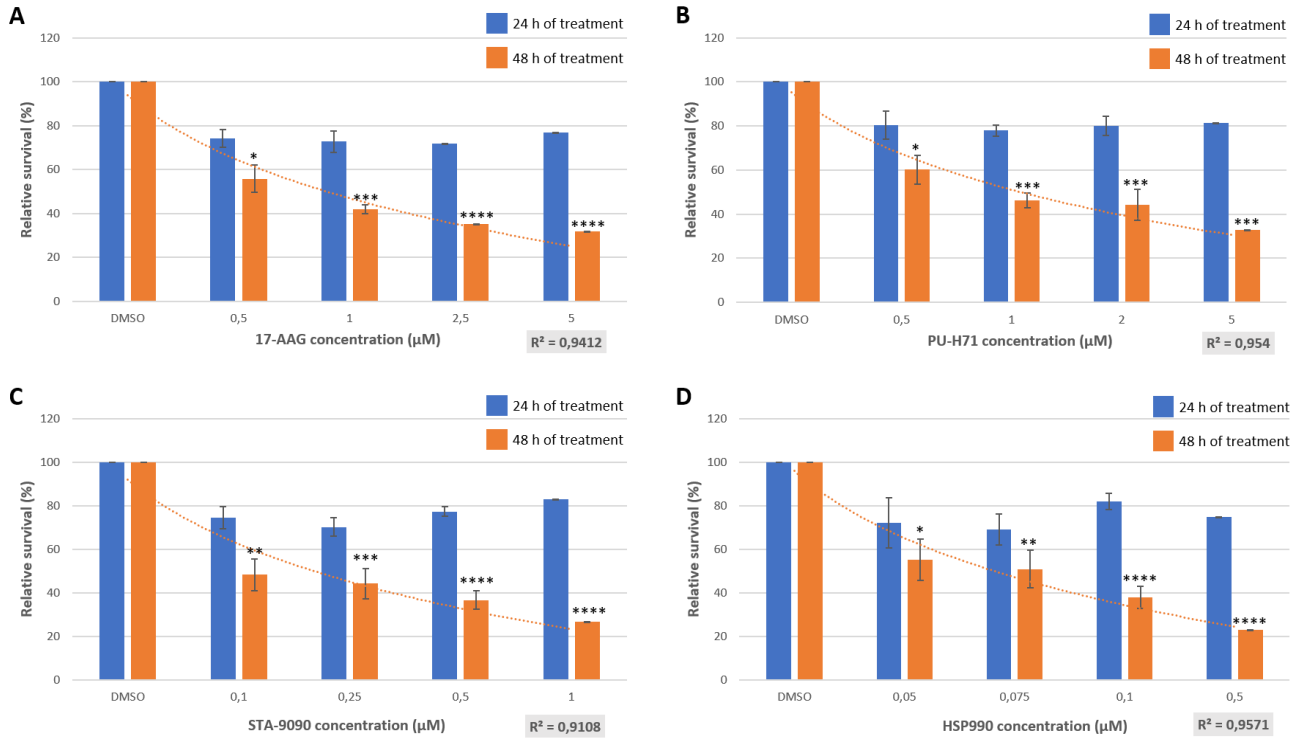


Fig. 1. Treatments with HSP90 inhibitors reduce cell viability in U2OS-WT cells. After plating 7000 cells in a 96-well plate and treating them with 17-AAG **(A)**, PU-H71 **(B)**, STA-9090 **(C)** and HSP990 **(D)** in shown concentrations for 24 h or 48 h, CCK8 reagent was added and absorbance was measured at 450 nm after 3 h of incubation with CCK8 reagent. Logarithmic trendline is shown in all graphs which their R^2 values. One way ANOVA, each treatment against control (DMSO), mean ± SEM shown. * $p \leq 0,1$; ** $p \leq 0,01$; *** $p \leq 0,001$; **** $p \leq 0,0001$. n= 1 for 17-AAG: 2.5 µM, 5 µM; PU-H71: 5 µM; STA-9090: 1 µM and HSP990: 0.5 µM. n= 3 for 17-AAG: 0.5 µM, PU-H71: 0.5 µM, 2 µM; STA-9090: 0.1 µM, 0.25 µM and HSP990: 0.05 µM, 0.075 µM. n= 4 for 17-AAG: 1 µM; PU-H71: 1 µM; STA-9090: 0.5 µM and HSP990: 0.1 µM.

Moreover, images of cells were taken to demonstrate whether HSP90 inhibitors induce cell cytotoxicity. We can observe that cells under treatment at specific concentrations, as specified in the figure legend, round up and become less confluent proving the cytotoxic effect of these compounds in comparison to control sample (*Fig. 2*). This effect is enhanced at increasing concentrations.

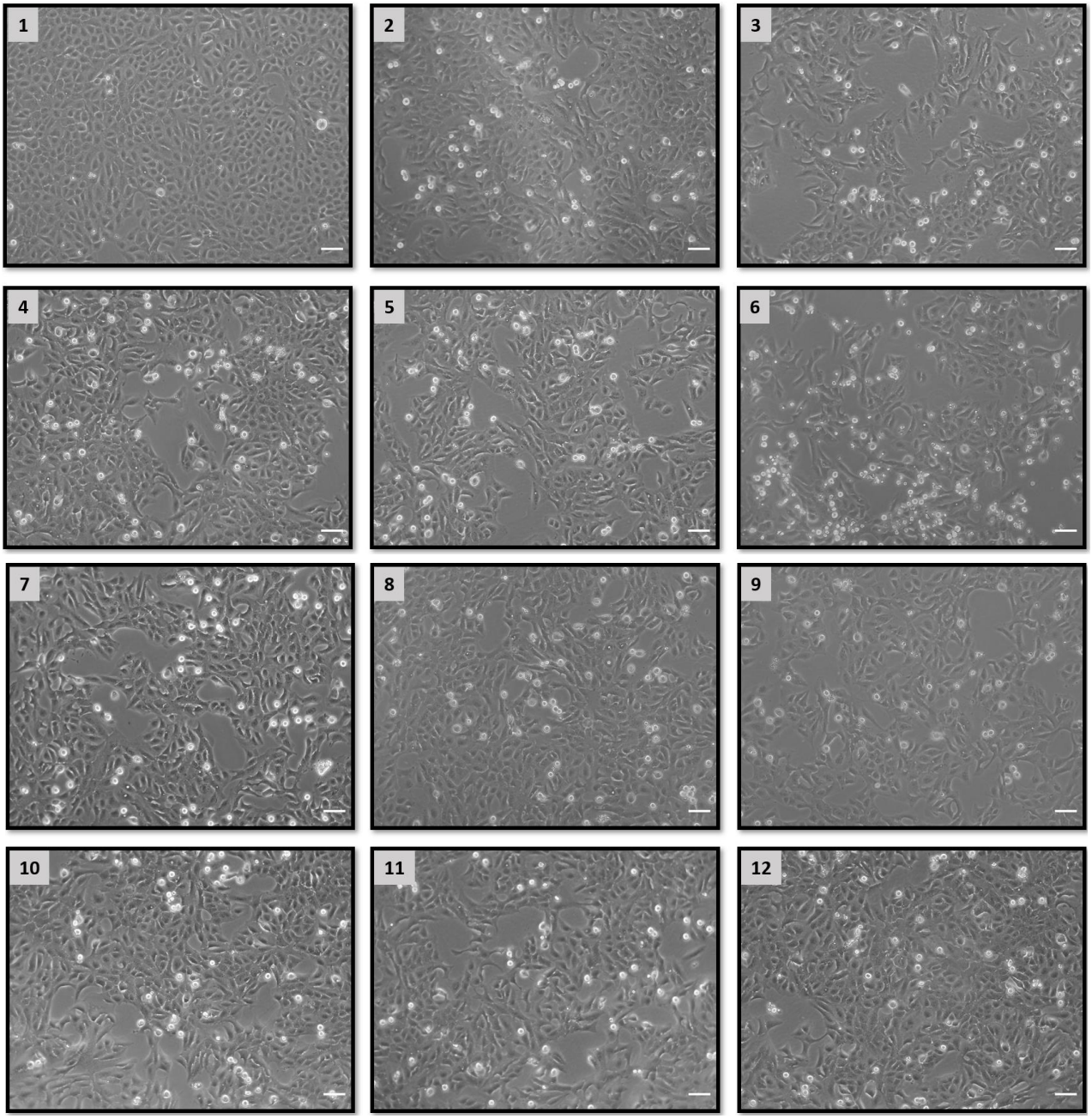


Fig. 2. HSP90 inhibitors have cytotoxic effects in U2OS-WT cells. Microscopical images of U2OS-WT cells under HSP90 inhibitor treatment at increasing concentrations were captured with an inverted bright field microscope. 10X magnification objective. **1:** DMSO (control), **2:** 17-AAG 0.5 µM, **3:** 17-AAG 1 µM, **4:** PU-H71 0.5 µM, **5:** PU-H71 1 µM, **6:** PU-H71 2 µM, **7:** STA-9090 0.1 µM, **8:** STA-9090 0.25 µM, **9:** STA-9090 0.5 µM, **10:** HSP990 0.05 µM, **11:** HSP990 0.075 µM, **12:** HSP990 0.1 µM. Grayscale edited with PowerPoint. Scale bar: 80 µm.

HSP90 inhibitors induce HSR

It was already shown that 17-AAG binds to HSP90 and inhibits its chaperoning function, therefore resulting in the degradation of HSP90's client proteins. This induces HSR, which is cytoprotective for cancer cells and therefore a problematic feature for an anticancer drug (Bagatell et al., 2000). This way, in order to test the HSP90 inhibitors, we determined whether they induce an HSR by treating U2OS-WT cells for 48 hours with PU-H71, STA-9090 and HSP990 (*Fig. 3a*).

When HSR is induced, HSF1 is hyperphosphorylated, trimerized, and consecutively, translocated to the nucleus. As a result, chaperones, such as HSP90 or HSP70, are synthesized (Prodromou, 2016). Therefore, hyperphosphorylation of HSF1 and upregulation of HSP70 protein levels were used as reference for the activation of HSR. As positive control for HSF1 hyperphosphorylation, 1 h heat shock (HS) treatment at 42 °C and for HSP70 upregulation HS + 3 h recovery (H + R) were used. However, although HSF1 hyperphosphorylation has also been used as a proxy before (Pesonen et al., 2021), since incubation time with inhibitors is 48 h and phosphorylation of residues do not last long (Salazar et al., 2005), there is no hyperphosphorylation of our samples. 17-AAG was also added as a positive control, since it is already known that it activates HSF1 and therefore HSR (Bagatell et al., 2000). In addition, β-tubulin was used as loading control.

After 48 h of incubation with inhibitors, we can see increased relative levels of HSP70, even more than 100 times than in HS, indeed all the treated samples have statistically significant increase in HSP70 signal level (*Fig. 3b*). In addition, client protein stability was assessed by checking p53 client protein expression under inhibitory conditions. Its levels changed with treatments. The overall signals of the treatments are higher than the control, but they decrease when inhibitor concentrations increase. On the other hand, quantification of HSF2 expression was done *(data not shown; n= 1)* and there is an increase of its expression after HSP90 inhibitor treatment, which increased at higher concentrations. Statistical analysis could not be done due to small number of samples. In addition, several bands of HSF2 could be seen.

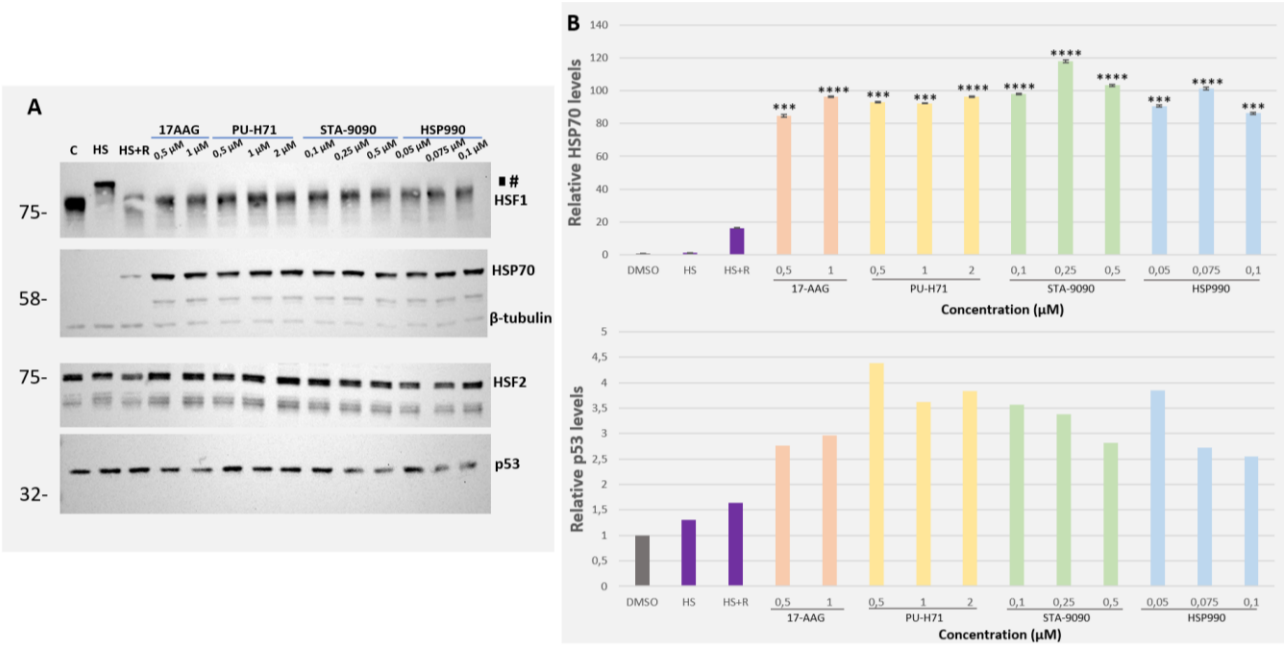


Fig. 3. Treatment with HSP90 inhibitors induce HSR. A) Immunoblot analysis of HSF1, HSP70, HSF2 and p53 expression. U2OS-WT cells were treated with DMSO (control), heat shock (HS, 42 °C, 1 h), 1 h HS with 3 h recovery at 37 °C (H+R), and 17-AAG, PU-H71, STA-9090 and HSP990 at different concentrations for 48 h. The HSF1 upper shift induced by HS (labeled with #) correspond to hyperphosphorylated HSF1 form. β-tubulin was used as a loading control. **B)** The amount of relative HSP70 protein related to β-tubulin (n= 4) and p53 (n= 2) were quantified with ImageJ. One way ANOVA, each treatment against control (DMSO), mean ± SEM shown. * $p \leq 0,1$; ** $p \leq 0,01$; *** $p \leq 0,001$; **** $p \leq 0,0001$.

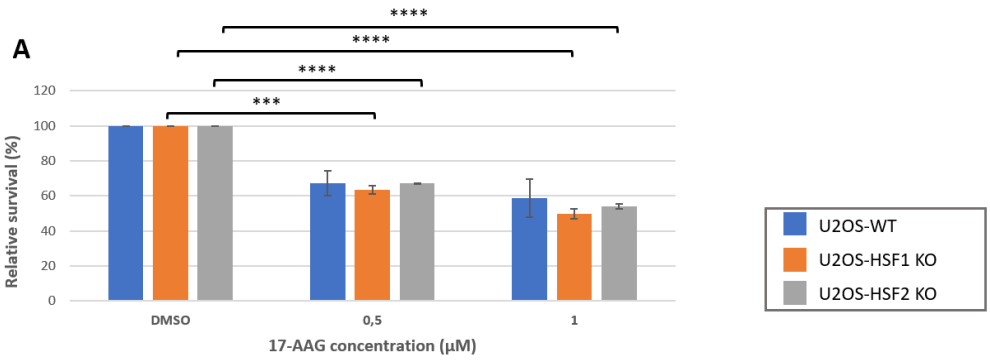
C1 and C2 TRAP-1 inhibitors reduce cell viability

As mentioned before, although some HSP90 inhibitors have entered in clinical trials, their advantages were limited because of their high toxicity and side effects in patients since HSP90 inhibitors inhibit all the HSP90 isoforms and impact the cellular environment (Neckers et al., 2018). Therefore, therapy based on the subcellular compartmentalization of HSP90, such as the mitochondrial isoform TRAP-1, is an attractive option to avoid those adverse events. Hence, TRAP-1 inhibitors might be a beneficial therapeutic target for cancer therapy (Lettini et al., 2017). To study whether TRAP-1 inhibitors reduce cell viability as HSP90 inhibitors, we performed cell viability assay with CCK8 reagent. At the same time, we wanted to demonstrate if there were differences between wild-type, HSF1 KO and HSF2 KO cells, since it is already known that KO cells are more sensitive to HSP90 inhibitors, being HSF1 KO the most sensitive (Bagatell et al., 2000; Joutsen et al., 2020).

Cells were treated with C1, C2, C3 and C4 TRAP-1 inhibitors, which had not been studied before, for 48 h, and afterwards, CCK8 reagent was added for 3 h and 4 h before measuring the absorbance. The results were obtained by measuring the mean between 3 h and 4 h. Statistical analysis was also performed with the mean value.

17-AAG was used as positive control, since it is known to reduce cell viability and there is a differential effect between WT and KO cells. HSF1 KO cells are the most sensitive to 17-AAG, but there is not statistically significant difference between WT and KO cells nor HSF1 KO and HSF2 KO cells. However, there is significant difference in the decrease of cell viability for both KO cells treated with 0.5 µM and 1 µM 17-AAG against the control (*Fig. 4a*).

When the same experiment was done with TRAP-1 inhibitors, it was seen that C1 and C2 reduced cell viability; for C1 the decrease was statistically significant for 5 µM in HSF2 KO and 10 µM in HSF1 KO against control; for C2 it was just in 10 µM in HSF2 KO against control. Suprisingly, they did not follow what it has been shown with 17-AAG, since WT cells were more sensitive than KO cell lines, having significant differences between WT and HSF2 KO (*Fig. 4b, c*). No decrease in cell viability was seen with C3 and C4; relative cell survival remains over 90% in all cell types (*Fig. 4d, e*).



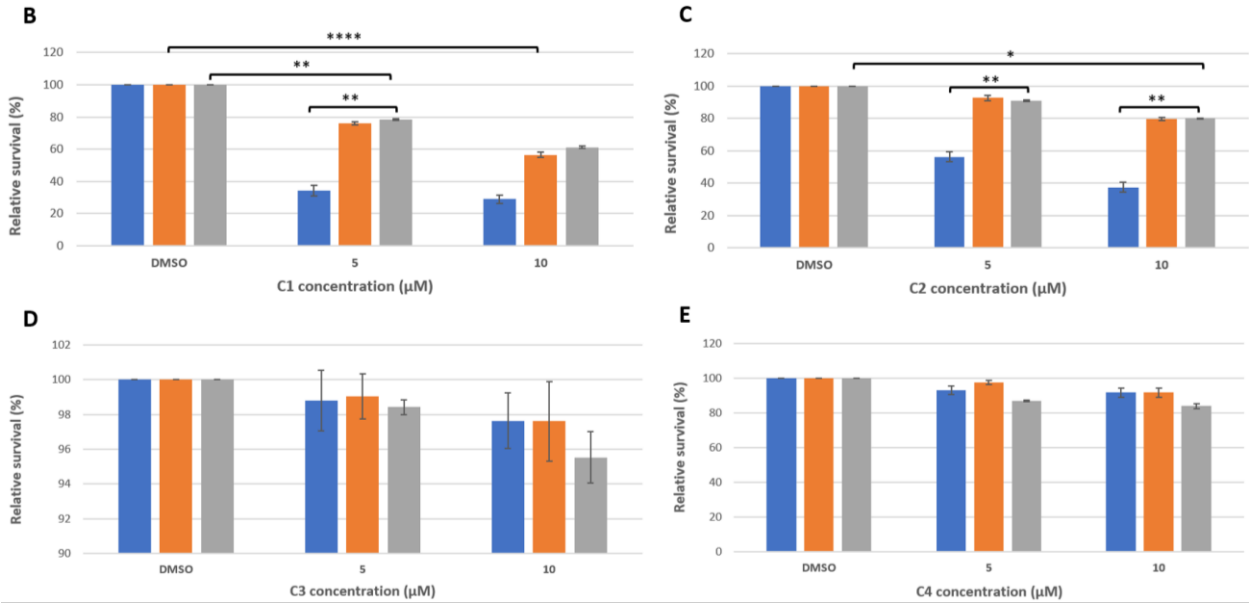


Fig. 4. TRAP-1 inhibitors C1 and C2 reduce cell viability of U2OS cells and there are differences between WT and KO cell lines. After plating 7000 cells in a 96-well plate and treating them with 17-AAG **(A)**, C1 **(B)**, C2 **(C)**, C3 **(D)** and C4 **(E)** in shown concentrations for 48 h, CCK8 reagent was added and absorbance was measured at 450 nm after 3 h and 4 h. Values correspond to the mean between 3 h and 4 h values. Two-way ANOVA, treatments against control (DMSO) and WT against KO cells, mean ± SEM shown. * $p \leq 0,1$; ** $p \leq 0,01$; *** $p \leq 0,001$; **** $p \leq 0,0001$. (n= 3).

DISCUSSION

In the progression of malignant transformation that leads to clinical cancer, targeting HSP90 has been seen as a beneficial treatment, since tumor cells depend on many proteins that at the same time are stabilized by this chaperone. Indeed, HSP90 is expressed at higher levels in many cancer types and enhances tumor growth and metastasis, as this chaperone can buffer cancer cells from environmental stressors by promoting stability to several pathways in the Hallmarks of cancer. Hallmarks include angiogenesis, invasion, evasion of growth suppressors, promotion of proliferative signaling and inactivation of pro apoptotic signals (Pillai et al., 2012). However, many inhibitors tested to this day induce HSR, stimulating the expression of chaperones that further stabilize the proteome and promote cellular protein homeostasis (also termed proteostasis) which is cytoprotective during cancer treatments (Pesonen et al., 2021; Roos-Mattjus et al., 2021).

The earlier inhibitors (i.e., 17-AAG) were not successful, and this clinical failure is believed to be due to their poor pharmaceutical properties, selectivity and toxicity profiles in patients (Ramanathan et al., 2007). In this thesis, we demonstrated with CCK8 assay that novel N-terminal HSP90 inhibitors PU-H71, STA-9090 and HSP990 reduce cell viability of U2OS-WT cells. After 2 h, 3 h and 4 h of incubation with CCK8 reagent, obtained absorbance values were compared *(data not shown*) and concluded that 3 h and 4 h of incubation resulted in more accurate values, since those values start to reach a plateau.

Cell viability refers to the percentage of live cells within a population in the culture, and it is considered an indirect quality measure in optimization problems, this is why it should be kept between a critical value (Kappatou et al., 2019). A reduction of 30-50% in cell viability was our threshold to obtain

when we were trying to set the optimal concentrations and treatment timings, since facing a cell viability over 80% could be due to the variability of the experiments. For instance, it is not possible to plate exactly 7000 cells per well every time or when plating some cells can die, and indeed, having different amounts of cells can totally change the results in CCK8.

The reduction seemed to be optimal after 48 h of incubation with such inhibitors, since after 24 h cell viability remained over 80%. We suggest that in 24 h, cells might be in an irreversible cell cycle arrest in response to a stress situation, meaning that cells are not actively dividing, but without leading to the possible apoptosis yet. This is because CCK8 reagent reacts with not only healthy cells but also viable cells that are metabolically active, as senescent cells, even if this may eventually result in apoptosis (Malavolta et al., 2018). Moreover, if we consider that eukaryotic cells divide approximately every 24-30 hours, after 24 h of treatment, control cells will not have completed the whole cell cycle. This means that if control cells are not doubling their number and treated cells are not dying, cell viability will remain stable (Cooper et al., 2000). This conclusion led us to optimize the incubation time for next CCK8 assays with U2OS cells. In addition, concentrations of HSP90 inhibitors were optimized after trial with lower concentrations, those that resulted in cell viability around 50-60%. This way, it is possible correlate cell behavior to cell number in response to the treatments, providing a more accurate picture of the cellular activity, metabolism and assessment of disrupted pathways and structures.

In addition, we demonstrate that the increment in inhibitors concentration resulted in a logarithmical decrease of cell viability, and it is expected that if concentration continues increasing a plateau will be reached. Therefore, HSP90 inhibitors induce a dose-dependent reduction of cell viability.

With cell viability decrease, one can not assume that apoptosis of U2OS cells is happening, since the reason for this decline could be another one, necrosis for example. However, it has been seen that PU-H71 induces apoptosis in triple negative breast cancers (TNBC) through the mitochondrial pathway by efficient inactivation and downregulation of proteins related with the Ras/Raf/MAPK pathway and G (2)-M phase of the cell cycle (Caldas-Lopes et al., 2009), STA-9090 induces apoptosis of a wide variety of human cancer cell lines (Ying et al., 2012), and HSP9090 elevates apoptosis related molecules (Menezes et al., 2012). Therefore, apoptosis could be a potential reason for the decrease of cell viability in U2OS cells, although we cannot assume this is certainly happening. To do so, we would need specific apoptosis markers or perform different analysis to show indicative characteristics of cells undergoing apoptosis.

Second, we demonstrate that the administered concentrations of PU-H71, STA-9090 and HSP990 have cytotoxic effects and induce HSR in U2OS-WT cells. We can see that in 1 hour there is an induction and expression of phosphorylated HSF1 in cells that have gone under HS treatment. However, when we let the cells rest for 3 h, phosphorylated HSF1 expression decreases, reaching the same expression as in the control. This explains why we do not see phosphorylated HSF1 in treated samples. These cells have been incubating together with the inhibitors for 48 h, so any phosphorylation that could have happened is no

longer available, since the post-translational modifications (PTM) of already synthesized proteins are constantly changing, which makes sense if we understand signaling pathways as a network that are constantly regulated to reach homeostasis (Salazar et al., 2005; Anckar et al., 2011).

Changes in transcriptional level of a protein, on the other hand, are considered long-term modulation, for the reason that upregulation or downregulation of proteins include the action of the whole transcriptional and translational machinery, and this is why we cannot see HSP70 expression in HS sample, which was taken after 1 h exposed to the stress. However, if we measure mRNA levels for HSP70, they should be high (Nelson et al., 2017). Nevertheless, both HS+R and treated samples show high relative levels of HSP70 expression, and this is because their transcription and translocation machinery had time to synthesize the protein. HSP70, which is expressed due to the HSF1 translocation to the nucleus and activation of its transcription, is an indicator of HSR activation (Anckar et al., 2011). Tubulin expression is decreasing in treated cells, although we cannot assume that cells are dying, but since at the same time HSP70 is overexpressed, we can assume that treated cells have a strong HSR. Make note that cells treated with 17-AAG show a lower relative level of HSP70 than STA-9090 which indicates that 17-AAG induces a weaker HSR. This data can be supported by the fact that previously it has been seen that geldanamycin based inhibitors, as 17-AAG, exhibit a lower potency than triazolone class of HSP90 inhibitors, as STA-9090, since they saw that 50% inhibitory concentrations (IC_{50}) for STA-9090 against malignant cell lines were 10-50 times lower than for 17-AAG (McCleese et al., 2009). Moreover, it needs to be considered that equal proteins amounts were not loaded in the wells. When only boiled in 3 × Laemmli, we would need to lyse the cell pellet and measure protein concentration to get equal number of proteins.

On the other hand, the decrease in p53 levels in a dose-dependent manner demonstrated that inhibition of HSP90 promotes instability and eventually degradation of client proteins. It is known that U2OS cells have a WT p53 (Menendez et al., 2013), so this ensures that HSP90 inhibition is the factor that promotes the degradation of p53, due to the instability of the protein that the inhibition of HSP90 supposse. However, at the same time, we see that these levels are higher levels than in the control sample. It could be because when we are inhibiting HSP90, it also results in proteasomal inhibition (Joutsen et al., 2020), blocking p53 proteasomal degradation and promoting its accumulation. Then, since p53 can be also degraded by proteasome-independent pathway (Nuaaman et al., 2013), the instability of p53 supported by HSP90 inhibition could lead to increase the degradation by this non proteasomal way.

Regarding HSF2 expression, we demonstrated that HSF2 levels are increasing due to HSP90 inhibitors treatment. We cannot really give an explanation for this since there is not much information available in the literature and most studies are limited to its role in the development. However, Santopolo et al., 2021 also saw that HSF2 expression increase in a HSF1 dependent manner upon exposure to prolonged proteotoxicity, proteasomal inhibition by bortezomib in U2OS cells, showing that HSF2 is absolutely essential for cell survival under these conditions. Hence, they concluded that HSF2 is needed to protect cells

against accumulation of unstable proteins. Since HSP90 inhibition also results in proteasomal inhibition due to the instability of HSP90 client proteins that participate in this protein-degradation machinery (Quadroni et al., 2015), our hypothesis is that HSF2 accumulates because the degradation rate is lower than synthesis rate. However, our experiment was done in one single biological repeat, so our results are not enough to do statistical analysis and accept this tendency. Moreover, trials with KO cells should be done to fully understand the trend.

In addition, different bands of HSF2 can be seen at 75 kDa marker, which correspond to the different isoforms of HSF2, HSF2-α and HSF2-β (Park et al., 2015). Those bands that can be seen below 75 kDa may be different forms of the HSF2 protein, such as protein cleavage or degradation.

Although this data is not shown in this thesis, it is considerably remarkable that in HSF1 KO cells, there is not HSP70 expression nor HSF1 phosphorylation. Hence, there is no HSR induction when HSF1 is not present. On the other hand, in HSF2 KO, HSP70 is still expressed, so this means that the cell is not dependent on HSF2 for HSR induction. However, the signal is weaker than in WT, which means that somehow HSF2 takes part in the pathway for HSR induction, and cells are more sensitive without HSF2 (Ostling et al., 2007).

As mentioned, theoretically we should do the biological assays 3 times to study and be able to accept the tendency. However, in some cases just 1 or 2 biological repeats were performed as stated previously. All the required biological repeats were not done due to lack of time. Hence, lab mates continued with this research, including experiments with KO cells, and results will be shown in their thesis.

Finally, we also elucidated several conclusions with TRAP-1 inhibitors trials. First, after optimization experiments with HSP90 inhibitors, we took the liberty to discard the 24 hours incubation experiment, and we directly incubated the cells with the inhibitors for 48 hours. We concluded that C3 and C4 inhibitors do not have any effect in U2OS cell viability in the performed concentrations for both WT and KO cells, since relative cell survival remained over 90%. However, it was seen that HSR was induced because HSP70 levels were increased when these compounds were used in WT (*data not shown*), although the signal was lower than for that obtained in 17-AAG. This could mean that C3 and C4 inhibit enough TRAP-1 molecules to promote a HSR, but their toxicity might not be strong enough to kill cells and neither for promoting a strong HSR as in first-generation HSP90 inhibitor.

On the other hand, C1 and C2 inhibitors gave results that cannot yet be explained due to the lack of information about them in the literature. In this case, we cannot see what it has been seen with HSP90 inhibitors: cells lacking HSF1 are more sensitive than WT cells in response to the proteotoxic stress induced by HSP90 inhibitors (Bagatell et al., 2000), and then, it has recently been demonstrated that, HSF2 KO cells are also more sensitive than WT cells (Joutsen et al., 2020). In this case, cell viability for WT is much lower than that for KO cells in all used concentrations. Even the relative survival for KO cells remains in some

cases over 70% and the difference between WT and KO is statistically significant. This means that the KO gives somehow an advantage to overcome the TRAP-1 inhibition. If we start to speculate, we could think that since TRAP-1 is related to protection against mitochondrial apoptosis by reducing oxidative stress (Ramos Rego et al., 2021), maybe these compounds do not have the same efficiency in KO cells, and therefore, TRAP-1 concentrations might remain higher than in WT, this way, protecting the cell from apoptosis. Another reason for higher TRAP-1 levels in KO cells may be that when cells are lacking HSF1 or HSF2 there is a change in their metabolism promoting the synthesis of TRAP-1, which is a cytoprotective protein. Qiao et al., 2017 observed that HSF1 deficiency led to decreased mitochondrial number, but at the same time, mitochondria were rounder and bigger in the livers of HSF1-deficient mice. Therefore, these damaged mitochondria may accumulate more TRAP-1 for protective purposes. Or it might be that already the basal levels of TRAP-1 are different for both types of cells. Or it could be that the inhibitors are more efficient in KO cells and these cells compensate this loss by increased mitochondrial accumulation of cytoprotective chaperones, protecting the cell.

Inhibition of HSP90 chaperones could be essential for cancer treatment. However, as mentioned, although many inhibitors have been synthesized and evaluated, none has been approved by FDA due to their toxic side effects. Isoform-specific inhibitors warn us that further study is needed in the future, as their use may be the solution to avoid the toxicity of the pan-inhibition. Moreover, other HSP90 isoforms should be considered when targeting their inhibition. Further trials should be done with HSF1 and HSF2 KO cell lines to fully understand their implication in HSR. In addition, in this thesis N-terminal inhibitors have only been mentioned, but trials with C-terminal inhibitors are ongoing and seem to be a promising class since they do not induce a HSR (Eskew et al., 2011). At the same time, this therapy should be considered to be combined with other targeted therapies, such as monoclonal antibodies or angiogenesis inhibitors.

CONCLUSION

HSP90 family chaperones are required for adaptation to stress conditions through HSR, as it takes part in the folding, activation, assembly, and therefore, stabilization of target proteins, better known as client proteins. Between them, we have a wide range of oncoproteins essential for malignant transformation of cells. Application of drugs that inhibit the ATPase activity of HSP90 results in the depletion of client proteins linked to proliferation, invasion and metastasis. Hence, inhibition of the folding machinery has been targeted as promising treatment of cancer. We show that PU-H71, STA-9090 and HSP990 induce dose-dependent decrease in U2OS-WT viability after 48 h of treatment, showing a logarithmic trendline, and they also produce cytotoxicity and promote HSR. Finally, due to low efficacy, adverse events and commercialization issues that general HSP90 inhibitors have, isoform-specific inhibitors were studied, such as TRAP-1 inhibitor. We demonstrate that between the studied ones C3 and C4 do not have effect in cell viability, whereas C1 and C2 do have effect, where WT cells surprisingly are the most sensitive. Nevertheless, novel inhibitors should be considered to evade upregulation of HSPs and the consecutive cytoprotective HSR activation that protects the proteome, supporting malignant transformation and tumor

development. Isoform-selective inhibitors seem to be an option to avoid the pan-inhibition of HSP90 inhibitors that produce adverse effects. In addition, C-terminal HSP90 inhibitors should be further assessed in future researches, as they seem not to induce HSR and might be another promising target for cancer therapy.

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