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# **The new platinum(IV) derivative LA-12 shows stronger inhibitory effect on Hsp90 function compared to cisplatin**

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# Abstract

## Background

Cisplatin and its derivatives are commonly used anti-cancer drugs. However, cisplatin has clinical limitations including serious side effects and frequent emergence of intrinsic or acquired resistance. Thus, the novel platinum(IV) complex LA-12 represents a promising treatment modality, which shows increased intracellular penetration resulting in improved cytotoxicity in various cancer cell lines, including cisplatin resistant cells.

## Results

LA-12 disrupts cellular proliferation regardless of the p53 status in the cells, however the potency of the drug is greatly enhanced by the presence of a functional p53, indicating several mechanisms of action. Similarly to cisplatin, an interaction of LA-12 with molecular chaperone Hsp90 was proposed. Binding of LA-12 to Hsp90 was demonstrated by Hsp90 immunoprecipitation followed by platinum measurement using atomic absorption spectrometry (AAS). An inhibitory effect of LA-12 on Hsp90 chaperoning function was shown by decrease of Hsp90-assisted wild-type p53 binding to p21<sup>WAF1</sup> promoter sequence *in vitro* and by accelerated ubiquitination and degradation of primarily unfolded mutant p53 proteins in cells exposed to LA-12.

## Conclusions

To generalize our findings, LA-12 induced degradation of other Hsp90 client proteins such as Cyclin D1 and estrogen receptor was shown and proved as more efficient in comparison with cisplatin. This newly characterised molecular mechanism of action opens opportunities to design new cancer treatment strategy profitable from

unique LA-12 properties, which combine DNA damaging and Hsp90 inhibitory effects.

## Background

Cisplatin [cis-diamminedichloroplatinum(II)] is a platinum-based anticancer drug commonly used for treatment of different types of cancer, including ovarian, testicular, head and neck or lung carcinomas [1]. Although cisplatin exerts significant anti-tumour activity, cisplatin-based therapies cause numerous side effects, such as nephrotoxicity, neurotoxicity and nausea. The intrinsic as well as acquired resistances to cisplatin-based therapy also represent substantial complications for the treatment of tumours.

For this reason, vast efforts were committed to develop novel platinum-based complexes to overcome platinum resistance, to reduce cisplatin side effects and to introduce novel mechanisms of anti-cancer action. Two derivatives, carboplatin (cis-diammine-(1,1-cyclobutanedicarboxylato)platinum(II)) and oxaliplatin (trans-R,R.cyclohexane-1,2-diammine)oxalatoplatinum(II)), have been introduced [2]. Taken together, these three drugs represent all of the available platinum drugs approved by the Food and Drug Administration for clinical use [3]. In addition to substantial side effects, these three drugs also require intravenous administration, therefore a new generation of platinum drugs represented by satraplatin (JM216) [(OC-6-43)-bis(acetato)amminedichloro(cyclohexylamine)platinum(IV)] has been generated. JM216 represents the first orally administered platinum compound which has been evaluated in clinical trials [2, 4, 5].

LA-12, [(OC-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum(IV)], is a hydrophobic platinum(IV) complex structurally similar to JM216, which

contains 1-adamantylamine instead of cyclohexylamine non-leaving ligand [6] which provides this drug different properties. LA-12 has shown a higher cytotoxicity than JM216 when tested on a panel of 14 cancer cell lines of various origin and different cisplatin sensitivity [6, 7] and no cross-resistance with cisplatin [6, 8]. LA-12 has also displayed (i) higher antitumor activity in comparison with cisplatin and JM216, (ii) favourable pharmacokinetics and (iii) relatively low acute toxicity in a panel of pre-clinical *in vivo* studies [9-11].

The cytotoxic mode of action of cisplatin is mediated by its interaction with DNA to form DNA adducts, primarily intra-strand crosslink adducts, which activate several signal transduction pathways, including those involving ATR, p53, p73, and MAPK, and culminate in the activation of apoptosis [12]. JM216 has a mechanism of action similar to that of cisplatin, inducing the formation of DNA adducts and inter- and intra-strand crosslinks and resulting in G2 arrest and induction of apoptosis [13]. The principle mechanisms of LA-12 effects are not fully understood to date, although there is some evidence that exposure to LA-12 can disrupt cell proliferation and induce apoptosis more potently than cisplatin in both p53 dependent and independent manners [14, 15]. Interestingly, LA-12 induces unique changes in the profile of gene expression compared to cisplatin, indicating a distinct mode of action resulting in the differential activation of both p53-dependent and p53-independent gene targets [16].

Besides the well known cisplatin DNA-mediated effect, the other mechanism of cisplatin action was described demonstrating that cisplatin may also disrupt the function of some proteins, including heat shock protein 90 (Hsp90) [17]. According to this study, cisplatin associates with the molecular chaperone Hsp90 and reduces its' chaperone activity. For example, this can result in the degradation of steroid receptors [18] and/or inhibition of interaction between Hsp90 and inositol hexakisphosphate

kinase-2 (IP6K2), which disrupts the inhibitory effect of Hsp90 on IP6K2, leading to increased diphosphoinositol pentakisphosphate (IP7) formation and sensitisation of cancer cells to apoptosis [19, 20].

Since it is supposed that LA-12 undergoes metabolic changes and serves actually as a pro-drug for chemotherapeutically active platinum analogues, it is possible that these active metabolites can interact with proteins analogous to cisplatin, resulting in stronger cytotoxic or cytostatic effects. According to this hypothesis we have analyzed whether LA-12 can bind to Hsp90 resulting in inhibition of its protective chaperoning function such as folding, stabilization, activation and assembly of its “client” proteins.

## **Results**

### **Time and dose dependent accumulation of LA-12 in cancer cells in comparison with cisplatin**

LA-12 and cisplatin uptake into cells was determined by AAS platinum amount measurement in lysates of cells treated with different doses of these drugs for different periods of time. We observed rapid and high initial accumulation of platinum after 0.5 hour incubation with LA-12 in all concentrations used, rising up to 4 hours of incubation. In subsequent time points, platinum level remained stable. The platinum amount in cells was also dependent on the concentration used.

In contrast, there was no platinum detected in response to 1  $\mu$ M cisplatin treatment. Compared to LA-12 exposure, platinum levels were detected only when 10  $\mu$ M and 50  $\mu$ M cisplatin doses were administered. In response to LA-12, platinum levels were 30 to 50 times higher depending on concentration used and time interval

(Figure 1). These results demonstrate that cellular uptake of LA-12 is faster and more effective compared to cisplatin.

### **LA-12 specifically interacts with Hsp90**

To assess the anticipated interaction of Hsp90 with LA-12, we measured in triplicate the amount of platinum bound to Hsp90 by AAS after immunoprecipitation of Hsp90 from H1299 cells treated with 10 and 50  $\mu$ M both LA-12 and cisplatin. We detected significant amounts of platinum after LA-12 treatment in contrast to cisplatin treatment, where only after using 50  $\mu$ M concentration was a limited amount of platinum detected (about detection limit of AAS) (Table 1). The presence of Hsp90 in the samples after immunoprecipitation was confirmed using western blot (Figure 2). These data show that LA-12 binds to Hsp90 more effectively than cisplatin *in vivo*, probably due to the higher intracellular levels of LA-12.

### **Inhibition of Hsp90 assisted p53 binding to promoter sequence of p21<sup>WAF1</sup> by LA-12**

The influence of LA-12 on the ability of wild-type p53 to specifically bind to its target sequence derived from the p21<sup>WAF1</sup> gene promoter was tested using an electrophoretic mobility shift assay (EMSA). It is known that human wild-type p53 binding activity is facilitated by molecular chaperone Hsp90 under physiological conditions and this process is inhibited by geldanamycin [21]. Thus in 22 °C wild-type p53 tetramers can bind to the p21<sup>WAF1</sup> promoter independently of Hsp90 status. Under physiological conditions (37 °C) in a control reaction without Hsp90, human wild-type p53 loses its ability to bind to the p21<sup>WAF1</sup> promoter; however in presence of human Hsp90 $\beta$  and ATP, wild-type p53 can develop transcriptionally active tetramers that bind to the p21<sup>WAF1</sup> promoter sequence. In consequence, when Hsp90 $\beta$  is

inhibited by 17-AAG, the affinity of wild-type p53 to p21<sup>WAF1</sup> promoter is disrupted. Similarly, in a case of LA-12 we also observed the disruption of Hsp90 $\beta$  activity leading to inhibition of wild-type p53 DNA binding affinity (Figure 3). Cisplatin could not be effectively compared to LA-12 and 17-AAG in this assay, as it directly disrupts the p53 binding to promoter sequence (not shown).

### **LA-12 inhibits Hsp90 assisted folding and initiates degradation of unfolded proteins**

Hsp90 is important for folding and stabilization/degradation of client proteins. In our previous work we demonstrated that particular p53 mutations show different ratios between native wild-type p53 conformations (recognized by PAb1620 antibody) and denatured conformations (recognized by PAb240 antibody) and highlighted an important role of Hsp90 to maintain the mutant p53 protein conformation and stability [22]. In the case of partially folded p53 mutants, such as R273H naturally expressed in MDA-MB-468 cells, the native conformation is maintained by Hsp90. Thus, inhibition of Hsp90 results in a decrease of R273H mutant p53 folded conformation and an increase of denatured conformation, although the overall protein levels are not changed. We found that LA-12 is also able to cause the same changes in R273H folded p53 mutant conformation without appreciably affecting the level of total p53 protein (Figure 4A). In the case of predominantly unfolded mutant p53 proteins, such as R175H naturally expressed in SK-BR-3 cells, inhibition of Hsp90 function results in their degradation, and the identical effect is seen after treatment with LA-12 (Figure 4B).



## **Mechanism of mutant p53 protein degradation in response to LA-12**

In our previous work we have shown that naturally unfolded mutants, such as p53 R175H in SK-BR-3 cells, form stable complexes with chaperones and that Hsp90 activity is crucial to prevent their degradation [22]. If the Hsp90 activity is blocked by 17-AAG, the denatured mutants develop complex with Hsp70 and CHIP proteins, where ubiquitin ligase CHIP is responsible for ubiquitination of unfolded p53 and ubiquitinated p53 protein is targeted for degradation in the proteasome [22, 23]. To investigate whether LA-12 also initiates unfolded p53 mutant degradation via activation of CHIP ubiquitin ligase, we compared mutant p53 levels in cells transiently transfected by CHIP and a dominant-negative CHIP-TPR truncated form, which contains the TPR domain (responsible for interactions with chaperones) but lacks the ubiquitination domain. In MDA-MB-468 cells with a partially unfolded p53 mutant that is independent of Hsp90 activity, there was no effect of 17-AAG or LA-12 in the presence or absence of CHIP or CHIP-TPR (Figure 5A). In mock-transfected SK-BR-3 cells, we observed a decrease of mutant p53 in response to both LA-12 and 17-AAG, as well as in cells transfected by CHIP. Importantly, transfection by CHIP-TPR decreased the rate of degradation of the unfolded mutant p53, indicating the role of CHIP ubiquitin ligase in degradation of R175H p53 mutant caused by LA-12 (Figure 5B).

## **LA-12 induces degradation of not only mutant p53 but also of other Hsp90 client proteins**

Accordingly we found that LA-12 is responsible for massive decrease of p53 mutant proteins that adopt a predominantly denatured conformation (BT-474 and SK-BR-3 cells); in cells which carry both native and denatured conformations of mutant p53 (T-47D, BT-549 and MDA-MB-468), the decrease of p53 level was

noticeable only after longer incubations (9 hours) with the drug. In MDA-MB-231 cells, where mutant p53 occurs predominantly in native conformation, no decrease of p53 level was observed even after prolonged incubation. Interestingly, apart from p53 protein, LA-12 also induces degradation of other Hsp90 client proteins, such as Cyclin D1 and Estrogen Receptor (Figure 6).

Importantly, the 10  $\mu$ M drug dose used in our experiments is equal to 1.95 mg/l platinum, which corresponds to the plasma levels of platinum shown in previous *in vivo* studies [10, 24]. The maximum platinum plasma concentration 1.54 and 6.25 mg/l in rats was observed within 1 hour after oral administration of 38.6 and 540 mg/kg LA-12, respectively [24]. Interestingly, maximum tolerated single oral dose of LA-12 in mouse model was 1000 mg/kg [10].

## Discussion

Platinum(IV) complexes represent a class of anticancer agents that are activated by reactions with reducing agents present in body liquids after their administration. Thus, the platinum(IV) complexes display potential advantages over their platinum(II) counterparts because of their greater stability and bioreductive activation, thereby allowing a greater proportion of the drug to arrive at the target intact. Thus, it is not surprising that the cellular uptake of LA-12 was faster and more effective than that of cisplatin (Figure 1).

Hsp90 is a molecular chaperone and is one of the most abundant proteins expressed in cells [25]. Molecular chaperone Hsp90 is a member of the heat shock protein family, which is up-regulated in response to stress and its *in vivo* function is ATP-dependent [26]. Hsp90 exists as a homodimer, which contains three domains. The N-terminal domain contains an ATP-binding site that binds the natural products

geldanamycin and radicicol. Hsp90 function depends upon the ability of the N-terminal domain to bind and hydrolyze ATP, which is regulated and coupled with the conformational changes of the Hsp90 dimer [27]. The middle domain exhibits high affinity for co-chaperones as well as client proteins [28]. The structure of the C-terminus of Hsp90 contains the MEEVD sequence, which is known to bind co-chaperones that contain multiple copies of the tetratricopeptide repeat (TPR), a 34 amino acid sequence [29]. Interestingly, the middle C-terminal part of Hsp90 has been implicated biochemically as the site of a putative second cryptic ATP-binding site on Hsp90 [26]. The contribution of this site to the overall regulation of chaperone function is not clear, but novobiocin has been reported to interact with this site and alter the conformation of the chaperone [30]. Importantly, cisplatin has also been found to bind to an Hsp90 site that overlaps the putative ATP/novobiocin binding site in this region, leading to inhibition of some Hsp90 activities [17, 26]. The observed higher affinity of Hsp90 to LA-12 may be explained by very specific properties of C-terminal domain of Hsp90. This domain contains several solvent-exposed hydrophobic residues, which are responsible for binding of client protein such as unligated glucocorticoid receptor [31]. Consequently the hydrophobic properties of LA-12 could explain its preferential binding to Hsp90 in contrast to cisplatin.

The ability of LA-12 to inhibit Hsp90 function was shown using several approaches. (i) EMSA, based on the fact that under physiological conditions the chaperone activity of Hsp90 is important for the transcriptional activity of genotypically wild-type p53 [21] clearly demonstrated LA-12 as potent inhibitor of Hsp90 chaperoning function. (ii) Determination of p53 conformation in cells expressing various p53 mutants, which were exposed to LA-12, revealed that the unfolded mutant p53 proteins that do not rely on Hsp90 activity are less stable than

folded mutant p53 proteins that require Hsp90 activity. These findings are in agreement with previous work showing that the half-life of p53 mutant positively correlates with their conformational stability [22].

It was shown that inhibition of Hsp90 results in ubiquitination and degradation of primarily unfolded p53 mutants. This MDM2-independent degradation of unfolded p53 mutant is mediated by chaperone interacting protein CHIP. Our results show that CHIP is responsible for LA-12 mediated degradation in the same way as 17-AAG, which supports our theories finding LA-12 as a specific Hsp90 inhibitor.

Accordingly to both higher cellular uptake of LA-12 and at least 10-fold higher presence of platinum bound to Hsp90 in cells exposed to LA-12 compared to cells treated by cisplatin (Table 1), a stronger suppressive effect of LA-12 on Hsp90 activity and function in relation to cisplatin could be expected. This statement was confirmed by the ability of LA-12 to influence the stability and degradation rate of other Hsp90 client proteins, such as estrogen receptor and cyclin D1 compared to cisplatin, which did not decrease the level of these proteins.

## Conclusions

In summary, our data indicate that cellular uptake of LA-12 is more effective compared to cisplatin resulting in more extensive binding of LA-12 to Hsp90 *in vivo*. This interaction is responsible for direct inhibition of Hsp90 chaperone function. These unique properties predict LA-12 as a novel promising anti-cancer therapeutic exploiting dual mode of action in inducing genotoxic damage and inhibiting Hsp90 activity.

# Methods

## Cell cultures

All human cancer cell lines were obtained from ATCC. Cell lines BT-474, MDA-MB-468, MDA-MB-231, T-47D, BT-549 and SK-BR-3 contain different p53 mutations (E285K, R273H, R280K, L194F, R249S and R175H, respectively), whereas the wild-type allele was lost in all these cell lines. MCF-7 cells contain wild-type p53, and H1299 cells are p53-null. Cells were periodically checked for morphology by microscope and regularly screened for mycoplasma using Hoechst staining.

Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Paisley, Scotland) supplemented with 10% fetal bovine serum (Invitrogen, Paisley, Scotland), 300 mg/l L-glutamine (Invitrogen, Paisley, Scotland), 100 U/ml penicillin (Invitrogen, Paisley, Scotland) and 0.1 mg/ml streptomycin (Invitrogen, Paisley, Scotland), in a humidified incubator at 37°C in 5% CO<sub>2</sub> atmosphere. Cells were grown to 80% confluence prior to experimental treatments.

## Chemicals

The following anti-neoplastic drugs were used in this study: LA-12, cisplatin (Pliva-Lachema a.s., Brno, Czech Republic), 17-AAG (Alexis Biochemicals, Lausen, Switzerland). LA-12 and cisplatin were dissolved in DMSO and diluted in cell medium to 0.5 mM stock solution and 17-AAG was dissolved in DMSO to 2 mM stock solution. Stock solutions of LA-12 and cisplatin were freshly prepared before use. The final concentration of DMSO in cell culture medium did not exceed 0.2 %.

## **SDS-PAGE and western blotting**

SDS-PAGE and western blotting were performed as described previously [16]. The following antibodies were used: DO-1 mouse monoclonal antibody directed towards the epitope <sup>20</sup>-SDLWKL-<sup>25</sup> within the p53 N-terminal region [32], CM-1 rabbit polyclonal antibody against human p53 protein (in house antibody), CHIP-11.1 mouse monoclonal antibody recognizing full length as well as truncated CHIP protein (in house antibody), Estrogen Receptor (clone SP1) rabbit monoclonal antibody (Lab Vision, Fremont CA, USA), CD1 mouse monoclonal antibody recognizing Cyclin D1 (in house antibody), EEV1-2.1 mouse monoclonal antibody against Hsp90 protein (in house antibody) and AC-40 monoclonal antibody recognizing actin (Sigma-Aldrich Inc. St. Louis, USA), which was used as a protein loading control. Final concentration of all antibodies used for immunodetection of proteins was 1 µg/ml in 5% milk containing 0.1% Tween 20 in PBS.

## **Immunoprecipitation**

Immunoprecipitation of conformationally different forms of p53 protein was performed using 1 µg of mouse monoclonal antibodies PAb1620 recognizing the wild-type conformation of human p53 protein at native form [33, 34] and PAb240 recognizing the mutant conformations of p53 at native form [35], as described previously [33, 35]. For immunoprecipitation of Hsp90 protein for platinum amount measurement by AAS, cells were lysed in 150 mM NaCl, 50 mM HEPES (pH 7.2) and 0.5% Tween 20 and sonicated. Protein concentrations were measured using Bradford's assay. 200 µl of cell lysate containing 400 µg of total protein was incubated overnight together with 2 µg of EEV2-1.1 mouse monoclonal antibody against Hsp90β protein (in house antibody) and AC88 mouse monoclonal antibody against Hsp90 protein (Stressgen Biotech Corp., Victoria, BC, Canada). 15 µl of G-

protein sepharose beads (GE Healthcare, Wien, Austria) was used to precipitate the antibody. After three washes in lysis buffer, beads were resuspended in 200 µl of 150 mM NaCl, 50 mM HEPES (pH 7.2) and 0.5% Tween 20 and boiled for 10 min.

### **Preparation of samples for AAS platinum amount measurement**

Cells were incubated for the given time intervals with different doses of LA-12 and cisplatin. Control and treated cells were collected by centrifugation, washed three times with ice-cold PBS, lysed in 150 mM NaCl, 50 mM HEPES (pH 7.2) and 0.5% Tween 20 and sonicated. Protein concentrations were measured using Bradford's assay. 200 µl of cell lysate were used for platinum amount measurement by AAS.

### **In vitro p53 DNA Binding Assay**

Human wild-type p53 and human Hsp90β were purified and activity of p53 was quantified by EMSA as described previously [36]. Simplified, the assay is based on the ability of human recombinant Hsp90 to restore the specific DNA binding activity of human recombinant p53 at 37°C *in vitro*. A preincubation with Hsp90 inhibitors prior to p53 inactivation at 37°C was carried out as described for geldanamycin [21].

## **List of abbreviations**

AAS: Atomic absorption spectrometry, EMSA: Electrophoretic mobility shift assay, CHIP: Carboxy terminus of Hsp70-interacting protein, MDM2: Mouse double minute 2 homolog, Hsp90: Heat shock protein 90

## **Competing interests**

The authors declare that they have no competing interests.

## Authors' contributions

VK designed, performed and analysed platinum accumulation experiments, performed immunoprecipitation experiments and prepared the manuscript, RH designed and performed degradation experiments and prepared the manuscript, DW performed EMSA experiments, EM performed CHIP transfection experiments, VH and DS performed AAS analysis, PM and PS contributed to the experimental design of the study, BV contributed to the conception and design of the entire study and the final editing of the manuscript. All authors read and approved the manuscript.

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## Tables

**Table 1 - Analysis of interaction of Hsp90 with LA-12**

Sample	Applied compound	Result ( $\pm$ SD) mg Pt/ml	Result ng Pt/mg protein
D-MEM	negative	-	
10 $\mu$ M cisplatin	10 $\mu$ M cisplatin	2.09 (0.05)	
50 $\mu$ M cisplatin	50 $\mu$ M cisplatin	9.93 (0.21)	
10 $\mu$ M LA-12	10 $\mu$ M LA-12	1.96 (0.01)	
50 $\mu$ M LA-12	50 $\mu$ M LA-12	9.14 (0.26)	
L1	negative	-	
L2	10 $\mu$ M cisplatin	0.040 (0.0004)	20
L3	50 $\mu$ M cisplatin	0.290 (0.005)	145
L4	10 $\mu$ M LA-12	1.98 (0.02)	990
L5	50 $\mu$ M LA-12	11.6 (0.26)	5500
P1	negative	-	
P2	10 $\mu$ M cisplatin	-	
P3	50 $\mu$ M cisplatin	< 0.02	
P4	10 $\mu$ M LA-12	0.034 (0.004)	
P5	50 $\mu$ M LA-12	0.222 (0.01)	
NC1	50 $\mu$ M cisplatin	-	
NC2	50 $\mu$ M LA-12	-	
AC1	50 $\mu$ M cisplatin	-	
AC2	50 $\mu$ M LA-12	-	

Platinum levels were determined by AAS in whole cell lysates (L1-5) of H1299 control cells and cells treated with 10  $\mu$ M and 50  $\mu$ M LA-12 or cisplatin for 9 hours. The protein concentration of cell lysates was 2 mg/ml. The amounts of platinum bound to Hsp90 was measured after immunoprecipitation of Hsp90 from H1299 control cells and cells treated with 10  $\mu$ M and 50  $\mu$ M LA-12 or cisplatin for 9 hours (P1-5). Immunoprecipitations without antibody (NC1-2) and with DO-1 antibody (AC1-2) in cells treated with 50  $\mu$ M LA-12 and cisplatin were performed as negative control for crossreactivity of platinum compounds with other reaction components.

Control AAS platinum measurements were performed in D-MEM cell medium containing 10  $\mu$ M and 50  $\mu$ M LA-12 or cisplatin. Results are mean  $\pm$  SD of triplicate experiments.

## Figures

### Figure 1 Platinum accumulation in H1299 cells

H1299 cells were treated with varying concentrations of LA-12 and cisplatin for indicated time intervals. Platinum levels in cell lysates were analysed by AAS. Results are mean  $\pm$  SD of triplicate experiments.

### Figure 2 Detection of Hsp90 protein

The levels of Hsp90 protein in H1299 whole cell lysates and after immunoprecipitation of Hsp90 in LA-12 and cisplatin treated cells were analysed by western blotting. EEV1-2.1 mouse monoclonal antibody was used for detection of Hsp90 protein.

### Figure 3 The Hsp90-dependent binding of wild-type p53 protein to p21<sup>WAF1</sup> gene promoter sequence is inhibited by LA-12 and 17-AAG

A) Wild-type p53 binding (0.125  $\mu$ M tetramer) to the promoter sequence at 22 °C (the main band) is not influenced by 4% DMSO (final concentration in the reaction) or LA-12 and 17-AAG dissolved in the corresponding DMSO amount. B) Hsp90 $\beta$  (3  $\mu$ M dimer) with 5 mM ATP (final concentration in all reactions) rescues the p53 activity from inactivation at 37 °C, in the presence of an increasing DMSO concentration, while LA-12 and 17-AAG inhibit Hsp90 $\beta$ . The LA-12 stock solution is 2x more concentrated than 17-AAG to compensate for the lower LA-12 activity. Thus both inhibitors are in corresponding DMSO amounts: 100, 200, 300  $\mu$ M LA-12 and

similarly 50, 100, 200  $\mu$ M 17-AAG introduce 2%, 4%, 6% DMSO concentration to the reaction, respectively.

#### **Figure 4 Analysis of p53 mutant protein conformation**

p53 mutant protein conformation was analysed in A) MDA-MB-468 cells containing mostly folded p53 R273H mutant and B) SK-BR-3 cells containing only unfolded p53 R175H mutant. Cells were treated for 9 hours with 10  $\mu$ M cisplatin, 10  $\mu$ M LA-12 and 4  $\mu$ M 17-AAG. Conformational changes of p53 were determined using immunoprecipitation with p53 conformation-specific mouse monoclonal antibodies PAb1620 (detects folded conformation) and PAb240 (detects unfolded conformation). CM-1 rabbit polyclonal antibody was used for detection of p53 protein.

#### **Figure 5 Analysis of p53 mutant protein degradation**

p53 mutant protein levels were analysed in A) MDA-MB-468 cells containing mostly folded p53 R273H mutant and B) SK-BR-3 cells containing unfolded p53 R175H mutant transfected with CHIP or CHIP-TPR protein by western blotting. Cells were treated for 9 hours with 10  $\mu$ M cisplatin, 10  $\mu$ M LA-12 and 4  $\mu$ M 17-AAG. CM-1 rabbit polyclonal antibody and CHIP-11.1 mouse monoclonal antibody were used for detection of p53 and CHIP proteins. Actin was used as loading control.

#### **Figure 6 Analysis of degradation of Hsp90 client proteins**

Protein levels of p53, cyclin D1 (CD1) and estrogen receptor (ER) were determined in cells treated for 3, 6 and 9 hours with 10  $\mu$ M cisplatin and 10  $\mu$ M LA-12 by western blotting. CM-1 rabbit polyclonal antibody, SP1 rabbit monoclonal antibody and CD1 mouse monoclonal antibody was used for detection of p53, ER and CD1 proteins, respectively. Actin was used as loading control.



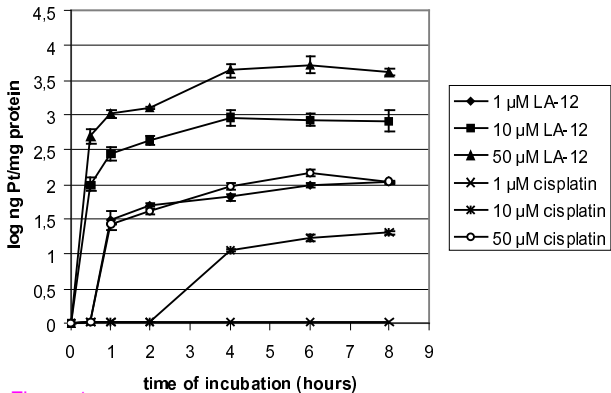


Figure 1

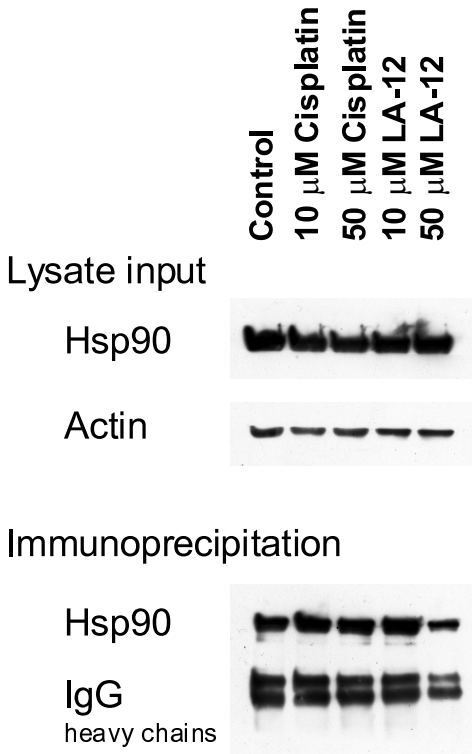
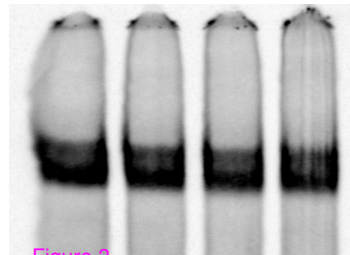


Figure 2

**A**

1h, 22°C

-	DMSO	LA-12	17-AAG
	4%	200	100

**B**

22°C

37°C

1h, 37°C, Hsp90 $\beta$ 

DMSO			LA-12			17-AAG		
2%	4%	6%	100	200	300	50	100	200

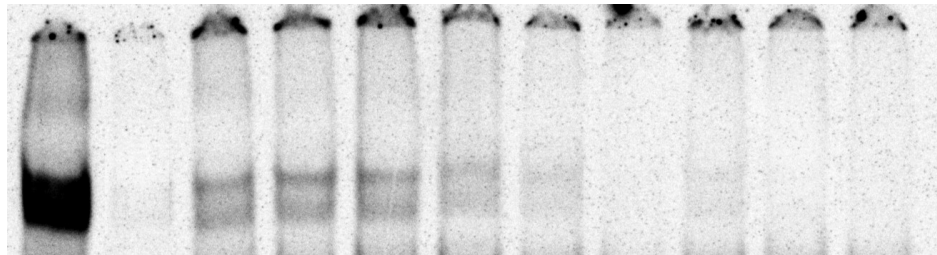


Figure 3

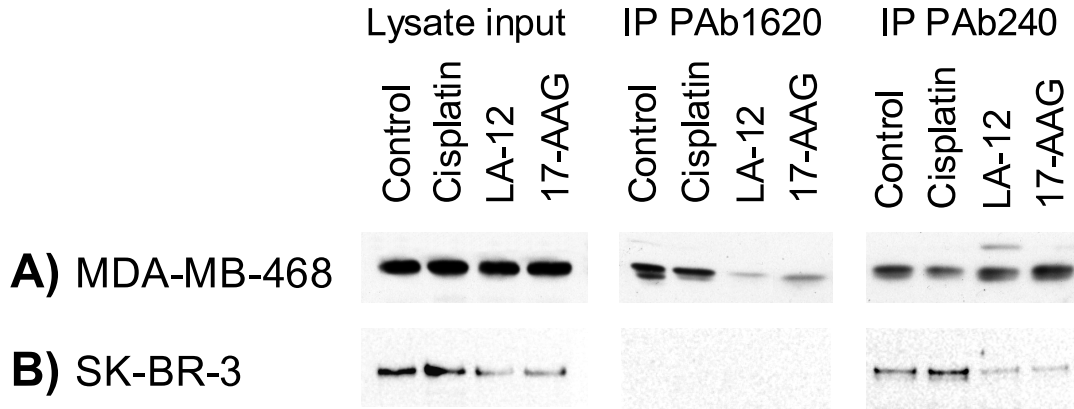
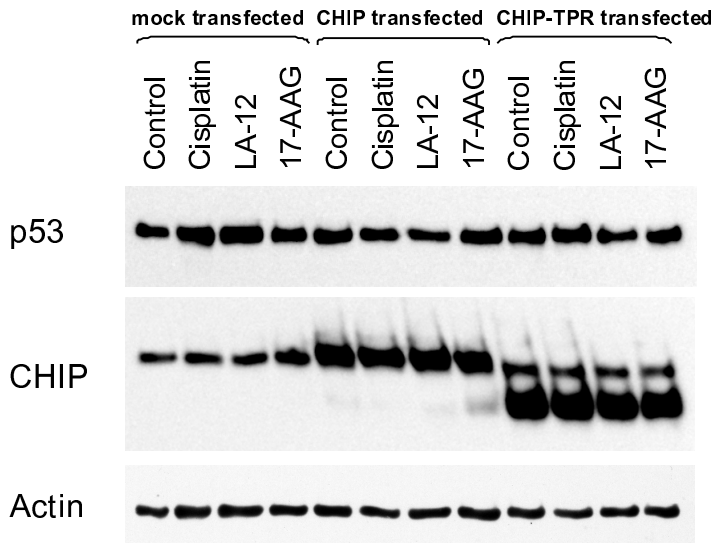


Figure 4

## A) MDA-MB-468



## B) SK-BR-3

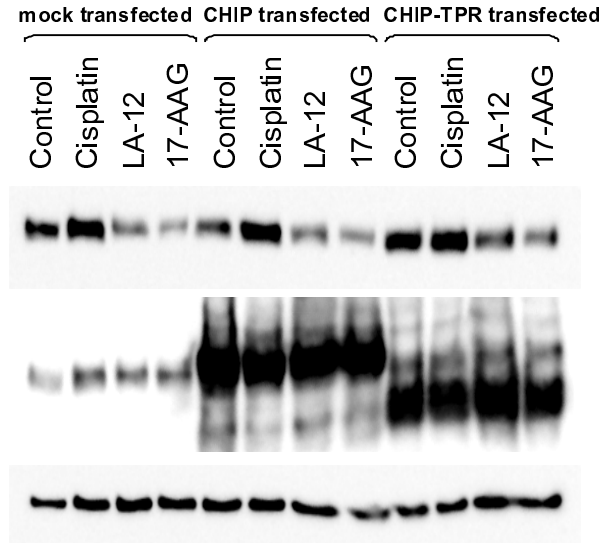


Figure 5

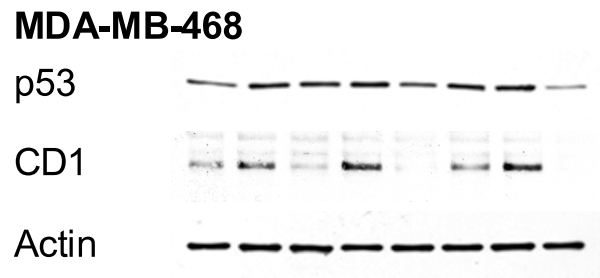
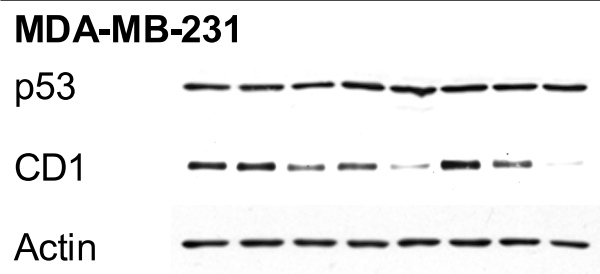
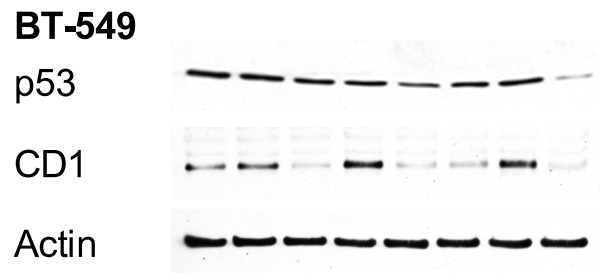
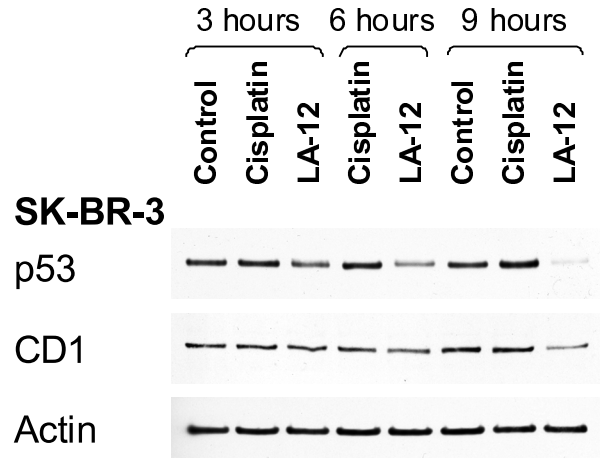
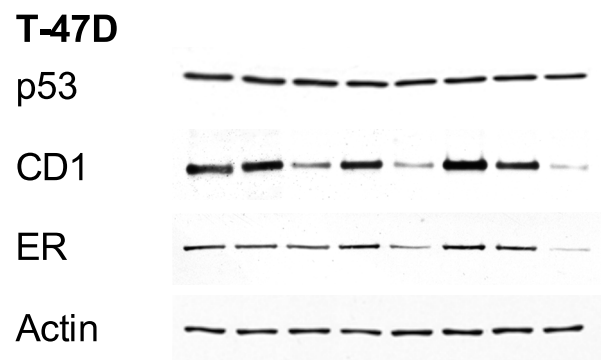
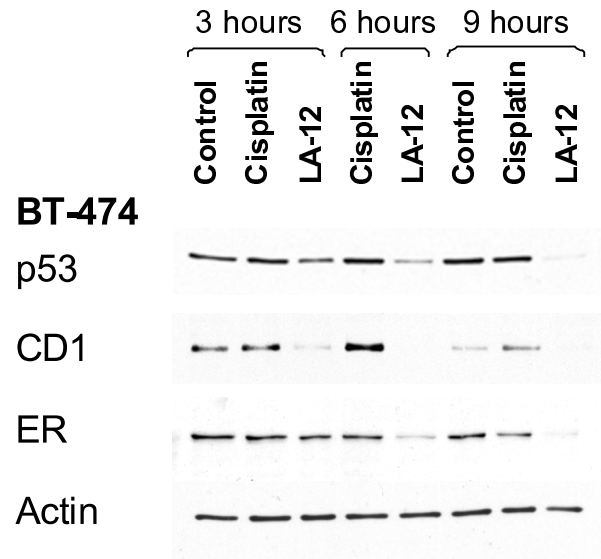


Figure 6