Heat Shock Protein 90 Regulates the Metaphase-Anaphase Transition in a Polo-Like Kinase-Dependent Manner

Guillermo de Cárcer
Molecular Oncology Programme, Centro Nacional de Investigaciones Oncológicas, Madrid, Spain

ABSTRACT

We have shown previously that the molecular chaperone heat shock protein 90 (Hsp90) is required for a proper centrosome function. Indeed, this Hsp90 function seems to be reflected in Polo-like kinase stability. Inhibition of Hsp90 in HeLa cells results in cell cycle arrest either in G2 stage or at the metaphase-anaphase transition. Here, we show that this inhibition leads to inactivation of the anaphase-promoting complex or cyclosome by both dephosphorylation and induction of the spindle assembly checkpoint. Hsp90 inhibition compromises two of the main mitotic kinases, Polo-like kinase 1 (Plk1) and cdc2. Interestingly, this mitotic arrest does not occur in certain tumor cell lines where Hsp90 and Plk1 are not associated. Those cells are able to process mitosis successfully and have an active Plk1 despite Hsp90 inactivation. Therefore, it seems that Hsp90 regulates completion of mitosis depending on its association with Plk1.

INTRODUCTION

Cell proliferation is a fundamental, highly regulated process. Evidences have accumulated that cell cycle components, regulatory proteins, and members of the ever-increasing mitogenic signal cascade associate with chaperones and stress proteins for different periods of time. Heat shock proteins, more specifically the heat shock protein 90 (Hsp90) family, show elevated levels in proliferating mammalian cells. They transiently associate with key molecules of the cell cycle control system (1). This supports the notion that chaperones and stress proteins play a role in the maintenance of the cell cycle and the signaling processes. More specifically, Hsp90 has been shown to be chaperone of several essential cell cycle regulator kinases such as p34/cdc2 (2, 3) and Polo-like kinases (4, 5). Interestingly, Hsp90 inhibition by geldanamycin (GA) and related ansamycins induces G1 arrest (6) and G2-M arrest in erythroleukemic cells (7).

Cell division is achieved by the progression through a series of events known as the cell cycle. To ensure that the original cell is not associated. Those cells are able to process mitosis successfully and have an active Plk1 despite Hsp90 inactivation. Therefore, it seems that Hsp90 regulates completion of mitosis depending on its association with Plk1.

by two WD-40 proteins, Cdc20 (also known as p55CDC or Fizzy) and Cdh1 (also known as Hct1 or Fizzy-related). These two regulators affect APC/C activity in early mitosis and late mitosis/G1, respectively (8, 9). Another main spindle checkpoint factor is Mad2, which gives the signal to hold the mitosis until all of the chromosomes are assembled at the metaphase plate and are properly attached to the spindle. Mad2 forms a ternary complex with the APC/C and Cdc20, and doing so inhibits APC/C function (10–12). In addition, APC/C is activated by phosphorylation through a mechanism including p34/cdc2 and Polo-like kinase (13, 14). Once the APC/C is active, there are two major mitotic events regulated by the APC/C: (a) sister chromatide separation; and (b) exit from mitosis. Sister-chromatid separation depends on the degradation of cohesin, the proteins that bind chromatides together. It has been shown recently that cohesin also needs to be phosphorylated by Polo-like kinases to be degraded (15). Finally, exit from mitosis depends on the APC/C-mediated degradation of cyclin B (16), the mitotic partner of p34/cdc2 kinase.

One of the major kinases involved in cell division and specifically in APC/C regulation is Polo-like kinase 1 (Plk1). This kinase was described firstly in Drosophila (17) as a major mitotic regulator kinase. Polo-like kinases are well conserved in all of the eukaryotes and associate transiently with several mitotic structures including spindle poles, kinetochores, the central spindle, and the midbody. Furthermore, mutations in Drosophila Polo cause abnormalities in mitosis (18), and Plk1 inactivation in mammalian cells has been found to induce a mitotic abnormality in generating aneuploidy (19). The maximal Plk1 kinase activity is reached in the G2-M phase of the cell cycle, and the function of Plk1 is considered necessary for mitotic cellular events such as spindle formation, chromosome segregation, and cytokinesis (20).

As mentioned before, Plk1 associates with Hsp90. This relationship between chaperon and kinase reflects its importance in centrosome function (4). Some tumor cells have the Plk1 gene mutated in such a way that the interaction between Plk1 and Hsp90 is disrupted (5). It seems that the Hsp90-Plk1 relationship could be important for cell division and may play a role in certain oncogetic events. This article shows that Hsp90 is involved in mitotic events in such a way that when Hsp90 is not active, mitotic cells arrest at the metaphase-anaphase transition and are unable to accomplish the cellular division. We show that Hsp90 inhibition induces dephosphorylation of the APC/C complex and activation of the spindle checkpoint machinery. Moreover, we show that this interaction of Hsp90 with the APC/C machinery is Polo-like kinase dependent. In fact, in certain tumor cell lines where Hsp90 is not associated with Plk1, Hsp90 inhibition does not arrest the metaphase-anaphase transition, and mitosis is successful. Interestingly, and in support of our previous results (4), inhibition of Hsp90 function results in centrosomes that are unable to nucleate microtubules, and, therefore, the spindle is not properly displayed. In contrast, in those cells where Hsp90 and Plk1 do not associate, centrosomes are fully active as microtubule-organizing centers, and the spindle is perfectly assembled.

MATERIALS AND METHODS

Cell Culture and Drug Treatments. HeLa cervical carcinoma cells, A549 lung adenocarcinoma cells, and HepG2 hepatoma cells were grown on Petri
dishes at 37°C in DMEM supplemented with 10% fetal bovine serum. Where indicated, 1.78 μM GA, 0.2 μM of nocodazole (Sigma), and 3 μM roscovitine (Sigma) were added to the culture medium.

**Antibodies.** The following antibodies were used: (a) mouse monoclonal anti-Plk1 cocktail (Zymed); (b) rabbit polyclonal anti-p55CDC (Santa Cruz Biotechnology); (c) mouse monoclonal anti-p34cdc2 (Sigma); (d) mouse monoclonal anti-α-tubulin (Sigma); (e) rat monoclonal anti-Hsp90 (Stressgen); (f) mouse monoclonal anti-cdc27 (Transduction Laboratories); (g) rabbit polyclonal antiphosphorylated histone 3 (Upstate Biotechnology); (h) mouse monoclonal anti-MPM-2 (Upstate Biotechnology); (i) mouse monoclonal anti-cyclin B1 (PharMingen); (j) mouse monoclonal anti-Mad2 (Transduction Laboratories); and (k) mouse monoclonal anti-γ-tubulin (Sigma).

**Cell Extracts and Western Blot Analysis.** Cells were scraped from Petri dishes, washed in PBS, and resuspended in lysis buffer [50 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, and 10% glycerol] supplemented with 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 25 mM sodium β-glycerophosphate, 1 mM sodium fluoride, and 1 mM sodium orthovanadate. Cell lysates were clarified by centrifugation in a top-bench centrifuge. The resulting proteins were separated in SDS-PAGE gels and transferred onto nitrocellulose membranes. Blots were incubated in blocking buffer (10% milk in PBS with 0.05% Tween-20) for 1 h and then incubated in PBS with 0.05% Tween-20 containing the primary antibody dilution for 1 h. They were then washed in PBS with 0.05% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for 45 min. After several washes, blots were developed with enhanced chemiluminescence reagent (Amersham).

**Coimmunoprecipitation Analysis.** Cell lysates (200–500 μg of protein) were incubated for 2 h at 4°C in a total volume of 200 μl with 4 μl of rabbit polyclonal anti-p55CDC (cdc20; Santa Cruz Biotechnology) followed by incubation of protein A beads for an additional hour (Amersham). Immunoprecipitates were collected by centrifugation, washed three times with the cell lysis buffer, and subjected to SDS-PAGE electrophoresis and immunoblot analysis.

**Immunofluorescence Microscopy.** Cells were grown onto coverslips, fixed in 4% formaldehyde in PBS for 10 min, and permeabilized in cold (−20°C) methanol. They were then incubated with glycine 20 mM/PBS for 10 min, followed by an additional incubation with BSA 3% in PBS for 20 min. Microtubules and centrosomes were stained with antibodies against α-tubulin (Amersham; N356) and γ-tubulin (Sigma; GTU88), respectively, and the corresponding dye-tagged secondary antibodies. Staining with antibodies against phosphorylated histone 3 monitored DNA condensation. Chromosomes were counter-stained with 4'-6'-diamidino-2-phenylindole (2 μg/ml). Finally, cells were mounted with Mowiol and analyzed with a Leica epifluorescence microscope.

**RESULTS**

**Hsp90 Inhibition Blocks Mitotic Progression.** Treatment of exponentially growing HeLa cells with the Hsp90-inhibitor GA arrests the cell cycle during G1 and G2 (6, 7). Moreover, it also leads to a mild but significant increase of the mitotic index in the order of 3- to 4-fold (21), suggesting that functional Hsp90 may also be required for exit from mitosis. To substantiate this conclusion, we studied the effect of GA on HeLa cells released from a nocodazole-induced mitotic arrest (Fig. 1A). In the absence of GA, >50% of the cells went out of mitosis during the first hour after the removal of nocodazole, and 2 h later, the mitotic index went down to nearly basal levels. In contrast, in the presence of GA, the mitotic index remained unchanged for 3 h after the removal of nocodazole (Fig. 1B). Consistent with this observation is that cyclin B remains stable after the release from nocodazole-induced arrest in GA-treated cells, although it follows fast degradation kinetics in control cells (Fig. 1C, center). Significantly, cdc27, one of the main components of the APC/C, presents much lower phosphorylation levels with GA treatment (Fig. 1C, top). It is known that APC/C has to be phosphorylated to increase its affinity to cdc20, and this fact plays a key role in the final APC/C activation and mitotic progression (8, 22). We then analyzed the state of the spindle checkpoint in GA-treated HeLa cells. When the spindle checkpoint is triggered, mitosis is arrested by Mad2 forming a ternary complex with cdc20 and the APC/C. Thus, immunoprecipitations were performed pulling down cdc20 and testing the presence of Mad2 in the complex (Fig. 1D). We found that although the amount of Mad2 that coprecipitates with cdc20 drops very significantly after the removal of nocodazole in control cells (Fig. 1D, Lane 3), it does not change in HeLa cells treated with GA, suggesting that the spindle checkpoint remains active even 3 h after the removal of nocodazole (Fig. 1D, Lane 5).

These observations suggest strongly that GA inhibits exit from mitosis through inhibition of the APC/C function and a strong activation of the spindle checkpoint machinery.

**Hsp90 Inhibition Affects MPM-2 Epitopes Phosphorylation.** Hsp90 has an assortment of kinases as client proteins. Hence, GA-induced Hsp90 inactivation may affect many kinases involved in mitotic progression. One method to test how mitotic phosphorylation levels are affected is by using the antibody MPM-2. MPM-2 antibody recognizes phosphoproteins ranging from 55 to 210 kDa. These proteins are abundant in mitotic cells and are cell-cycle-regulated (23, 24). Indeed, MPM-2-recognized proteins are enriched greatly at spindle structure, and, more specifically, at the spindle poles, and are characterized by containing two different phosphoepitopes containing phosphothreonine. Finally, MPM-2 antibodies are able to detect mitotic phosphoproteins in different organisms such as yeast, plants, Caenorhabditis elegans, fruit fly, Xenopus, and mammalian cells, indicating that these epitopes are highly conserved throughout evolution (25).

The MPM-2 phosphorylation state was determined in HeLa cells obtained at the moment of the nocodazole release, in the presence or absence of GA. We found that phosphorylation of these specific mitotic phosphoepitopes was compromised severely in GA-treated mitotic cells in which some of the phosphoepitopes were present at a much lower level, and others were not detectable (Fig. 2A). Significantly, cdc27 is one of the phosphoproteins belonging to the MPM-2 group (26), and its phosphorylation stage is compromised concordantly with the experiment shown in Fig. 1C.

Multiple kinases are able to phosphorylate MPM-2 epitopes, including mitotic kinases such as cdc2 and Plk1 (27, 28). Both kinases require Hsp90 for their stability and function, a conclusion we have confirmed with the nocodazole-arrested GA-treated cells used in our assays (Fig. 2B). We found that the kinase activity of Plk1 and cdc2 at time point 0 (12 h in GA) drop to 47% and 61%, respectively, in GA-treated cells. Remarkably, Drosophila heterozygotic Polo mutants show a 50% reduction in Polo activity and present severe mitotic aberrations (29). Previous results showed that after microinjection of HeLa cells with anti-Plk1 antibodies, little if any MPM-2 reactivity is observed on spindle poles (19). Moreover, the level of MPM-2 immunoreactivity has been correlated directly to the severity of the Polo mutant alleles in Drosophila melanogaster (30).

In conclusion, reduction in MPM-2 phosphorylation levels might be
because of the cdc2 and/or Polo inactivation and, therefore, support the possibility of a mitotic failure.

**GA Stops Mitosis in a Plk1-Dependent Manner.** It seems clear that GA induces mitotic arrest, and one of the main reasons could be the affection in cdc2 and/or Polo kinase function. Interestingly, inhibition of cdc2 has already been shown to be of no relevance for exit from mitosis once it has already initiated. In other words, treatment of mitotic HeLa cells with the cdc2 inhibitor roscovitine does not affect the mitotic process, and chromatides segregate (31). Roscovitine is a strong and specific inhibitor of Cdks, with the strongest Ki (0.65 μM) for cdc2 (32, 33). We have confirmed this result with the conditions used in our assays. Roscovitine does not inhibit exit form mitosis in HeLa cells as assayed by cyclin B degradation (Fig. 3) and microscopy (data not shown).

**Fig. 1.** A, the scheme shows the combination of drugs applied to mammalian cells to study the effects of geldanamycin (GA) in synchronized mitotic cells. GA needs at least 12 h for heat shock protein 90 inactivation reflected in Polo-like kinase 1 activity and stability. Given that mitosis is too short compared with GA treatment, cells were treated initially with nocodazole for 6 h to increase cell levels in G2-M. Then, GA was added for 12 h at the same time, nocodazole was washed out and cells released into mitosis. With this strategy, most of the cells affected by GA were at the beginning of mitosis, and heat shock protein 90 is certainly inhibited. Control cells (no GA) are treated only with DMSO (GA solvent). B, HeLa cells were incubated after the procedure described in A. Once the nocodazole was washed out, cells were time coursed and immunostained with antibodies against phospho-histone 3. Mitotic cells were counted compared with the total amount of cells. C, a portion of HeLa cells from the same experiment were homogenized, and extracts were immunoblotted with antibodies against cdc27 and cyclin B. At the end of the nocodazole, arrest control cells show how cdc27 has a maximum bandshift because of phosphorylation, and this phosphorylation decays gradually after the release (Lanes 2–6). In contrast, GA-treated cells present a tremendous reduction of phosphorylated cdc27 (Lanes 7–11). GA also affects cyclin B degradation in comparison with control cells. Tubulin was blotted as loading control. Lane 1 shows the levels for the proteins analyzed in an exponential cell culture. D, protein extracts from HeLa cells were cdc20 immunoprecipitated and immunoblotted with Mad2 antibody. Mad2 strongly binds cdc20 under nocodazole arrest (Lanes 2 and 4) but detaches from cdc20 as if mitosis was accomplished under normal conditions (Lane 3). Under GA treatment, Mad2 still bonds cdc20 even 3 h after nocodazole release, indicating an active spindle checkpoint (Lane 5).

**Fig. 2.** A, mitotic cells obtained from nocodazole treatment in the presence or absence of geldanamycin (GA) were homogenized. Protein extracts were blotted with MPM-2 antibody, antiphosphohistone 3, and anti-cdc27. GA-treated cells show clearly a reduction in mitotic phosphoepitopes. Cdc27 presents a dramatic dephosphorylation (no bandshift) under GA induction. Histone 3 phosphorylation levels are higher in cells released from nocodazole (Lanes 2 and 3) in comparison with asynchronous cells (Lane 1), showing that those cells are in mitosis. Tubulin was blotted as loading control. B, the same extracts were used for Polo-like kinase 1 and cdc2 in vitro kinase assays. Both activities of kinase are clearly reduced under GA induction. Plk1, Polo-like kinase 1; Bars, ±SD.
Polo-like kinase has been shown to regulate APC/C activity by phosphorylation (14), and Plk1 activity is essential to prevent premature inactivation of the APC/C-cdc20 complex in the early Xenopus cell cycle (34). Indeed, Descombes and Nigg (13) described that the exit of Xenopus egg extracts from M-phase arrest requires active Plx (Xenopus Plk1) and that Plx is required for activation of APC. Furthermore, Shirayama et al. (35) reported that APC-dependent destruction of Clb2 (yeast cyclin B) depends on Cdc5 (yeast Plk1). This evidence coupled with the roscovitine result suggests that GA-induced Plk1 degradation is responsible for the observed mitotic arrest.

Given all of the above observations, and notwithstanding the possible role of other unsuspected Hsp90-dependent proteins, we decided to test whether the mitotic arrest induced by GA in cells released from nocodazole arrest could be a consequence of the inactivation of Plk1. To this end, we took advantage of two cancer cell lines, A549 and HepG2. These cells carry a functional mutant form of Plk1, the stability of which is not dependent on Hsp90 (5). We first assayed the kinase activity of this stable Plk1 immunoprecipitated from nocodazole-arrested cells with and without GA. Plk1 kinase activity is indeed much more stable in A549 than in HeLa cells after GA treatment (Fig. 4A). The same conclusion applied to HepG2 cells (data not shown). We then studied whether these mutant cell lines arrest in mitosis in the presence of GA. We found that neither A549 nor HepG2 cultures accumulate cells in mitosis after a 24-h treatment with GA but HeLa cells do it (Fig. 4B). We then examined whether these
cells can exit mitosis in the presence of GA. We found that 3 h after the release from nocodazole-induced mitotic arrest, 70% of A549 cells exit mitosis (Fig. 4C), whereas essentially 100% of mitotic HeLa cells remain arrested as shown in Fig. 1B. Interestingly, roscovitine was tested in A549 and HepG2 cells to see whether cdc2 inhibition could disrupt mitosis once it was initiated. As in HeLa cells, A549 and HepG2 cells progress through mitosis in the presence of roscovitine and, moreover, in the presence of GA and roscovitine together (Supplementary Data, Fig. 2). Therefore, the roscovitine effect seen in HeLa cells does not seem to be specific for this cell line.

Consistent with these observations, GA-induced A549 cells present certain cdc27 phosphorylation levels, and, significantly, this phosphorylation is gradually reduced during mitotic progression, indicating the cells are exiting mitosis. Indeed, although in a slower fashion than control cells, cyclin B is degraded corroborating the mitotic progression (Fig. 4D).

Finally, the spindle checkpoint machinery was assayed in A549 and HepG2 cells, checking whether Mad2 binds to cdc20 (Fig. 4E). In non-GA-treated A549 and HepG2 cells, Mad2 coprecipitates with cdc20 in the presence of nocodazole (Fig. 4E, Lanes 2 and 4). This is consistent with observations that reveal that these two cell lines have functional spindle checkpoint machinery, and they arrest in the presence of microtubule poisons (data not shown). However, 3 h after the nocodazole release, the amount of Mad2 that coprecipitates with cdc20 drops considerably, even in the presence of GA (Fig. 4E, Lanes 3 and 5), corroborating that at least these two cancer cell lines are able to carry out mitosis even with Hsp90 repressed.

Interestingly, previous data shows how centrosomes are inactivated in vitro when Hsp90 is inhibited (4) and HeLa cells present severe mitotic aberrations (21). Therefore, the following question is asked: can GA-treated A549 and HepG2 cells organize spindles?

To address this issue, an aliquot of these GA-treated cells were analyzed by immunofluorescence (Fig. 5). A549 cell line showed to be not sensitive to GA, in relation to spindle organization. As soon as nocodazole is removed, the centrosomes in these cells start to nucleate microtubules, and mitotic spindles are assembled (Fig. 5, E–H). Similar results were obtained with HepG2 cells (data not shown). Worthy of note is that A549 cells immunofluorescence shown in Fig. 5 is an aliquot of cells taken from the same experiment shown in Fig. 4C, which shows clearly how mitosis progresses in time immediately after nocodazole release. In contrast, in the presence of GA, 100% of mitotic HeLa cells show a severe mitotic aberration (Fig. 5, A–D). There are some polymerized microtubules; they are in disarray and their organization is not always mediated by the centrosomes that behave like weak microtubule-organizing centers. Moreover, the spindle cannot be reassembled in GA-treated cells even after 3 h from the removal of nocodazole, and this probably triggers the spindle checkpoint and leads to mitotic arrest. All of these HeLa mitotic figures correlate with the mitotic quantification data shown in Fig. 1B.

**DISCUSSION**

Hsp90 inhibition by GA generates growth inhibition and apoptosis in several cancer cell lines. More specifically, GA induces cell growth arrest at G1 and G2-M transition. Unpublished results from our laboratory showed how serum-starved HeLa cells treated with GA and then released in fresh serum are unable to achieve cell division. In addition, these GA-treated cells accumulate cyclin B; therefore, the cycle arrest has to be far beyond S phase and before mitosis, thus, G2. It has been proposed that this G2 arrest might be due to the fact that Hsp90 is related to cdc2, and its stability and activity are compromised with GA induction (3).

Concomitantly, the fact that Plk1 stability is affected when Hsp90 is inactive might be additional evidence to reinforce the idea of G2 arrest. It is accepted that Plk1 plays a key role for entry into mitosis, and recent experiments demonstrate how Plk1 is essential in the phosphorylation and nuclear import of cyclin B to trigger mitosis (36). Moreover, Plk1 is a target of the DNA damage checkpoint to inhibit the entry into mitosis (37).

Interestingly, when exponential-growing HeLa cells are treated with GA, the number of cells in mitosis increases, specifically at metaphase, by about three to four times. Hence, although cells are unable to enter into mitosis if treated with GA, the fact that cells arrest as well in mitosis shows that Hsp90 inhibition is somehow disrupting the mitotic process once initiated.

GA-induced mitotic arrest has escaped to other researchers, because they reported a G2-M arrest by flux cytometry. Using this technique, mitotic cells cannot be distinguished from G2 cells. Results presented here show clearly how Hsp90 inhibition leads to a mitotic arrest in HeLa cells compromising the function of essential mitotic factors such APC/C and activating the mitotic spindle checkpoint. Although the activation of the checkpoint machinery seems to be the main reason behind mitotic arrest, dephosphorylation of certain APC/C components led us to study whether some mitotic kinases are involved in this phenotype.

We found how cdc2 and Polo-like kinase activity is compromised
by GA during the mitotic stages and how this inhibition may lead to a dramatic decrease in MPM-2 phosphoepitopes reactivity, showing clearly that the mitotic progression is affected. Moreover, we found how centrosomal activity is disrupted in vivo and how GA-treated cells are not able to assemble mitotic spindles. These observations are consistent with previous results, where we showed in vitro that the centrosome nucleation capability is inhibited by GA in a Polo kinase-dependent manner, and the addition of recombinant Polo-like kinase resume the microtubule-organizing center activity (4). Furthermore, the presence of Cdc20, Mad2, and the APC/C at centrosomes at the spindle, centromeres, and midbody (38–40) suggest that APC/C activity is required at all of these cellular locations. Remarkably, Plk1 can be found in all of those structures, and this colocalization may be essential in ensuring APC/C functions correctly.

The fact that on division cdc2 does not seem to be essential for mitotic progress suggested that the observed mitotic phenotype might be a consequence of Plk1 inactivation. To address this issue, we focused our attention to certain cancer cell lines, where Polo kinase is mutated and unrelated to Hsp90 (5). The hepatoma cell line HepG2 and the lung adenocarcinoma cell line A549, among others, have a mutated Plk1 gene product that does not bind to Hsp90 although is fully functional. Interestingly, Plk1 levels and function in such cells are not compromised when Hsp90 is inhibited by GA. Hence, both cell lines are appropriate tools to test whether Plk1 inactivation could be the reason behind the mitotic arrest under GA induction.

Consistent differences with HeLa cells are observed when A549 and HepG2 cells are treated with GA. Both cell lines have Plk1 activity and do not arrest at the metaphase-anaphase transition although Hsp90 inhibition. Moreover, A549 cells progress through mitosis correctly, with a slight delay compared with nontreated cells. There are main differences with HeLa cells concerning cdc27 phosphorylation. A549 cells under GA treatment show lower levels of cdc27 phosphorylation, but this reduction is ~30%, whereas in HeLa cells it increases up to 70% reduction. More importantly, the phosphorylated-cdc27 pool gradually reduces alongside mitotic progression in A549 cells. Finally, GA does not activate the spindle checkpoint machinery, and spindle formation is normal in A549 and HepG2 cells, demonstrating clearly that Hsp90 inhibition does not influence mitotic progression in such cancer cell lines. A possible explanation for these phenotypes might be that Plk1 kinase activity is not essential in A549 and HepG2 cells. This possibility can be discarded because interference RNA against Plk1 induces apoptosis in A549 cells and many other cancer cell lines (41). Therefore, results presented in this article suggest that Hsp90 may specifically regulate mitosis via Plk1 and not other kinases.

Worth mentioning is that all of these assays were carried out in transformed cell lines. We wanted to reveal whether primary cell lines responded in the same way as cancer cells. The Hs68 primary cell line (human newborn foreskin fibroblasts) was used for this purpose, and similar phenotypes were observed (Supplementary Data, Fig. 1). As in HeLa, Hs68 cells do not organize proper spindles, show severe centrosomal disruption in the presence of GA, and the mitotic progression is stopped. Therefore, Hsp90 inhibition disrupts cell division, and Plk1 is destabilized, regardless of the cell transformation stage.

It has been reported that G2-M block occurs after treatment with a GA derivative (17AAG) exclusively in retinoblastoma (RB)-deficient cells (6). Nevertheless, other evidences showed that the requirement for RB deficiency seems to be cell line dependent; as in other studies with human colon cancer and glioblastoma cell lines, a G2-M block was observed that was independent of RB status (42, 43). The only colon cell line known not to arrest at G2-M was the wild-type p53 cell line HCT116 (43). However, GA was shown previously to cause a G2-M block in an isogenic cell line model either with or without functional p53 (44). It seems likely that G2-M-induced arrest by GA derivatives is not dependent on p21 or RB status but other factors. As mentioned previously, many G2 kinases, such as wee1, myt1, Plk1, and cdc2 have been recognized as HSP90 client proteins, and degradation of these could clearly influence regulation of the G2-M checkpoint and induce inappropriate mitotic entry and mitotic catastrophe. Therefore, depletion of these G2 kinases might lead to a G2-M block, independent of RB status.

GA and its derivatives, concretely 17AAG, show antiproliferation activity in human tumor xenograft models. The 17AAG is being evaluated currently in Phase I clinical trials. Recently, Maloney and Workman (45) have published an interesting review describing how Hsp90 inhibitors may influence the six hallmark traits of cancer. These hallmarks are described as essential phenotypic traits that are required for development of full malignant phenotype (46). One of these hallmarks is related with the insensitivity of cancer cells to antitumor signals, where Plk1 kinase is directly related. There is a well-documented correlation between mammalian Plk1 expression and carcinogenesis. Indeed, mammalian Plk1 is overexpressed in a variety of human tumors, and its inhibition induces apoptosis in such tumor cells (47). Therefore, inhibition of Plk1 function could be an important application for cancer therapy.

A remarkable issue regarding Hsp90 inhibitors and cancer therapy is how these compounds increase the effectiveness of taxanes as anticancer drugs (48, 49). In some cases, this synergism allows cells to undergo apoptosis, although taxanes alone cannot induce such an effect (50). Taxanes are highly cytotoxic because of their ability to disrupt the microtubule network, induce cell cycle arrest, and finally, apoptosis. Indeed, taxanes induced cell arrest at the metaphase-anaphase transition activating the spindle checkpoint machinery. Interestingly, recent evidence shows that certain cancer cells present mutations in some spindle checkpoint genes (51). Partial inactivation of the mitotic checkpoint is associated with a relative resistance to spindle inhibitors, whereas checkpoint-competent cells initiate an apoptotic response when the checkpoint is challenged for a sufficient period. Unfortunately, this is the opposite of the desired response for tumor therapy. If certain cancers are indeed checkpoint defective, how might the efficacy of drugs that perturb microtubule assemblies be improved? One solution could be to use other drugs disrupting other key mitotic elements. The fact that GA inhibits mitotic progression in a Plk1-dependent manner might explain how Hsp90 inhibitors facilitate the action of other anticancer drugs and, more importantly, could be an important issue concerning cytotoxicity resistance of certain tumors.

Data presented here confirms Hsp90 as being crucial to the centrosome function in mammalian cells, which may be attributed to the degradation of Plk1. As well as influencing the onset of mitosis, Plk1 degradation may inhibit exit from mitosis through deregulation of the anaphase-promoting complex. Additional study is necessary to understand whether any other common mechanism may be responsible for arresting cells in a particular stage of the cell cycle after Hsp90 inhibition. Nonetheless, it is probable that the mechanisms controlling checkpoint control will depend on the genetic background of the examined cell line and may indeed involve a number of regulatory cell cycle mechanisms.

We certainly cannot discard other possible spindle proteins affected by the Hsp90 inhibition. In fact, Hsp90 is related with many factors involved in cell division (1). Early reports showed that Hsp90 antibodies decorate the spindle structure in mammalian cells (52), and furthermore, tubulin is a known Hsp90 client protein (53, 54), and as a consequence, it is possible that Hsp90 inhibitors may act as a novel class of microtubule inhibitors. Therefore, it may be that there is
another kind of induction on the microtubule network leading to a mitotic failure.

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