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Curcumin Derivative C212 Inhibits Hsp90 and Eliminates Both Quiescent and Growing Leukemia Cells

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Abstract

Relapsed leukemia following initial therapeutic response and remission is difficult to treat and causes high patient mortality. Leukemia relapse is due to residual quiescent leukemia cells that escape conventional therapies and later reemerge. Eliminating not only growing but quiescent leukemia cells is critical to effectively treating leukemia and preventing its recurrence. Such therapeutic agents, however, are lacking in the clinic. Here we report that a 4-arylmethyl derivative of the natural anticancer compound curcumin demonstrates a dual effect in eliminating both growing and quiescent leukemia cells. This curcumin derivative, C212, on the one hand, inhibits growing leukemia cells at a higher efficacy than curcumin by inducing apoptosis and cell cycle arrest; it, on the other hand, kills quiescent leukemia cells that are resistant to conventional chemotherapy drugs. Furthermore, C212 drives leukemia cells into and kills them at deep quiescence, avoiding the potential risk associated with awaking therapy-resistant subpopulation of quiescent leukemia cells. Lastly, we show that C212 induces apoptosis and drives cells into deep dormancy at least partially by binding to and inhibiting Hsp90, leading to client protein degradation and protein aggregation. Further elucidating the molecular mechanisms underlying the dual function of C212 in eliminating both growing and quiescent leukemia cells will aid the development of novel therapies against leukemia relapse.

Keywords: Leukemia, relapse, quiescence, dormancy, curcumin derivative, Hsp90, apoptosis, protein aggregation

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Introduction

Conventional anti-cancer drugs target fast-growing cells and induce cell death or cell cycle arrest. These drugs are ineffective against slow-growing or quiescent cancer cells that do not exhibit active DNA replication and cell division. Accordingly, quiescent cancer cells, including but not limited to cancer stem cells, escape conventional chemotherapies; they often reemerge after a period of dormancy, causing cancer relapse and metastasis that are exceedingly difficult to treat and leading to high patient mortality[1-3].

The ability to target and eliminate both growing and quiescent cancer cells is critical to treating cancer and preventing cancer recurrence. We now have a long list of chemotherapy drugs that kill growing cancer cells, but still lack therapeutic agents that effectively eliminate quiescent cancer cells in the clinic. How to eradicate quiescent slow-growing cancer cells has only begun to be explored, primarily from metabolic and epigenetic angles[4-7]. Recently, we have identified two natural compounds, ergosterol peroxide and ganodermanondiol, from the medical mushroom *Ganoderma lucidum*. We have shown that these two compounds eliminate quiescent slow-cycling cells by pushing cells to shallow quiescence and exposing them to cytotoxic effects[8]. Awaking quiescent cancer cells to kill presents a potential strategy to prevent cancer recurrence[9]. However, if some of these quiescent cancer cells develop and acquire therapy resistance during dormancy[10, 11], waking them can be risky.

In this study, we show that a curcumin derivative, C212, exhibits potent cytotoxicity against not only growing leukemia cells but quiescent counterparts without waking them up. Curcumin (diferuloylmethane) is the primary natural polyphenol found in the rhizome of *Curcuma longa* (turmeric) and others Curcuma spp[12]. It exhibits pharmacological effects on metabolic,

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cardiovascular, neurological, hepatic, and respiratory disorders[12, 13]; it also inhibits cancer growth in the reproductive, digestive, urinary, pulmonary, nervous, skeletal, skin, lymphatic, and immune systems, attributing to its immunomodulatory, anti-inflammatory, antioxidant, pro-apoptotic, and antiangiogenic properties[14, 15]. At the molecular level, curcumin interacts with multiple cellular pathways: it inhibits NF-κB, Akt/PI3K, and MAPK pathways and enhances p53 activity, to name a few[15, 16].

Recent work, including ours, showed that curcumin suppresses tumor growth by inhibiting heat shock protein 90 (Hsp90) and down-regulating Hsp90 client proteins[17-19]. Hsp90 is a molecular chaperone that is highly conserved and abundant in cells of virtually every organism; it interacts with and assists the proper folding of a large and diverse group of client proteins that are critical to cellular functions, many of which are kinases and transcription factors[20]. Increased activities of Hsp90 and client proteins enhance cellular homeostasis against stressful conditions—and in the case of cancer, enhance cancer cell survival[21, 22]. Accordingly, small molecular drugs that target Hsp90 have shown anticancer effects[23-25].

Following the anticancer effect of curcumin, especially in inhibiting Hsp90, we have designed and synthesized curcumin derivatives that preserve Hsp90 inhibition and increase compound bioavailability and anticancer efficacy[19, 26, 27]. Here we report that a novel curcumin derivative C212 binds to Hsp90 and inhibits Hsp90 function, inducing client protein degradation and cell apoptosis. C212 exhibits a dual function in eliminating both growing and quiescent cancer cells, as we show in two leukemia cell lines HL-60 and K562. Furthermore, C212 eliminates quiescent leukemia cells in their deep dormancy and thus avoids the risk of prematurely waking them up, presenting an attractive approach to prevent leukemia recurrence.

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Result:

C212 inhibits growing cancer cell viability by inducing apoptosis and G2/M arrest

C212, (1E,6E)-4-(2-Chlorinebenzyl)-1,7-bis(3-pyridine)-1,6-heptadiene-3,5-dione (Fig. S1A), was synthesized as one of several 4-arylmethyl curcumin analogues in our previous study[28]. Here we applied C212 and curcumin to a variety of cancer cell lines, including leukemia (HL-60 and K562), colon cancer (HCT116, SW620, and HT29), breast cancer (MCF-7), gastric cancer (SGC7901), and liver cancer (HepG2). We found that both C212 and curcumin dose-dependently suppressed cell viability in these cancer cell lines as tested in MTS assays (Fig. 1 A-H). Furthermore, C212 exhibited a lower IC50 (the concentration required to inhibit cell growth by 50%) than that of curcumin in each tested cell line, showing significantly stronger anticancer effects (Fig. 1 A-H and Table 1). Among the tested cell lines, leukemia cells (HL-60 and K562) as a group were the most sensitive to C212 (Table 1) and thus were focused on in the further study.

The inhibitory effect of C212 on leukemia cell viability is at least twofold. First, C212 dose-dependently induced apoptosis in HL-60 and K562 cells. With an increasing C212 concentration, 1) the subpopulation of cells exhibiting the characteristic externalization of inner membrane phospholipids (Annexin V-positive) and membrane compromise (PI-positive) increased monotonically in K562 (Fig. 2A, top) and HL-60 cells (Fig. 2A, bottom); 2) the subpopulation of cells exhibiting the characteristic depolarized mitochondrial membrane potential (with reduced red fluorescence of JC-1 staining) increased monotonically in K562 (Fig. 2B, top) and HL-60 cells (Fig. 2B, bottom); 3) the amounts of released cytochrome c and cleaved forms of PARP and caspase-9, caspase-3 and caspase-7 increased in K562 (Fig. 2C) and HL-60 cells (Fig. 2D). Second, C212 dose-dependently arrested the cell cycle. With an increasing C212 concentration, the subpopulation

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of cells arrested at the G2/M phase increased monotonically in K562 (Fig. S1B, top) and HL-60 cells (Fig. S1B, bottom), consistent with the correspondingly downregulated Cdc2 and Cyclin B1 protein levels in the cell (Fig. S1C).

C212 eliminates quiescent leukemia cells in contrast to chemotherapy drugs

Different from growing cells, quiescent cells are resistant to conventional chemotherapies that target cell proliferation machinery such as DNA replication and cell division. To test the cytotoxicity of C212 on quiescent leukemia cells, we first induced HL-60 and K562 cells to quiescence or a slow-growing state by serum starvation: a 12-hour serum starvation reduced the percentage of actively replicating (EdU+%) HL-60 cells from 98.3% in growing condition to 8.1% (Fig. 3A); for K562 cells, which appeared to be less sensitive to serum growth signals than HL-60, a 36-hour serum starvation reduced EdU+% from 98.8% in growing condition to 22.5% (Fig. 3B). As expected, chemotherapy drugs were inefficient in killing quiescent and slow-growing leukemia cells: the lethal concentrations to kill half of quiescent cell population (LC50q) with paclitaxel, topotecan, and doxorubicin treatments were typically several folds higher than those of proliferating cell population (LC50p) in both HL-60 (Fig 3 C-E and Table 2) and K562 cells (Fig 3 G-I and Table 2). By contrast, C212 exhibited the same LC50q and LC50p (2.6 μM) in HL-60 cells, suggesting its equal effectiveness in killing both quiescent and proliferating HL-60 cells. Moreover, C212 showed even stronger cytotoxicity in quiescent K562 cells than proliferating counterparts—its LC50q (2.7 μM) was about 3-fold lower than LC50p (7.6 μM). Put together, C212 appeared to effectively eliminate quiescent leukemia cells in contrast to commonly used chemotherapy drugs.

C212 drives leukemia cells into deep quiescence

It has been previously reported that arsenic trioxide (As2O3) and certain cytokines (IFN-α, G-CSF)

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were able to sensitize quiescent leukemia cells to chemotherapy cytotoxicity by reducing quiescence depth and waking them up[29]. Correspondingly, we examined whether C212 altered cell quiescence depth and killed quiescent leukemia cells. To measure quiescence depth, we first induced leukemia cells into quiescent or a slow-growing state by serum starvation (for 12 hours in HL-60 and for 36 hours in K562, same as in Fig. 3); we then stimulated cells with serum (2.5%) and checked the percentages of cells that exited quiescence and re-entered the cell cycle (EdU+): deeper quiescent cells would be harder to exit quiescence and thus have smaller EdU+% than shallower cells. When C212 was included in the last segment of serum starvation and through the serum stimulation phase (indicated with a red dash line, Fig 4 A and B), the EdU+% upon serum stimulation decreased in a C212 dose-dependent manner in HL-60 cells (Fig. 4A), from 50.5% (0 μM) to 13.3% (1.5 μM, sublethal according to Fig. 3F); in K562 cells that are less sensitive to serum signals than HL-60, the relatively high EdU+% background in serum starvation condition decreased in a C212 dose-dependent manner, as well as the EdU+% upon serum stimulation (Fig. 4B) which decreased from 33.5% (0 μM) to 8.3% (2 μM, sublethal according to Fig. 3J). These results indicated that C212 treatment at increasing sublethal doses drove leukemia cells into deeper quiescence, and thus a smaller subpopulation of quiescent cells was able to exit quiescence and re-enter the cell cycle given the same serum-stimulation condition (2.5%).

We further examined whether the effect of C212 to drive leukemia cells to deeper quiescence was exerted during serum starvation or stimulation or both. Our previous studies showed that quiescence depth is determined by the serum threshold to activate an Rb-E2F bistable gene switch in the cell; such a serum threshold can be modulated by various cellular activities in quiescence (during serum starvation) and quiescence exit (serum stimulation)[30, 31]. When C212 was included in the last

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segment of serum starvation but not the serum stimulation phase, cell cycle re-entry was not affected in HL-60 cells (EdU+% upon serum stimulation showed no statistically significant difference with or without C212; Fig. 4C) and was affected only slightly in K562 cells (EdU+% upon serum stimulation showed a statistically significant difference only at 2 μM C212 over control; Fig. 4D). When C212 was included in the serum stimulation phase but not in the last segment of serum starvation, cell cycle re-entry was affected significantly in both HL-60 and K562 cells and EdU+% upon serum stimulation decreased in a C212 dose-dependent manner (Fig. 4 E and F). Put together, our data suggested that C212 drove leukemia cells into deep quiescence, and it exerted this quiescence-deepening effect primarily by increasing the serum threshold during quiescence exit.

C212 inhibits Hsp90 inducing client protein degradation and protein aggregation

As found in our previous work[17], curcumin binds to the α-helix domain of Hsp90 at multiple residues (Fig. S2 A and C) and inhibits the function of Hsp90 in properly folding client proteins—e.g., kinases that participate in cell proliferation. C212 was also predicted to bind to Hsp90 based on molecular docking analysis between C212 and the C- and N-terminus of Hsp90 (Fig. S2 B and D). When we carried out fluorescence quenching experiments with purified Hsp90 protein, we found that C212 dose-dependently quenched the intrinsic fluorescence of Hsp90 (excitation/emission = 280/337 nm), which was accompanied with blueshifts of λem curves (Fig. 5A). Specifically, the titration curves of C212 with the N-terminus, middle (M-)region, and C-terminus of Hsp90 yielded estimated dissociation constants (K_d) of 36.3, 36.9, and 15.3, respectively (Table 3), suggesting that C212 interacted with Hsp90.

Through binding to Hsp90, C212, like curcumin, was expected to interfere with the Hsp90 function in folding and stabilizing client proteins. Indeed, in both K562 and HL-60 cells treated with

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C212, protein levels of Hsp90 clients Raf, Akt, Erk, and Mek, particularly in their phosphorylated forms, decreased in a C212 dose-dependent manner (Fig. 5 B & C). The C212-induced decreases of Hsp90 client protein levels were at least partially dependent on proteasome-mediated degradation, which could be rescued by co-treating cells with a proteasome inhibitor MG132 (Fig. S2E).

We also found that C212 treatment induced the formation of protein aggregates. As seen in both HL-60 (Fig. 5D) and K562 cells (Fig. 5E) induced to quiescence by serum starvation, the degree of protein aggregation (as measured by ProteoStat staining) increased in a C212 dose-dependent manner. This result was consistent with the notion that C212 interferes with Hsp90 function, which may lead to the accumulation of misfolded proteins. Non-degraded misfolded protein and aggregates are linked to diseases such as aging-related neurodegeneration and systemic amyloidosis[32-34]. Very recently, protein aggregates have been identified to drive deep cellular dormancy in both bacteria and neural stem cells[35, 36]. The role of C212 to inhibit Hsp90 and induce protein aggregation thus likely contributes to its role inducing deep quiescence in leukemia cells (Fig. 5F).

Discussion

In this study, we found that C212, a 4-arylmethyl curcumin derivative demonstrates a dual anticancer effect: it not only inhibits the viability of growing cancer cells by inducing apoptosis and G2/M cell cycle arrest, but eliminates quiescent leukemia cells that are resistant to conventional chemotherapy drugs such as paclitaxel, doxorubicin, and topotecan that preferentially kill fast-growing cells. This dual effect of C212 presents a novel class of cancer therapeutic strategy, particularly in preventing leukemia recurrence caused by residual quiescent leukemia cells.

Quiescence is a reversible cellular dormancy state that can persist over prolonged periods. Leukemia relapse, despite initial therapeutic responses and remissions, is often due to the

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reemergence of residual quiescent cells, including leukemia stem cells[19, 37-40]. Therefore, effective therapies targeting and eliminating quiescent leukemic cells are critical to preventing leukemia relapse. In this regard, cytokines such as IFN-α and G-CSF, as well as arsenic trioxide As2O3, have been shown to wake up quiescent leukemia stem cells and sensitize them to chemotherapy[29]. However, some quiescent leukemia cells during long-term dormancy under stressful conditions can undergo genetic or epigenetic changes and further develop resistance to follow-up therapies[10, 11]. Awakening these quiescent leukemia cells will cause a risk.

C212 presents a safer strategy to prevent leukemia relapse as it pushes quiescent leukemia cells into and kills them at a deep dormancy, instead of waking them up. The effect of C212 to induce deep quiescence is at least partially due to its binding to Hsp90 and inhibiting Hsp90 function. Hsp90 is an evolutionarily conserved molecular chaperone that participates in stabilizing and activating more than 200 client proteins[20, 41]. Inhibiting Hsp90 function leads to misfolded client proteins that are often insoluble and aggregate under physiological conditions. Accumulation of protein aggregates has been recently found to drive deep cell dormancy in both neural stem cells and bacteria[35, 36]. Consistently, C212 dose-dependently induces protein aggregation (Fig. 5 D and E) and drives quiescent leukemia cells into deeper dormancy at sublethal doses (Fig. 4).

Previously, we have found that by inhibiting Hsp90, curcumin and its derivatives induce the degradation of client protein $P210^{bcr/abl}$ and correspondingly the growth arrest and apoptosis of leukemia cells[17, 19, 27]. Similarly, C212 binds to and inhibits Hsp90, induces the degradation of Hsp90 client proteins in a dose-dependent manner in leukemia cell lines K562 and HL-60 (Fig. 5 B and C). Consistent with the apoptosis-inducing effect of inhibiting Hsp90[42], C212 induces apoptosis in K562 and HL-60 cells (Fig. 2).

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Inhibiting Hsp90 does not solely account for the effects of C212 in eliminating quiescent leukemia cells. When we targeted to inhibit Hsp90 using 17-AAG, a classic Hsp90 inhibitor, we found proliferating cells, instead of quiescent cells, were preferentially eliminated in both leukemia cell lines K562 and HL-60 (Fig. S3). This result suggests that the preferential killing effects of C212 on quiescent leukemia cells involve cellular targets beyond Hsp90, which is consistent with a broad spectrum of molecular targets known to curcumin[15, 16]. Further identifying the molecular mechanisms of C212 in eliminating both quiescent and growing leukemia cells will help further develop and improve treatment strategies against leukemia relapse.

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Materials and methods:

Reagents. C212 was synthesized in our laboratory as described previously[28]. Paclitaxel was purchased from LC Laboratories (P-9600), Topotecan from Sigma (T2705), Doxorubicin from Cayman (15007), and 17-AAG from APExBIO (A405410). The cloning, expression, and purification of the histidine (His)-targeted yeast full-length Hsp90 (1-732, 90 kDa), N-terminus of Hsp90 (N-Hsp90, 1-236, 25 kDa), middle region of Hsp90 (M-Hsp90, 272-617, 40 kDa), and C-terminus of Hsp90 (C-Hsp90,629-732, 15 kDa) were performed as described in previous work[43].

Cell culture and Quiescence entry/exit. Human leukemia cell lines K562 and HL-60 were cultured and passaged in RPMI-1640 medium (Corning, 10040CV) supplemented with 10% bovine growth serum (BGS; Hyclone, SH30541.03). To induce quiescence or slow-growing state, growing leukemia cells were spun down, washed once with and plated (in 12-well plates) in the starvation medium: HL-60, serum-free Dulbecco's Modified Eagle Medium (DMEM; Corning, 15-013-CV, without glutamine); K562, serum- and amino acid-free Earle's balanced salt solution EBSS (Gibco, 24010043). HL-60 cells were then cultured in starvation medium for 12 hours, and K562 cells for 36 hours. To induce quiescence exit and cell cycle re-entry, cells were switched to serum stimulation medium: HL-60, DMEM (with glutamine) containing 2.5% BGS; K562, EBSS containing 2.5% BGS.

Cell viability MTS assay. Leukemia cells were seeded in 96-well plates and cultured in 100 µl medium for the time indicated. Cells were then treated with C212 or chemotherapy drugs for 48 hours; 20 µl CellTiter stock solution (Promega, G3510) was added into each well, followed by a 3-hour incubation at 37 °C. The absorbance of each well was measured at 490 nm, with the absorbance of wells containing medium and CellTiter only set as the background control ($A_{background}$)

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and the absorbance of wells containing cells treated with vehicle set as the vehicle control ($A_{control}$). Cell viability = $(A_{treatment} - A_{background})/(A_{control} - A_{background})*100\%$.

Cell proliferation assays. To assess DNA content, leukemia cells were spun down and resuspended in 500 µl 70% ethanol for overnight at -20 ℃; cells were then washed once with 300 µl cold DPBS, resuspended in 300 µl DNA-staining solution containing propidium iodide (PI; Sigma, P4170) and RNase A (Sigma, R6513) at final concentrations of 50 µg/ml in DPBS, incubated in the dark for 30 min at 37 ℃, followed by PI intensity measurement using flow cytometry. To assess EdU incorporation during DNA synthesis after cell cycle re-entry from quiescence, 2 µM EdU was included in serum stimulation medium for 43 and 24 hours, respectively, for HL-60 and K562 cells, followed by click-iT EdU assay according to the manufacturer's protocol (Life Technologies, C10418)[30]; EdU intensity was measured using flow cytometry.

Apoptosis assays. The annexin V/FITC-PI staining assay was carried out according to the manufacturer's protocol (BD, 556547). Briefly, leukemia cells were spun down, washed once in DPBS, resuspended in 100 µL Annexin-V/PI staining buffer, and kept in the dark for 15 min at 37 ℃; cells were then washed once with 200 µl wash buffer provided by the kit, followed by FITC-PI fluorescence intensity measurement using flow cytometry. To assess mitochondrial membrane potential (MMP) depolarization during early apoptosis, leukemia cells were spun down, resuspended in 300 µL JC-1 staining solution (KeyGEN Biotech, KGA603), and kept in the dark at room temperature for 10 min; cells were washed twice with the wash buffer provided by the kit, followed by JC-1 fluorescence intensity measurement using flow cytometry. JC-1 is a cationic dye that accumulates in mitochondria and forms red fluorescent aggregates at high MMP; a decrease of JC-1 red fluorescence indicates MMP depolarization.

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Western blot. Whole-cell lysates were prepared with RIPA lysis buffer as described previously[19]. Approximately 40 mg of protein lysate per sample was loaded and separated in 8-12% SDS-PAGE gel and then transferred to PVDF membrane (Roche, 03010040001); membrane was blocked with 5% non-fat dry milk for 1 hour and then incubated with primary antibodies at 4 °C overnight, followed by secondary antibody incubation for 2 hours at room temperature and imaging. Primary antibody were purchased from Cell Signaling Technology (CST): Caspase 9 (9508S), Caspase 7 (9492S), Caspase 3 (9662S), Cleaved-Caspase 9 (7237S), Cleaved-Caspase7 (8438S), Cleaved-Caspase 3 (9664S), Akt (4691S), p-AKT (4060S), Raf 9422S), p-Raf (9421S), Mek (4694S), p-Mek (2338S), Erk (4695S), p-Erk (4377S), Cyclin B1 (4135S), Cdc2 (9116S), p-Cdc2 (4539S), β-actin (4970S), β-actin (MA1-140); secondary antibody goat anti-rabbit IgG-HRP was purchased from Santa Cruz Biotechnology (sc-7074).

Hsp90-interaction analysis. For the molecular docking analysis, chain A of the crystal structure of an Hsp90-Sba1 closed chaperone complex was extracted from Protein Data Bank (PDB, ID: 2CG9), from which structures of Hsp90 C- and N-terminus were derived[44]; the bindings between Hsp90 domains and curcumin/C212 were simulated using the Surflex-Dock program (SYBYL-X v1.3). For the quenching assay of intrinsic Hsp90 fluorescence, a C212 solution (0 to 50 μM in 0.2% DMSO) was successively added into 2.0 mL Hsp90 solution (5.0 μM in PBS, pH 7.6); fluorescence intensity was recorded from 290 to 500 nm at 303 Kelvin using a Cary Eclipse spectrofluorometer (Varian/Agilent).

Protein aggregation assay. The accumulation of protein aggregates in the cell was assessed using the ProteoStat Aggresome detection kit (Enzo, ENZ-51035-K100) according to the manufacturer's protocol. Briefly, leukemia cells were spun down, fixed with 200 μl 4% paraformaldehyde at room

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temperature for 20 min, and permeabilized with 200 µl assay buffer containing 0.5% Triton-X100 and 3 mM EDTA in PBS on ice for 20 min. ProteoStat staining solution was added to cell suspension at 1:5000 and incubated at room temperature for 30 min, followed by ProteoStat fluorescence measurement using flow cytometry.

Statistical analysis. Data were analyzed using Prism 6.0 (GraphPad) and Origin 8.5 (OriginLab). Statistical significance was determined in 1-tailed *t* test.

Disclosure statement:

The authors declare no competing interest.

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Reference

1. Senft, D.; Jeremias, I., Tumor Cell Dormancy-Triggered by the Niche. *Dev Cell* 2019, 49, 311-312.
2. Aguirre-Ghiso, J. A.; Sosa, M. S., Emerging Topics on Disseminated Cancer Cell Dormancy and the Paradigm of Metastasis. *Annual Review of Cancer Biology* 2018, 2, 377-393.
3. Summers, M. A.; McDonald, M. M.; Croucher, P. I., Cancer Cell Dormancy in Metastasis. *Cold Spring Harbor perspectives in medicine* 2019.
4. Lemons, J. M.; Feng, X. J.; Bennett, B. D.; Legesse-Miller, A.; Johnson, E. L.; Raitman, I.; Pollina, E. A.; Rabitz, H. A.; Rabinowitz, J. D.; Coller, H. A., Quiescent fibroblasts exhibit high metabolic activity. *PLoS biology* 2010, 8, e1000514.
5. Roesch, A.; Vultur, A.; Bogeski, I.; Wang, H.; Zimmermann, K. M.; Speicher, D.; Korbel, C.; Laschke, M. W.; Gimotty, P. A.; Philipp, S. E.; Krause, E.; Patzold, S.; Villanueva, J.; Krepler, C.; Fukunaga-Kalabis, M.; Hoth, M.; Bastian, B. C.; Vogt, T.; Herlyn, M., Overcoming intrinsic multidrug resistance in melanoma by blocking the mitochondrial respiratory chain of slow-cycling JARID1B(high) cells. *Cancer cell* 2013, 23, 811-25.
6. Sharma, S. V.; Lee, D. Y.; Li, B.; Quinlan, M. P.; Takahashi, F.; Maheswaran, S.; McDermott, U.; Azizian, N.; Zou, L.; Fischbach, M. A.; Wong, K. K.; Brandstetter, K.; Wittner, B.; Ramaswamy, S.; Classon, M.; Settleman, J., A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell* 2010, 141, 69-80.
7. Viale, A.; Pettazzoni, P.; Lyssiotis, C. A.; Ying, H.; Sanchez, N.; Marchesini, M.; Carugo, A.; Green, T.; Seth, S.; Giuliani, V.; Kost-Alimova, M.; Muller, F.; Colla, S.; Nezi, L.; Genovese, G.; Deem, A. K.; Kapoor, A.; Yao, W.; Brunetto, E.; Kang, Y.; Yuan, M.; Asara, J. M.; Wang, Y. A.; Heffernan, T. P.; Kimmelman, A. C.; Wang, H.; Fleming, J. B.; Cantley, L. C.; DePinho, R. A.; Draetta, G. F., Oncogene ablation-resistant pancreatic cancer cells depend on mitochondrial function. *Nature* 2014, 514, 628-32.
8. Dai J, M. M., Everetts NJ, et al. , Elimination of quiescent slow-cycling cells via reducing quiescence depth by natural compounds purified from Ganoderma lucidum. *oncotarget* 2017.
9. Fujimaki, K.; Yao, G., Crack the state of silence: Tune the depth of cellular quiescence for cancer therapy. *Molecular & cellular oncology* 2018, 5, e1403531.
10. De Angelis, M. L.; Francescangeli, F.; La Torre, F.; Zeuner, A., Stem Cell Plasticity and Dormancy in the Development of Cancer Therapy Resistance. *Frontiers in oncology* 2019, 9, 626.
11. Rossari, F.; Zucchinetti, C.; Buda, G.; Orciuolo, E., Tumor dormancy as an alternative step in the development of chemoresistance and metastasis - clinical implications. *Cellular Oncology* 2019.
12. Esatbeyoglu, T.; Huebbe, P.; Ernst, I. M.; Chin, D.; Wagner, A. E.; Rimbach, G., Curcumin—from molecule to biological function. *Angewandte Chemie International Edition* 2012, 51, 5308-5332.
13. Pulido-Moran, M.; Moreno-Fernandez, J.; Ramirez-Tortosa, C.; Ramirez-Tortosa, M., Curcumin and Health. *Molecules* 2016, 21, 264.
14. Anand, P.; Sundaram, C.; Jhurani, S.; Kunnumakkara, A. B.; Aggarwal, B. B., Curcumin and cancer: an "old-age" disease with an "age-old" solution. *Cancer Lett* 2008, 267, 133-64.
15. Shanmugam, M. K.; Rane, G.; Kanchi, M. M.; Arfuso, F.; Chinnathambi, A.; Zayed, M. E.; Alharbi, S. A.; Tan, B. K.; Kumar, A. P.; Sethi, G., The multifaceted role of curcumin in cancer prevention and treatment. *Molecules* 2015, 20, 2728-69.
16. Kunnumakkara, A. B.; Anand, P.; Aggarwal, B. B., Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. *Cancer Letters* 2008, 269, 199-225.
17. Wu, L. X.; Xu, J. H.; Huang, X. W.; Zhang, K. Z.; Wen, C. X.; Chen, Y. Z., Down-regulation of p210(bcr/abl) by curcumin involves disrupting molecular chaperone functions of Hsp90. *Acta pharmacologica Sinica* 2006, 27, 694-9.

18. Giommarelli, C.; Zuco, V.; Favini, E.; Pisano, C.; Dal Piaz, F.; De Tommasi, N.; Zunino, F., The enhancement of antiproliferative and proapoptotic activity of HDAC inhibitors by curcumin is mediated by Hsp90 inhibition. *Cellular and molecular life sciences* 2010, 67, 995-1004.

19. Wu, L.; Yu, J.; Chen, R.; Liu, Y.; Lou, L.; Wu, Y.; Huang, L.; Fan, Y.; Gao, P.; Huang, M.; Wu, Y.; Chen, Y.; Xu, J., Dual inhibition of Bcr-Abl and Hsp90 by C086 potently inhibits the proliferation of imatinib-resistant CML cells. *Clinical cancer research* 2015, 21, 833-43.

20. Taipale, M.; Krykbaeva, I.; Koeva, M.; Kayatekin, C.; Westover, K. D.; Karras, G. I.; Lindquist, S., Quantitative analysis of HSP90-client interactions reveals principles of substrate recognition. *Cell* 2012, 150, 987-1001.

21. Trepel, J.; Mollapour, M.; Giaccone, G.; Neckers, L., Targeting the dynamic HSP90 complex in cancer. *Nature reviews. Cancer* 2010, 10, 537-49.

22. Takayama, S.; Reed, J. C.; Homma, S., Heat-shock proteins as regulators of apoptosis. *Oncogene* 2003, 22, 9041-7.

23. Zhang, T.; Hamza, A.; Cao, X.; Wang, B.; Yu, S.; Zhan, C. G.; Sun, D., A novel Hsp90 inhibitor to disrupt Hsp90/Cdc37 complex against pancreatic cancer cells. *Molecular cancer therapeutics* 2008, 7, 162-70.

24. Butler, L. M.; Ferraldeschi, R.; Armstrong, H. K.; Centenera, M. M.; Workman, P., Maximizing the Therapeutic Potential of HSP90 Inhibitors. *Molecular cancer research* 2015, 13, 1445-51.

25. Solit, D. B.; Chiosis, G., Development and application of Hsp90 inhibitors. *Drug discovery today* 2008, 13, 38-43.

26. Chen, C.; Liu, Y.; Chen, Y.; Xu, J., C086, a novel analog of curcumin, induces growth inhibition and down-regulation of NFkappaB in colon cancer cells and xenograft tumors. *Cancer biology & therapy* 2011, 12, 797-807.

27. Fan, Y. J.; Zhou, Y. X.; Zhang, L. R.; Lin, Q. F.; Gao, P. Z.; Cai, F.; Zhu, L. P.; Liu, B.; Xu, J. H., C1206, a novel curcumin derivative, potently inhibits Hsp90 and human chronic myeloid leukemia cells in vitro. *Acta pharmacologica Sinica* 2018, 39, 649-658.

28. Yang Liu, M. Y., Qundan Wu, Lixian Wu and Jianhua Xu, Synthesis and Evaluation of 4-arylmethyl Curcumin Analgues as Potent Hsp90 Inhibitors. *Letters in Drug Design & Discovery* 2014, 11, 993-99.

29. Essers, M. A.; Trumpp, A., Targeting leukemic stem cells by breaking their dormancy. *Molecular oncology* 2010, 4, 443-50.

30. Kwon, J. S.; Everetts, N. J.; Wang, X.; Wang, W.; Della Croce, K.; Xing, J.; Yao, G., Controlling Depth of Cellular Quiescence by an Rb-E2F Network Switch. *Cell reports* 2017, 20, 3223-3235.

31. Yao, G.; Lee, T. J.; Mori, S.; Nevins, J. R.; You, L., A bistable Rb-E2F switch underlies the restriction point. *Nature cell biology* 2008, 10, 476-82.

32. Pratt, W. B.; Gestwicki, J. E.; Osawa, Y.; Lieberman, A. P., Targeting Hsp90/Hsp70-based protein quality control for treatment of adult onset neurodegenerative diseases. *Annual review of pharmacology and toxicology* 2015, 55, 353-71.

33. Tyedmers, J.; Mogk, A.; Bukau, B., Cellular strategies for controlling protein aggregation. *Nature reviews. Molecular cell biology* 2010, 11, 777-88.

34. Luo, W., Sun, W., Taldone, T. et al., Heat shock protein 90 in neurodegenerative diseases. *Mol Neurodegeneration* 2010, 10, 1750-1326.

35. Pu, Y.; Li, Y.; Jin, X.; Tian, T.; Ma, Q.; Zhao, Z.; Lin, S. Y.; Chen, Z.; Li, B.; Yao, G.; Leake, M. C.; Lo, C. J.; Bai, F., ATP-Dependent Dynamic Protein Aggregation Regulates Bacterial Dormancy Depth Critical for Antibiotic Tolerance. *Molecular cell* 2019, 73, 143-156 e4.

36. Leeman, D. S.; Hebestreit, K.; Ruetz, T.; Webb, A. E.; McKay, A.; Pollina, E. A.; Dulken, B. W.; Zhao, X.; Yeo, R. W.; Ho, T. T.; Mahmoudi, S.; Devarajan, K.; Passegué, E.; Rando, T. A.; Frydman, J.; Brunet, A., Lysosome activation

clears aggregates and enhances quiescent neural stem cell activation during aging. *Science* 2018, 359, 1277-1283.

37. Ebinger, S.; Ozdemir, E. Z.; Ziegenhain, C.; Tiedt, S.; Castro Alves, C.; Grunert, M.; Dworzak, M.; Lutz, C.; Turati, V. A.; Enver, T.; Horny, H. P.; Sotlar, K.; Parekh, S.; Spiekermann, K.; Hiddemann, W.; Schepers, A.; Polzer, B.; Kirsch, S.; Hoffmann, M.; Knapp, B.; Hasenauer, J.; Pfeifer, H.; Panzer-Grumayer, R.; Enard, W.; Gires, O.; Jeremias, I., Characterization of Rare, Dormant, and Therapy-Resistant Cells in Acute Lymphoblastic Leukemia. *Cancer cell* 2016, 30, 849-862.

38. Elrick, L. J.; Jorgensen, H. G.; Mountford, J. C.; Holyoake, T. L., Punish the parent not the progeny. *Blood* 2005, 105, 1862-1866.

39. Graham, S. M.; Jørgensen, H. G.; Allan, E.; Pearson, C.; Alcorn, M. J.; Richmond, L.; Holyoake, T. L., Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood* 2002, 99, 319-325.

40. Talpaz, M.; Estrov, Z.; Kantarjian, H.; Ku, S.; Foteh, A.; Kurzrock, R., Persistence of dormant leukemic progenitors during interferon-induced remission in chronic myelogenous leukemia. Analysis by polymerase chain reaction of individual colonies. *The Journal of clinical investigation* 1994, 94, 1383-9.

41. Li, J.; Soroka, J.; Buchner, J., The Hsp90 chaperone machinery: conformational dynamics and regulation by co-chaperones. *Biochimica et biophysica acta* 2012, 1823, 624-35.

42. Lanneau, D.; de Thonel, A.; Maurel, S.; Didelot, C.; Garrido, C., Apoptosis versus cell differentiation: role of heat shock proteins HSP90, HSP70 and HSP27. *Prion* 2007, 1, 53-60.

43. Chen JJ, G. Q., He XM, Yang SX, Chen C, Zhang LR. , Study on a screening model for inhibitor of Hsp90 ATPase activity. *Jounal of Xiamen University* 2010, 49, 711–6.

44. Ali, M. M.; Roe, S. M.; Vaughan, C. K.; Meyer, P.; Panaretou, B.; Piper, P. W.; Prodromou, C.; Pearl, L. H., Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex. *Nature* 2006, 440, 1013-7.

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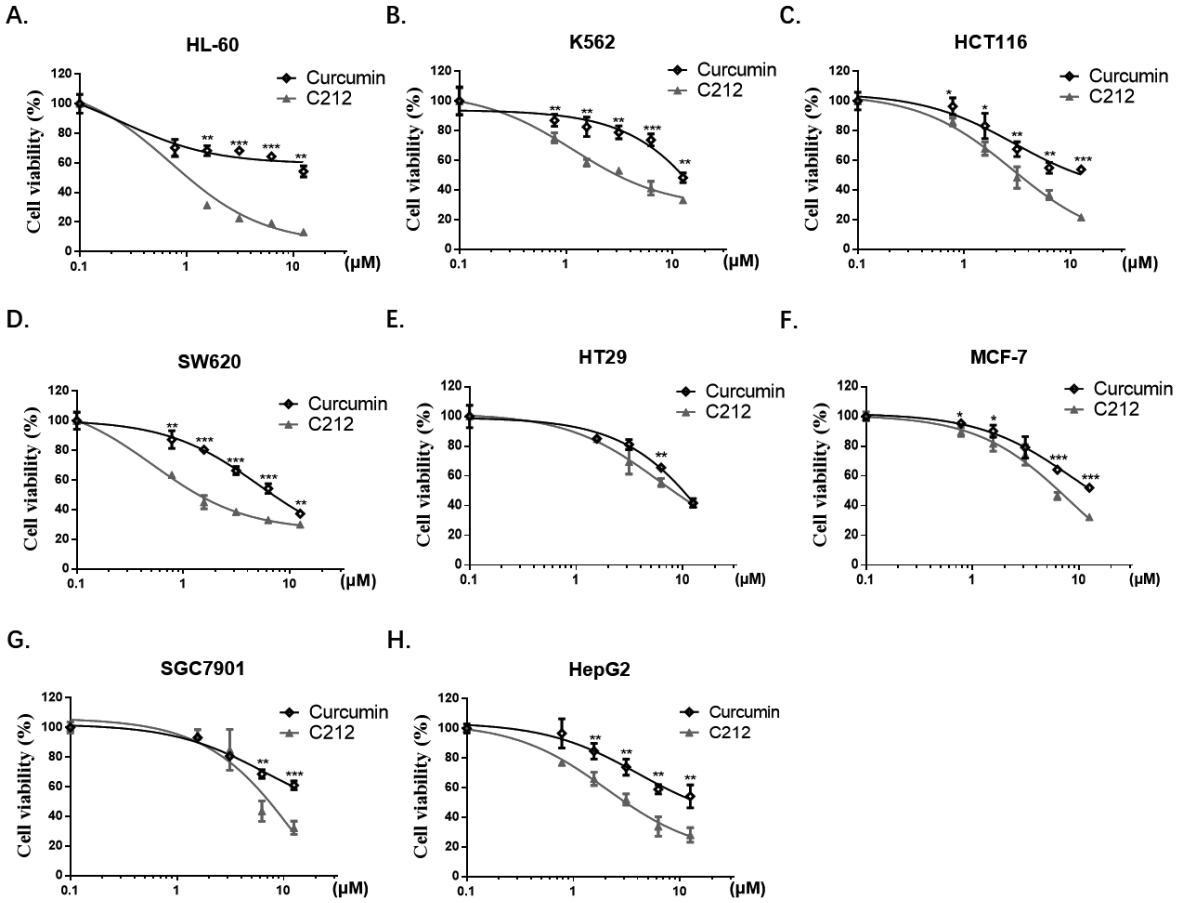

Fig. 1. C212 inhibits the viability of growing cancer cells. (*A*-*H*) Growing cancer cells seeded in 96-well plates were treated with curcumin and C212, respectively, at the indicated doses for 48 hours. Cell viability was measured with MTS assay in leukemia cell lines HL-60 and K562 (*A* and *B*), colon cancer cell lines HCT116, SW620, and HT29 (*C*, *D*, and *E*), breast cancer cell line MCF-7 (*F*), gastric cancer cell line SGC7901 (*G*), and liver cancer cell line HepG2 (*H*). Error bar, SEM (n = 3); **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (1-tailed *t* test).

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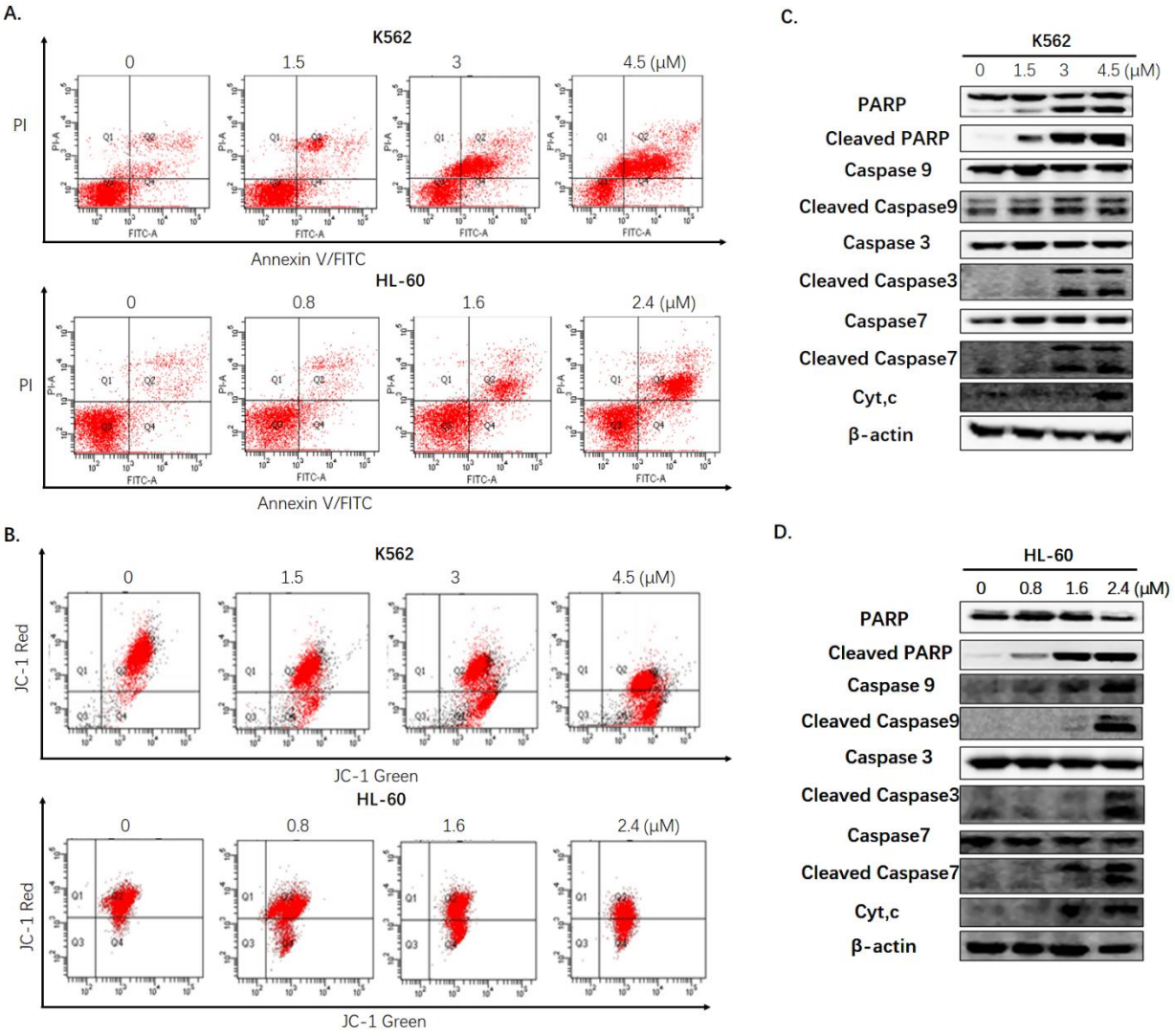

Fig. 2. C212 induces leukemia cell apoptosis. Growing leukemia cells K562 and HL-60 were treated with C212 at the indicated doses for 24 hours and subjected to apoptosis assay with Annexin-V & PI staining (*A*), mitochondrial membrane potential MMP assay with JC-1 staining (*B*), and immunoblotting of apoptosis marker proteins (in K562, *C*; in HL-60, *D*).

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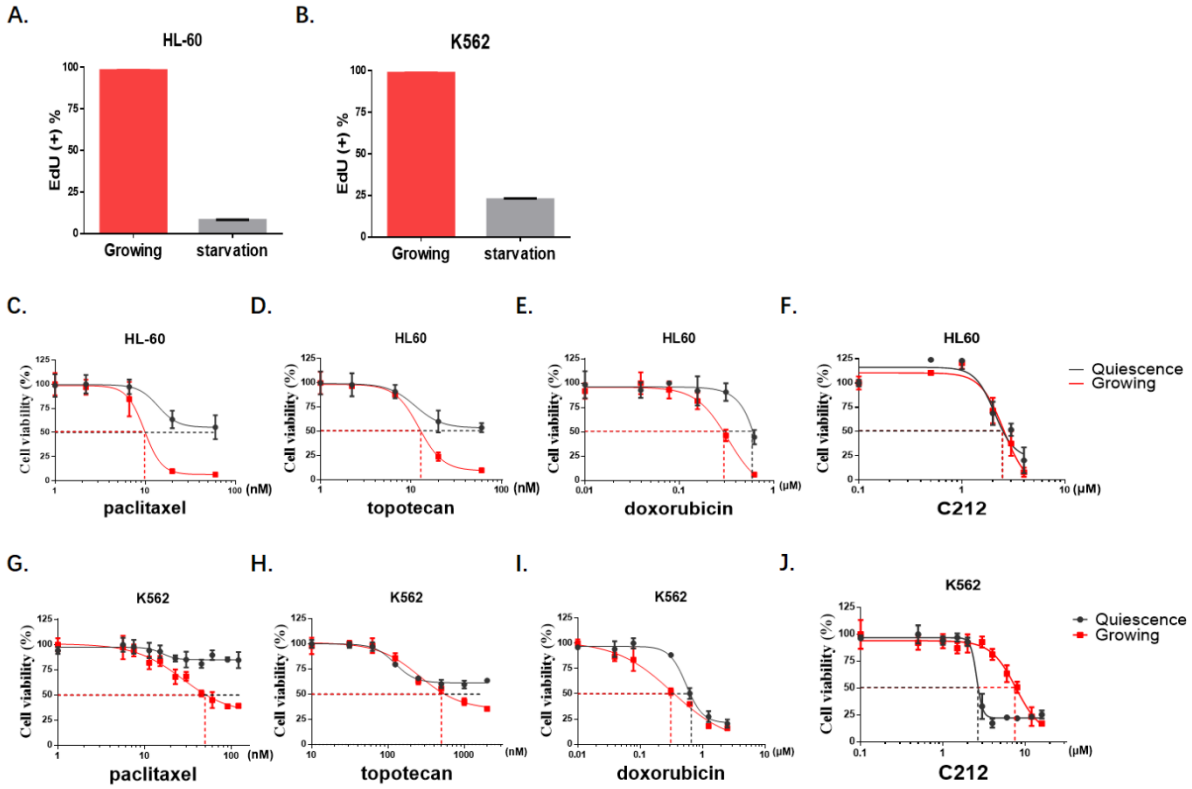

Fig. 3. C212, in contrast to chemotherapy drugs, effectively eliminates dormant leukemia cells. (*A, B*) Serum starvation-induced quiescence and slow-growing state. HL-60 (*A*) and K562 (*B*) cells were deprived of serum for 12 and 36 hours, respectively, to induce quiescence/slow-growth; EdU (2 μM) was added to the medium and cells were further incubated for 43 hours (*A*) and 24 hours (*B*), followed by a Click-iT assay to assess EdU incorporation in cells. Growing: control cells not deprived of serum but otherwise under the same treatment. Error bar, SEM (n = 2). (*C-J*) Growing and serum starvation-induced (as in *A* and *B*) quiescent cells (HL-60, *C-F*; K562, *G-J*) were treated with paclitaxel, topotecan, doxorubicin, and C212 at the indicated doses for 48 hours and subjected to MTS cell viability assay. Error bar, SEM (n = 3).

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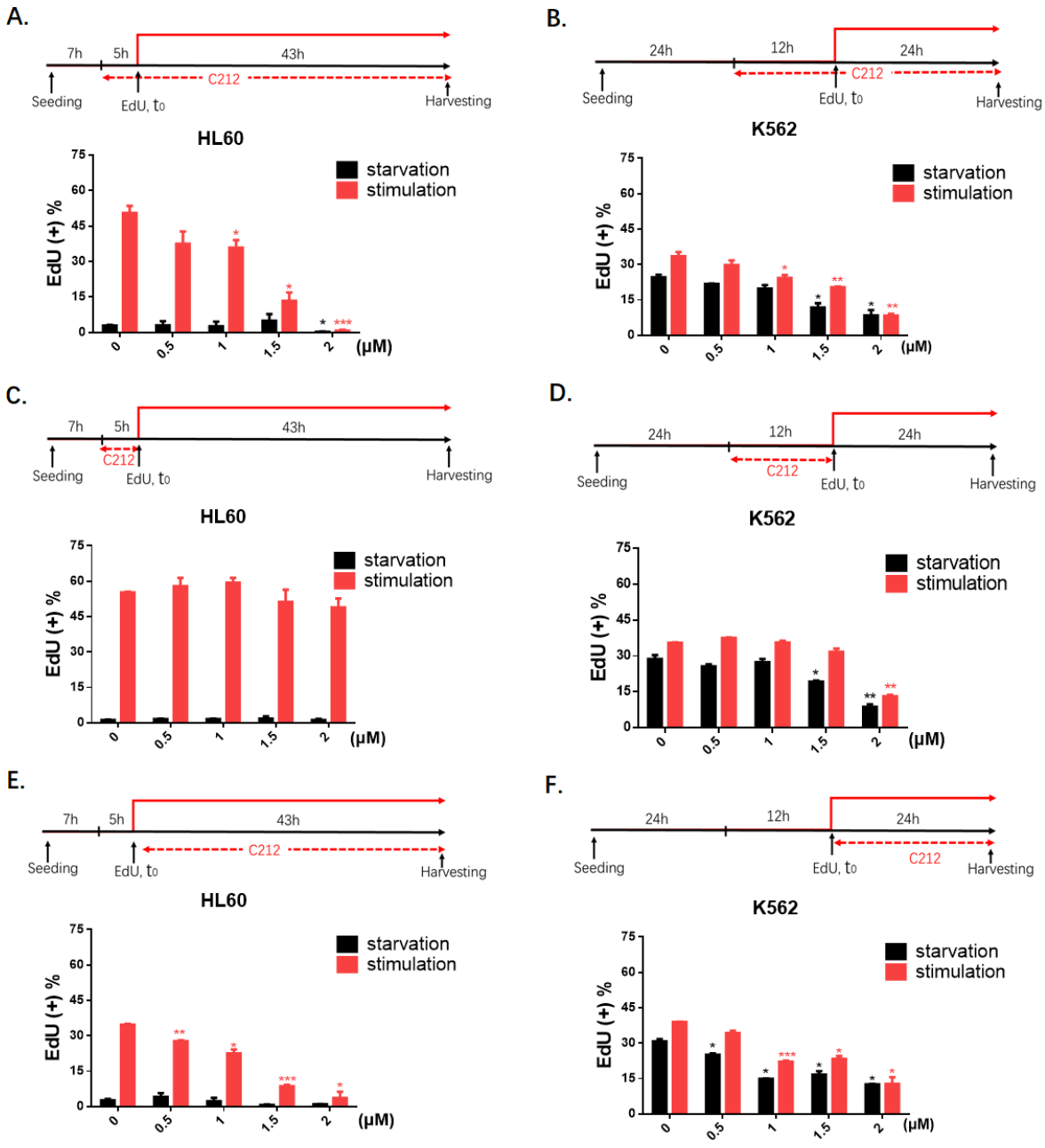

Fig. 4. C212 drives leukemia cells into deep quiescence. (*A* and *B*) Leukemia cells were serum-starved for the indicated durations to induce quiescence/slow-growth as in Fig. 3 (before t0); cells were then either kept in starvation condition (indicated by a black line) or stimulated with 2.5% serum (indicated by a red line) for 43 hours in HL-60 cells (*A*) or 24 hours in K562 cells (*B*), followed by cell harvesting and Click-iT EdU assay. C212 at indicated doses was included in the last segment of starvation and though serum stimulation, as indicated with a red dash line. EdU (2 μM) was added between t0 and harvesting. Error bar, SEM (n = 2); $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (1-tailed *t* test). (*C* and *D*) Same as *A* and *B*, except that C212 was included only in the last segment of starvation (as indicated with a red dash line). (*E* and *F*) Same as *A* and *B*, except that C212 was included only during serum stimulation (as indicated with a red dash line).

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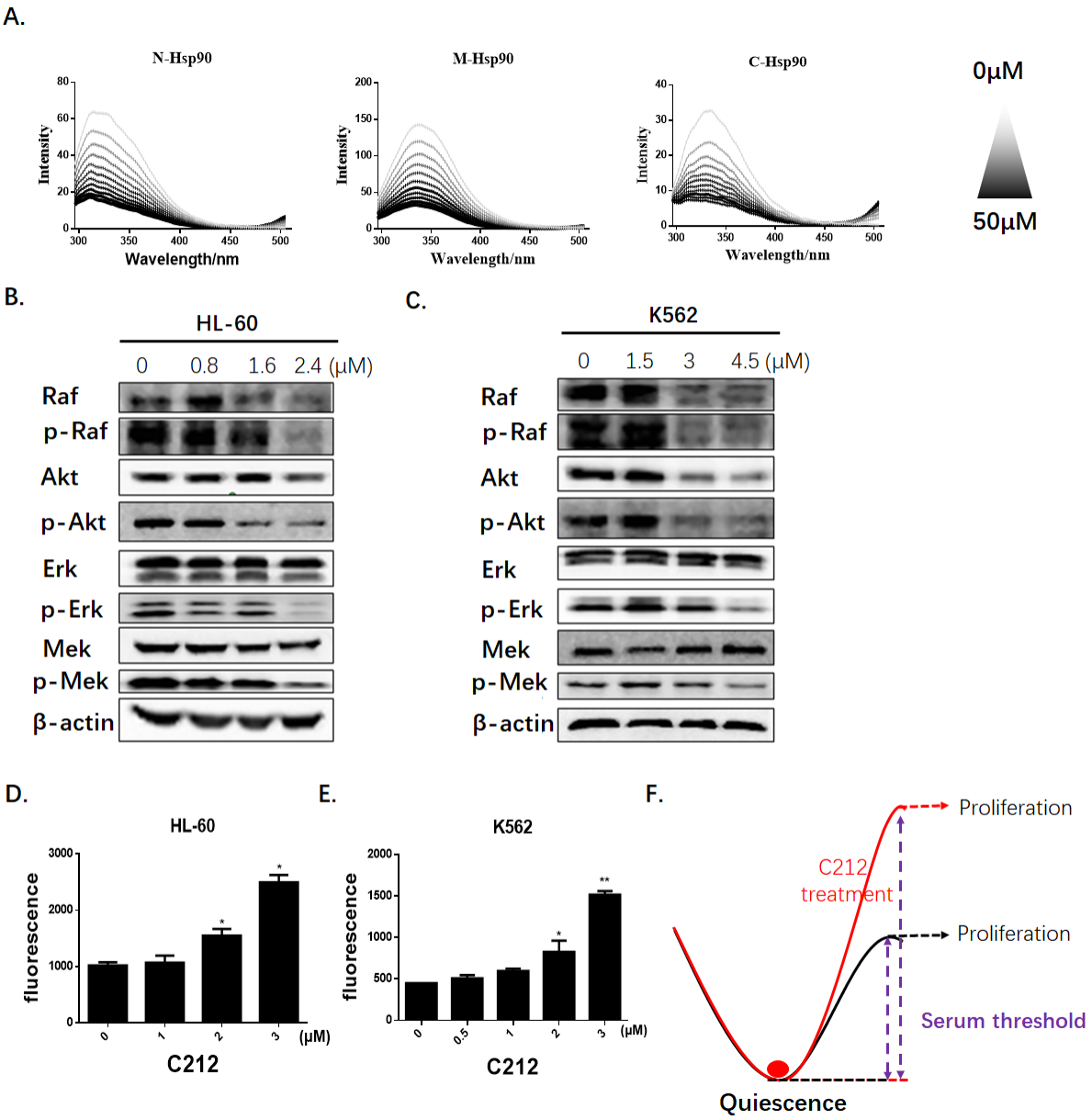

Fig. 5. C212 decreases Hsp90 client protein levels and induces protein aggregates. (*A*) Concentration-dependent quenching effect of C212 (0-50 μM) on the intrinsic fluorescence of N-, M-, and C-domains of Hsp90. Y- and X-axis indicate fluorescence intensity and emission wavelength, respectively. Each curve represents the average of triplicate measurements. (*B, C*) Leukemia cells (HL-60, *B*; K562, *C*) were treated with C212 at the indicated doses for 24 hours, followed by immunoblotting of Hsp90 client proteins in whole-cell lysate. (D, E) HL-60 (D) and K562 (*E*) cells that were serum-starved as in Fig. 3 to induce quiescence/slow-growth were further treated with C212 at the indicated doses for 30 and 36 hours, respectively, then subjected to fluorescence-based protein aggregation assay using ProteoStat. Error bar, SEM (n = 2); $*P < 0.05$, $**P < 0.01$ (1-tailed *t* test). (*F*) A proposed model of C212 that deepens quiescence of leukemia cells and increases the serum threshold for quiescence exit.

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Table 1. IC50 values of C212 and curcumin on cancer cell lines (48-hour treatment).

Cell line	C212 (μM)	Curcumin (μM)
HL-60	2.1±0.0	8.7±0.4
K562	3.6±0.1	13.8±0.8
HCT-116	3.4±0.4	18.8±0.3
SW620	1.5±0.0	7.2±0.4
HT-29	8.1±0.2	14.5±0.6
MCF-7	6.3±0.3	18.9±0.5
SGC7901	6.7±0.2	28.3±0.7
HepG2	3.4±0.1	16.7±0.4

Table 2. LC50 values of chemotherapy drugs and C212 on quiescent and growing leukemia cells (48-hour treatment).

	HL60		K562	
	Growing	Quiescent	Growing	Quiescent
Paclitaxel (nM)	10.0	>60	50	>120
Topotecan (nM)	14.2	>60	500	>2000
Doxorubicin (μM)	0.3	0.6	0.3	0.7
C212 (μM)	2.6	2.6	7.6	2.7

Table 3. Dissociation constant (Kd) values of C212 with different Hsp90 domains.

Compound	Fmax	Kd (μM)	R^2
N-Hsp90	80.8±1.1	36.3±1.0	0.999
M-Hsp90	195.8±2.5	38.7±0.9	0.999
C-Hsp90	32.8±0.9	15.3±1.2	0.995

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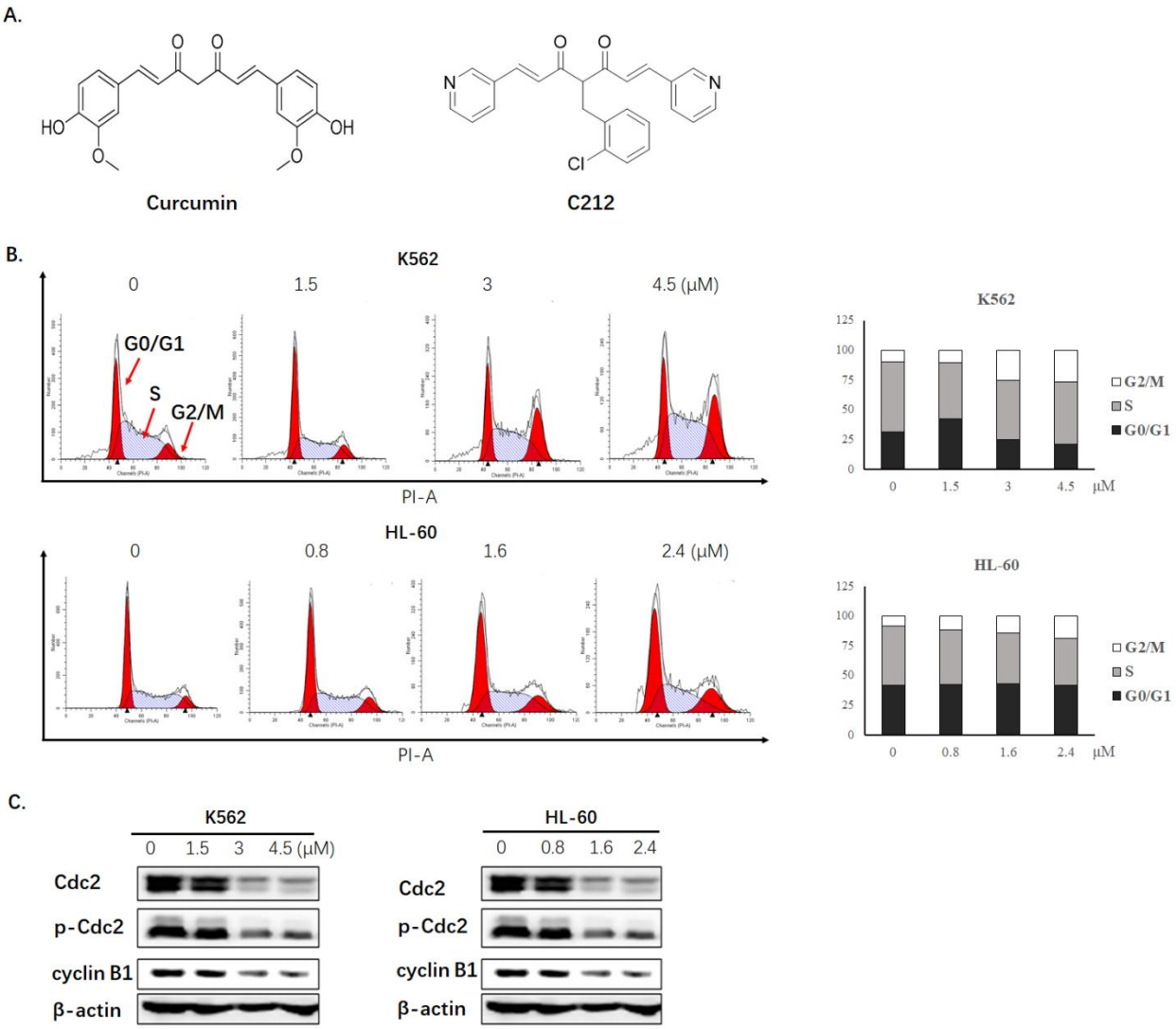

Fig. S1. (*A*) Chemical structures of curcumin and C212. (*B* and *C*) Growing leukemia cells were treated with C212 at the indicated doses for 24 hours and subjected to cell-cycle analysis with PI staining of DNA content (*B*; top, K562; bottom, HL-60) and immunoblotting of Cdc2 and cyclin B1 proteins (*C;* left, K562; right, HL-60).

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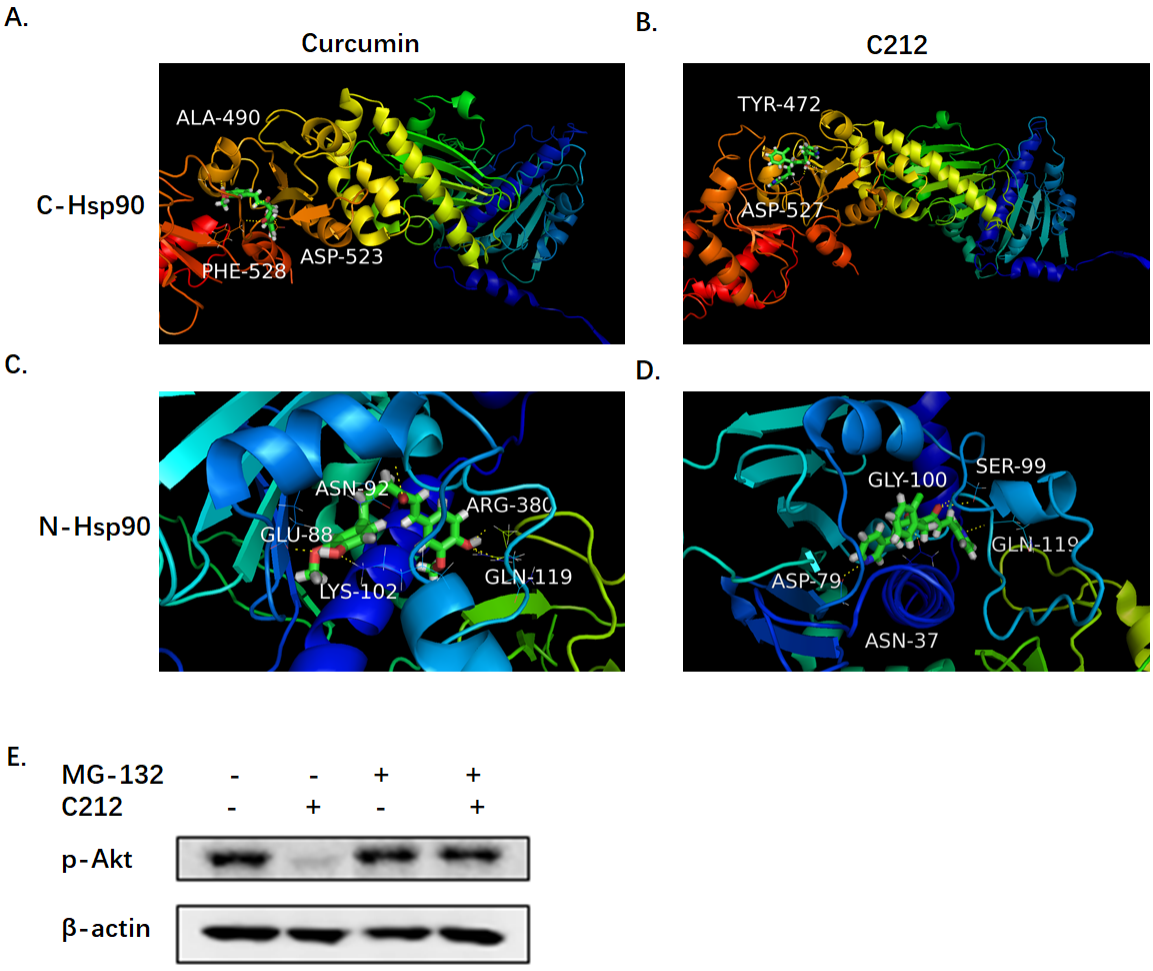

Fig. S2. (*A-D*) Molecular docking simulation of the binding of curcumin (*A* and *C*) and C212 (*B* and *D*) to Hsp90 at the C-terminus (*A* and *B*) and N-terminus (*C* and *D*). (*E*) Growing HL-60 cells were pretreated with or without C212 (2.4 μM) for 2 hours, followed by co-treatment with or without MG132 (1 μM) for 12 hours; whole-cell lysate was then subjected to immunoblotting.

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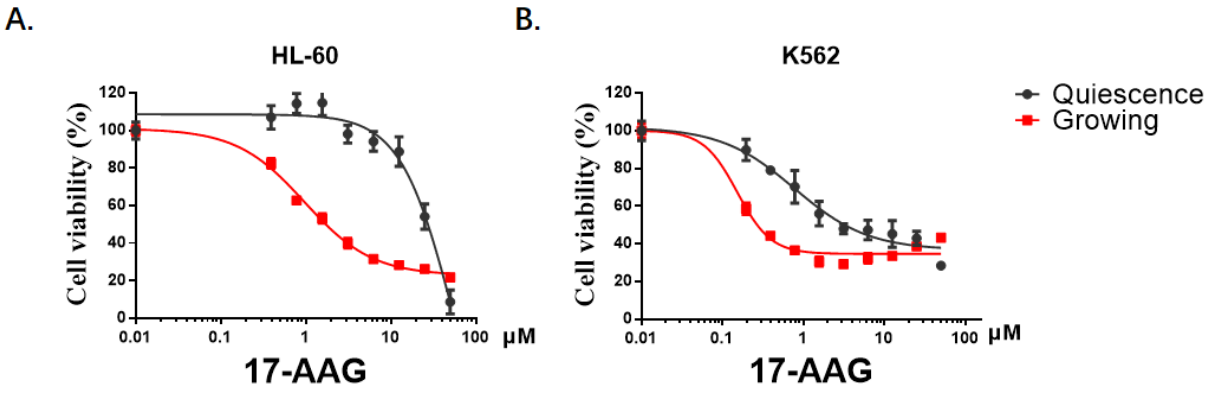

Fig. S3. Growing and quiescent/slow-growing HL-60 (*A*) and K562 (*B*) cells were treated with 17-AAG at the indicated doses for 48 hours, followed by MTS cell viability assay. Error bar, SEM (n = 3).