Cyclin-dependent kinase 8 mediates chemotherapy-induced tumor-promoting paracrine activities

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Conventional chemotherapy not only kills tumor cells but also changes gene expression in treatment-damaged tissues, inducing production of multiple tumor-supporting secreted factors. This secretory phenotype was found here to be mediated in part by a damage-inducible cell-cycle inhibitor p21 (CDKN1A). We developed small-molecule compounds that inhibit damage-induced transcription downstream of p21. These compounds were identified as selective inhibitors of a transcription-regulating kinase CDK8 and its isoform CDK19. Remarkably, p21 was found to bind to CDK8 and stimulate its kinase activity. p21 and CDK8 also cooperate in the formation of internucleolar bodies, where both proteins accumulate. A CDK8 inhibitor suppresses damage-induced tumor-promoting paracrine activities of tumor cells and normal fibroblasts and reverses the increase in tumor engraftment and serum mitogenic activity in mice pretreated with a chemotherapeutic drug. The inhibitor also increases the efficacy of chemotherapy against xenografts formed by tumor cell/fibroblast mixtures. Microarray data analysis revealed striking correlations between CDK8 expression and poor survival in breast and ovarian cancers. CDK8 inhibition offers a promising approach to increasing the efficacy of cancer chemotherapy.

transcriptional damage response | senescence | tumor microenvironment | nucleolus | chemical genomics

Chemotherapy and radiation therapy not only kill tumor cells but also induce tumor-promoting paracrine activities in the tumor environment, which may decrease treatment efficacy and contribute to de novo carcinogenesis. These paracrine effects include the promotion of tumor formation (1), stimulation of angiogenesis (2, 3), metastasis (4), tumor resistance to chemotherapy (5), and secretion of multiple tumor-promoting cytokines in vivo (6) and in vitro (7). These damage responses also occur in the stromal components of solid tumors (endothelial cells and fibroblasts), where they are mediated by p53 (8, 9). Tumor-promoting secretory phenotypes have been associated with cell senescence induced by DNA damage or aging (10–15). The DNA damage- and senescence-associated secretory phenotype results at least in part from increased transcription of genes encoding secreted proteins. This transcriptional response is observed in drug-treated cells before the development of the senescent phenotype and is "fixed" at the highest level in cells that become senescent (11).

Transcriptional activation of some tumor-promoting genes in drug-damaged cells was decreased upon the knockout of p21 (CDKN1A), a cell-cycle inhibitor induced, primarily by p53, in response to different types of damage and at the onset of senescence (11). p21 expression from an inducible promoter in HT1080 fibrosarcoma cells activated transcription of multiple damage-responsive tumor-promoting genes and produced mitogenic and antiapoptotic activities in conditioned media (10). p21 expression up-regulates not only cancer-associated genes but also different proteins implicated in age-related diseases (10), and it stimulates viral promoters, including those of HIV and CMV (16, 17). p21 binds several members of the cyclin-dependent kinase (CDK) family. The best-known CDKs (CDK1, CDK2, CDK4/6) mediate cell-cycle progression, but many others function as regulators of transcription or RNA processing but not of the cell cycle (18). p21 usually inhibits CDK activity, although it may stimulate CDK4/6 (19). Aside from the CDKs, p21 interacts with many transcription factors and cofactors (20). p21-induced transcription was shown to be mediated in part through transcription factor NF-κB (16, 17), but the mechanism of NF-κB stimulation by p21 is not yet fully understood.

The ability to reproduce transcriptional damage response and its paracrine effects by inducible p21 expression in HT1080 cells, without DNA damage (10), offers a unique system to identify "druggable" mediators of this pathway downstream of p21. We have now generated a class of noncytotoxic small molecules that inhibit p21-induced transcription and that were identified as selective inhibitors of CDK8 and its isoform CDK19 (21, 22). CDK8 is an oncogenic CDK family member that plays no role in cell-cycle progression but regulates several transcriptional programs involved in carcinogenesis (23) and the stem-cell phenotype (24). We have discovered that p21 interacts with CDK8 and, surprisingly, stimulates its activity, thereby explaining why p21 activates transcription. The CDK8 inhibitor not only suppressed the induction of transcription downstream of p21 but also blocked different chemotherapy-induced tumor-promoting paracrine activities of normal and tumor cells, in vitro and in vivo. In agreement with this tumor-supporting function of CDK8, its expression showed a striking correlation with treatment failure in human cancers. These results suggest that CDK8 inhibitors may become a unique class of anticancer drugs that increase the efficacy of cancer therapy by blocking chemotherapy-induced production of tumor-promoting secreted factors.

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Results

Development of Small-Molecule Inhibitors of p21-Induced Transcription. High-throughput screening (HTS) for downstream inhibitors of p21-activated transcription used HT1080 p21-9 cells with isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible p21 (10, 25), carrying a construct that expresses GFP from the CMV promoter. CMV was chosen as the strongest of the p21-stimulated promoters (17), providing sufficient signal intensity for HTS. A total of 62 of >100,000 compounds from diversified small-molecule libraries inhibited CMV-GFP induction by p21. Five of these hits were closely related 4-aminoquinazolines, designated SNX2-class compounds. The introduction of a carbonitrile group at position 6 during subsequent structure optimization greatly increased the efficacy of these compounds (Fig. 1*A*), one of which, designated Senexin A, was used for biological studies. Senexin A inhibited CMV-GFP induction by p21 when GFP expression was normalized either by relative cell number (Fig. 1*B*) or by the protein amount (Fig. 1*C*), an assay that compensates for p21-induced increase in cell size and autofluorescence. p21 was shown to activate NF-κB–dependent transcription (17), and Senexin A inhibited p21-stimulated activity of the consensus NF-κB–dependent promoter (17) (Fig. 1*D*). Senexin A had no effect on p21 induction by IPTG (Fig. 1*E*), on cell growth with or without p21 (Fig. S1*A*), or on p21-induced senescent phenotype [increased cell size, flattening, and senescence-associated β-galactosidase activity (26)] (Fig. S1*B*). Though Senexin A partly decreased the basal CMV promoter activity (Fig. 1 *B* and *C*), it had almost no effect on the basal activity of the NF-κB–dependent promoter (Fig. 1*D*). Together with the lack of growth inhibition (Fig. S1*A*), this result indicates that Senexin A does not affect overall transcription. Microarray analysis of the effects of p21 and Senexin A showed that Senexin A did not affect the inhibition of gene expression by p21 (Fig. 1*F*, *Lower Left*) and did not interfere with p21-mediated inhibition of large sets of genes belonging to Gene Ontology (GO) categories of mitosis and DNA replication (Fig. 1*G*). In contrast, many (but not all) p21-inducible genes were induced less when p21 was expressed in the presence of Senexin A (Fig. 1*F*, *Upper Right* and Fig. S2), including GO categories of proteolysis and extracellular space (Fig. 1*G*). Hence, Senexin A inhibits only p21-induced transcription but not other biological effects of p21.

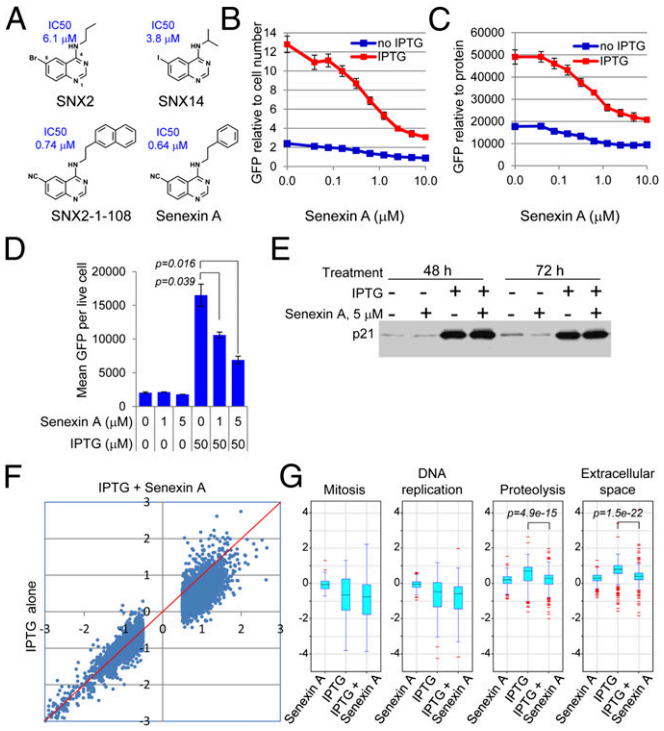

Fig. 1. Effects of Senexin A on the induction of transcription by IPTG-inducible p21. (*A*) Structures of some SNX2-class compounds. SNX2 and SNX14 were isolated through HTS; Senexin A and SNX2-1-108 were generated through chemical optimization. (*B*) Effects of Senexin A on CMV-GFP expression in HT1080-p21-9 cells, untreated or treated for 48 h with 50 μM IPTG (quadruplicate assays). *y* axis: GFP fluorescence normalized by Hoechst 33342 DNA staining (a measure of relative cell number). (*C*) Same as in *A*, except that GFP fluorescence was normalized by sulphorhodamine B staining (a measure of protein amount). (*D*) Effects of Senexin A on GFP expression from NF-κB–dependent consensus promoter in HT1080 p21-9 cells, untreated or treated for 72 h with 50 μM IPTG. *y* axis: Mean GFP fluorescence per live cell (measured by flow cytometry). (*E*) Immunoblotting analysis of p21 protein in HT1080 p21-9 cells, untreated or treated with 50 μM IPTG and 5 μM Senexin A, singly and in combinations. (*F*) Effects of Senexin A on the inhibition and induction of gene expression in HT1080-p21-9 cells treated with 50 μM IPTG alone (*x* axis) or with 50 μM IPTG and 5 μM Senexin A (*y* axis) for 48 h (microarray data). Fold changes in gene expression are plotted as log2; genes showing IPTG-induced fold changes with log2 < 0.5 are excluded. See Fig. S2 for quantitative PCR confirmation of gene expression changes. (*G*) Box-whisker plots of fold changes in the expression of all of the genes in the indicated GO categories in cells treated with Senexin A, IPTG, or IPTG plus Senexin A.

Transcriptional Effect of SNX2-Class Compounds Is Mediated by CDK8 Inhibition. The structure of SNX2-class compounds resembles known protein kinase inhibitors, and therefore we tested their effects on kinome panels. Indeed, SNX14, a compound originally discovered through HTS, inhibited many of 442 kinases screened by an ATP site-dependent competition binding assay (27) (Fig. 2*A*). In contrast, the optimized carbonitrile derivatives showed striking selectivity for only two closely related kinases, CDK8 and CDK19, as shown in Fig. 2*B* and Table S1 for SNX2-1-108. CDK8 and CDK19 inhibition showed excellent correlations with biological activity of SNX2-class compounds (Fig. S3*A*). Senexin A inhibited CDK8 and CDK19 ATP site binding with Kd50 of 0.83 μM and 0.31 μM, respectively (Fig. S3*B*) and CDK8 kinase activity with IC_{50} of 0.28 μM (Fig. 2*C*). To test if Senexin A inhibits cellular CDK8 functions, we have measured its effects on known biological activities of CDK8. CDK8 stimulates Wnt/β-catenin (28, 29), and we have found that Senexin A inhibits β-catenin–dependent transcription in HCT116 colon carcinoma cells (Fig. 2*D*). Another effect of CDK8 is positive regulation of transcriptional serum response (30). The induction of transcription factor EGR1 upon serum starvation, followed by readdition of serum, was strongly inhibited by Senexin A in HT1080 cells (Fig. 2*E*), as expected for a CDK8 inhibitor.

To test if CDK8/19 inhibition is responsible for the effect of Senexin A on p21-induced transcription, we asked if this effect of Senexin A can be reproduced by unrelated CDK8/19 inhibitors. Aside from pan-tropic CDK inhibitors, the only compound reported to inhibit CDK8/19 is a steroidal alkaloid cortistatin A (31). As predicted, an equipotent synthetic version of cortistatin A (32) inhibited CMV-GFP induction by p21 in HT1080 p21-9 cells (Table S2). Cortistatin A inhibits not only CDK8/19 but also ROCK kinases, an activity probably responsible for its antiproliferative effect on endothelial cells (31). In contrast, Senexin A, a selective CDK8/19 inhibitor, did not inhibit ROCK and did not share cortistatin A's strong antiendothelial cell activity (Table S2). We then asked if the effect of Senexin A can be reproduced by shRNA knockdown of CDK8 and CDK19. Lentiviral vectors expressing the corresponding shRNAs decreased CDK8 and CDK19 RNA and protein expression (Fig. S4 *A*–*D*). Fig. 2*F* shows the effects of CDK8 and CDK19 knockdown on mean fluorescence intensity of GFP expressed from the CMV promoter in HT1080 cells. The knockdown of CDK8 alone or of both CDK8 and CDK19 decreased p21-induced CMV-GFP expression. The knockdown of CDK19 alone did not have this effect

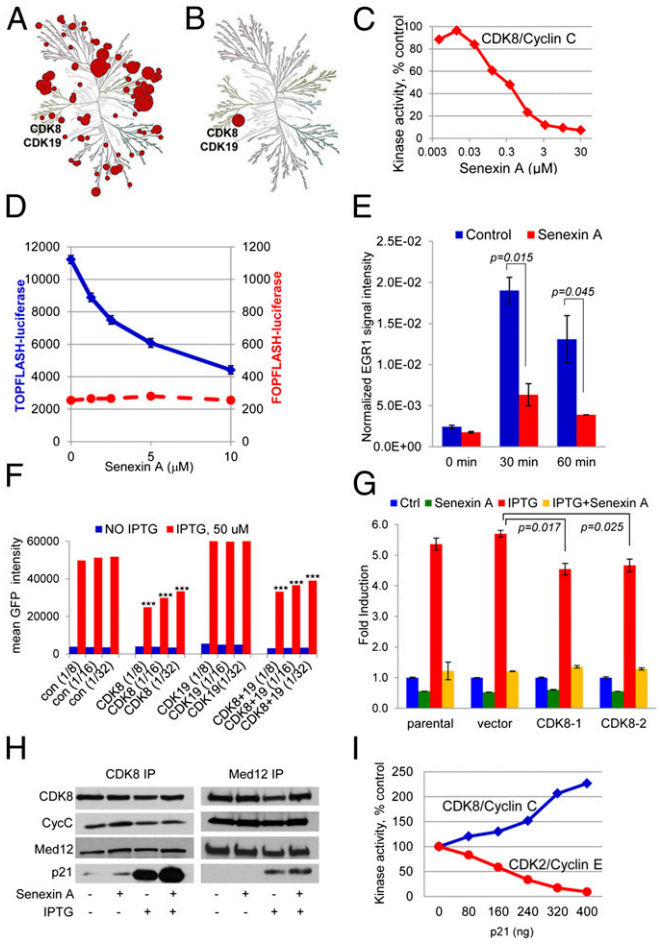

Fig. 2. Identification of CDK8 as a mediator of p21-induced transcription and target of SNX2-class compounds. (*A*) Effects of 10 μM SNX14 on the activity of 442 kinases, measured by ATP binding competition assay. The kinases are displayed in the form of an evolutionary dendrogram; red circles indicate the inhibited kinases. (*B*) Same analysis as in *A* conducted with 2 μM SNX2-1-108. (*C*) Effect of Senexin A on CDK8/cyclin C kinase activity in a cell-free assay. (*D*) Luciferase expression from β-catenin–dependent promoter (TOPflash) or its β-catenin–independent version (FOPflash) in HCT116 cells, after 48 h treatment with Senexin A (quadruplicate assays). (*E*) Quantitative PCR analysis of EGR1 mRNA expression in HT1080 p21-9 cells upon serum starvation for 48 h, followed by readdition of serum for the indicated periods of time, in the presence or absence of 5 μM Senexin A (triplicate assays). Primer sequences are listed in Table S3. (*F*) Mean fluorescence intensity of CMV-GFP expression in live (propidium iodide-negative) cells, infected with the indicated dilutions of pLKO.1 lentiviral vector (control) or with pLKO.1-expressing shRNAs targeting CDK8 or CDK19, singly or in combination, with or without 3-d treatment with 50 μM IPTG. ***Decrease relative to cells infected with control lentivirus, with $P = 0$ (two-tailed t test). (*G*) CMV-GFP expression in cells, untransduced or transduced with a control lentivirus or with lentiviruses expressing two different shRNAs against CDK8, with or without 48-h treatment with 50 μM IPTG or 5 μM Senexin A, alone or in combination. Bars represent fold changes in mean fluorescence intensity of the GFP-expressing live cells in test samples relative to the same untreated cells, in biological triplicates. (*H*) Coimmunoprecipitation analysis of CDK8, Med12, cyclin C, and p21 in HT1080 p21-9 cells, untreated, or treated for 48 h with 10 μM Senexin A and 50 μM IPTG, singly or in combination. (*I*) Dose-dependent effects of recombinant p21 on CDK2/cyclin E and CDK8/cyclin C kinase activities in cell-free assays (a representative from three experiments).

(Fig. 2*F*), but HT1080 cells express hardly any CDK19 protein (Fig. S4*C*). In the experiment in Fig. 2*G*, CDK8 knockdown with two different shRNAs was followed by the analysis of CMV-GFP induction by p21 in the presence or absence of Senexin A. Both shRNAs significantly decreased the induction of CMV-GFP expression by IPTG-induced p21 in the absence of Senexin A but had no effect on CMV-GFP induction in the presence of Senexin A (Fig. 2*G*), verifying that CDK8 is the target of Senexin A responsible for the inhibition of transcription downstream of p21.

p21 Stimulates CDK8 Kinase Activity and Cooperates with CDK8 in the Formation of Internucleolar Bodies. We have used immunoprecipitation to determine if p21 interacts with CDK8, its binding partner cyclin C, and CDK8-binding Mediator protein Med12. Both CDK8 and Med12 antibodies coprecipitated p21 with CDK8, cyclin C, and Med12 from extracts of HT1080 p21-9 cells (Fig. 2*H*). We have also analyzed the effects of p21 on kinase activities of CDK2 (the principal p21-inhibited kinase) and CDK8 in cell-free assays. As expected, recombinant p21 exerted concentration-dependent inhibition of CDK2/cyclin E kinase activity. In contrast, p21 stimulated CDK8/cyclin C kinase (Fig. 2*I*). The surprising CDK8 activation by p21 explains why an effect of p21, a protein conventionally described as a pleiotropic CDK inhibitor, was counteracted by CDK8 inhibition.

We have also used immunofluorescence confocal microscopy to analyze the effects of p21 and Senexin A on subcellular localization of CDK8 and p21 (Fig. S5*A*). Both proteins were found predominantly in the nuclei of HT1080 p21-9 cells. Aside from the nucleoplasm, p21 was highly concentrated within internucleolar bodies (INoBs), where p21 was reported to accumulate upon DNA damage (33). Remarkably, we found that p21 expression led not only to the protein's accumulation in INoBs, but actually caused INoB formation, as demonstrated by time-lapse video microscopy (Fig. S5*B* and Movie S1) and by the drastic increase in the fraction of INoB-containing cells upon p21 expression (Fig. S5*C*). CDK8 also becomes concentrated in the INoBs upon p21 induction, where it colocalizes with p21 (Fig. S5*A*). The addition of the CDK8 inhibitor greatly decreased INoB formation and nucleolar localization of both CDK8 and p21 (Fig. S5*A* and *C*). Hence, p21 and CDK8 cooperate in the formation of INoBs, where these proteins coaccumulate.

Role of p21 and the Effects of a CDK8 Inhibitor on Paracrine Tumor-Promoting Effects of DNA Damage. After identifying CDK8/19 inhibitor Senexin A as an inhibitor of transcription downstream of p21, we tested the effects of p21 and Senexin A on paracrine antiapoptotic activities of HCT116 cells treated with a DNA-damaging drug doxorubicin. This analysis used an assay that measures the ability of these cells to protect apoptosis-sensitive C8 murine-transformed fibroblasts from apoptosis in low-serum media (10). Coculture with HCT116 cells increased C8 cell survival in low serum, and this paracrine activity was strongly increased when HCT116 were pretreated with doxorubicin (Fig. 3*A*). This response to doxorubicin was abolished in HCT116 derivatives with the knockout of either p21 (34) or its positive regulator p53 (35) (Fig. 3*A*), demonstrating that p21 is required for damage-induced antiapoptotic activity. When wild-type HCT116 were treated with doxorubicin or carrier in the presence of Senexin A, their paracrine activity was drastically diminished (Fig. 3 *B* and *C*), but Senexin A had no effect on the antiapoptotic activity of $p21^{-/-}$ cells (Fig. 3*B*). Senexin A also decreased the expression of many secreted tumor-promoting factors in doxorubicin-treated wild-type HCT116 cells, as determined using an antibody array that measures the levels of 55 secreted proteins related to angiogenesis and other aspects of tumor growth. Notably, Senexin A did not inhibit doxorubicin-induced expression of Maspin, a tumor suppressor protein that is upregulated by damage through a p21-independent pathway (11) (Fig. S6). p21 immunoblotting showed that Senexin A moderately decreased p21 induction in HCT116 cells treated with 150 nM doxorubicin (Fig. 3*D*), but the magnitude of this reduction was much less than the effect of Senexin A on the antiapoptotic

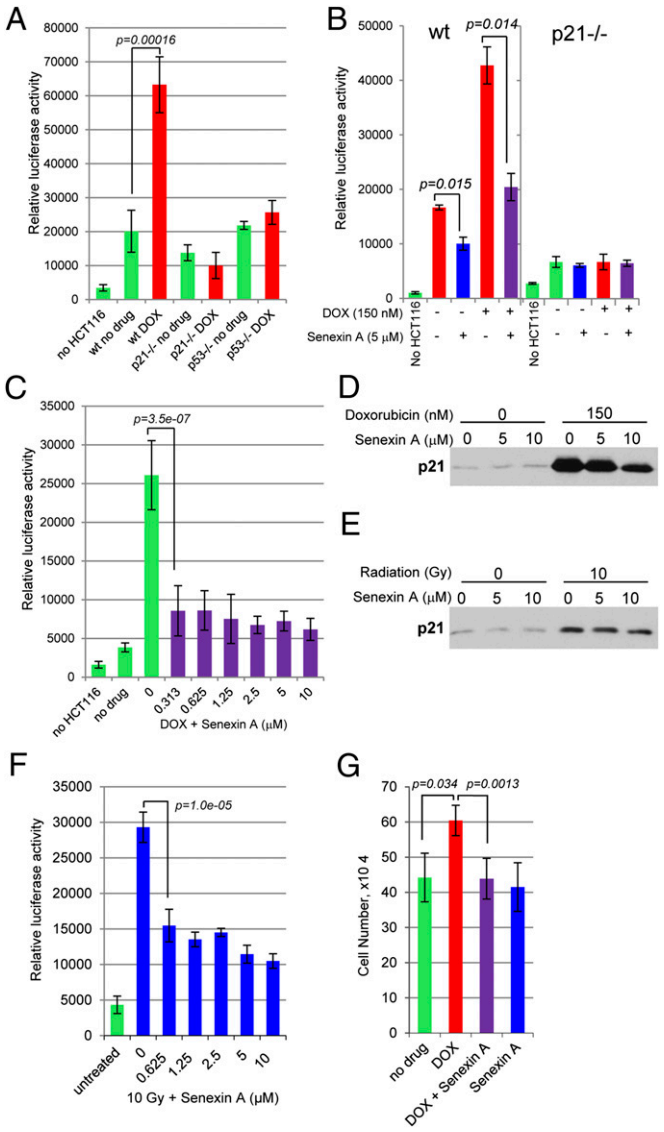

Fig. 3. Effects of p21 and CDK8 inhibitor on paracrine tumor-promoting activities. (*A*) Survival of luciferase-labeled C8 cells in low-serum media without coculture or in coculture with wild-type, p53-null, or p21-null HCT116 cells, untreated or pretreated for 72 h with 150 nM doxorubicin (quadruplicate assays). (*B*) Same assays as in *A* conducted with wild-type and p21-null HCT116 cells in the presence or in the absence of 5 μM Senexin A. (*C*) Same assays as in *A* and *B* conducted with wild-type HCT116 cells, untreated or pretreated for 72 h with 150 nM doxorubicin in the absence or presence of Senexin A (eight replicate assays). (*D*) Effects of Senexin A on p21 expression in HCT116 cells, untreated or treated for 72 h with 150 nM doxorubicin. (*E*) Effects of Senexin A on p21 expression in WI38 fibroblasts, untreated or exposed 3 d earlier to 10 Gy ionizing radiation. (*F*) Same assays as in *A*–*C* conducted with human WI38 fibroblasts, untreated or exposed 3 d earlier to 10 Gy ionizing radiation, in the absence or presence of Senexin A (triplicate assays). (*G*) A549 cell growth in conditioned media from MEF that were either untreated or treated for 24 h with 200 nM doxorubicin, singly or in combination with 5 μM Senexin A (triplicate assays). MEF-conditioned media were collected 48 h after removing the drugs; A549 cells were counted after 48 h.

activity (Fig. 3 *B* and *C*). Senexin A did not affect the senescent phenotype of doxorubicin-treated HCT116 cells (Fig. S7*A*).

We have also tested the effects of Senexin A on damage-induced tumor-promoting activities of normal fibroblasts. In contrast to its effect on damage-induced p21 expression in HCT116 cells, this compound had no effect on radiation-induced p21 expression in human WI38 fibroblasts (Fig. 3*E*), although it appeared to attenuate morphological changes in these cells (Fig. S7*B*). Irradiation of WI38 fibroblasts strongly increased their ability to protect C8 cells from apoptosis in low-serum medium, but this effect was greatly diminished when the fibroblasts were irradiated in the presence of Senexin A (Fig. 3*F*). In a different assay, doxorubicin treatment significantly increased mitogenic activity secreted into conditioned media of mouse embryo fibroblasts (MEF), but Senexin A abolished this damage response (Fig. 3*G*). Hence, the CDK8 inhibitor drastically decreases different damage-induced tumor-promoting paracrine activities of both tumor cells and normal fibroblasts.

CDK8 Inhibitor Reverses Chemotherapy-Induced Paracrine Tumor-Promoting Activities in Vivo. The extensive paracrine effects of DNA damage in cell culture prompted us to test the systemic effect of chemotherapeutic treatment on xenograft tumor growth in mice. Tumor-free C57BL/6-derived SCID mice were injected i.p. with a single dose of doxorubicin or carrier control. Five days later, mice received s.c. injection of 2×10^6 human A549 lung carcinoma cells, and tumor take was measured over 4 wk. As shown in Fig. 4*A*, A549 xenografts showed much better engraftment in mice pretreated with doxorubicin than in the untreated mice. However, this tumor-promoting effect of chemotherapy was fully reversed when doxorubicin treatment was followed by five daily injections of Senexin A (Fig. 4*A*). (Senexin A, administered at the same dose over 5 d, showed no detectable toxicity and no significant effects on body weight, organ weights, or blood cell counts in C57BL/6 mice).

We hypothesized that this systemic tumor-promoting effect of DNA-damaging drugs could manifest itself through the secretion of mitogenic factors into the blood of treated animals. To test this hypothesis, we compared mitogenic activities of sera from C57/BL6 mice that were either untreated or injected i.p. with doxorubicin, with or without Senexin A, by adding mouse sera to serum-free media used to culture A549 cells. Sera from doxorubicin-treated mice significantly increased the growth of lung cancer cells relative to sera from untreated mice. This effect of doxorubicin treatment was completely abolished, however, when doxorubicin injection was followed by in vivo administration of Senexin A (Fig. 4*B*).

We also tested Senexin A for in vivo chemosensitization of xenografts formed by tumor cells admixed with MEF. MEF were previously shown to exert chemoprotective activity, which required functional p53-mediated damage response and was associated with a secretory phenotype (9). SCID mice were injected s.c. with A549 cells mixed with MEF 1:1. Once tumors became palpable, mice were treated by a single i.p. injection of doxorubicin, with five daily injections of either carrier or Senexin A. Senexin A treatment strongly improved the response of A549/MEF tumors to doxorubicin (Fig. 4*C*). Hence, CDK8 inhibition blocks tumor-promoting paracrine activities induced by DNA-damaging chemotherapeutic drugs both in vitro and in vivo.

Clinical Correlations of CDK8 Expression. Our finding that CDK8 mediates paracrine tumor-promoting effects of DNA-damaging chemotherapeutic drugs suggests that CDK8 expression could be associated with chemotherapy failure and poor survival. CDK8 is involved in colon carcinogenesis, and higher CDK8 expression has been correlated with negative prognosis in colorectal and gastric cancers (23). To test the impact of CDK8 expression in cancers where this gene has not been implicated in carcinogenesis, we have used an online survival analysis tool that evaluates the effect of a gene on prognosis using microarray gene expression data from multiple studies on breast cancer (2,897 cases) and ovarian cancer (1,107 cases) (36). High expression of CDK8 showed a striking correlation with poor relapse-free survival in breast cancer patients ($P = 3 \times 10^{-15}$); very strong correlations

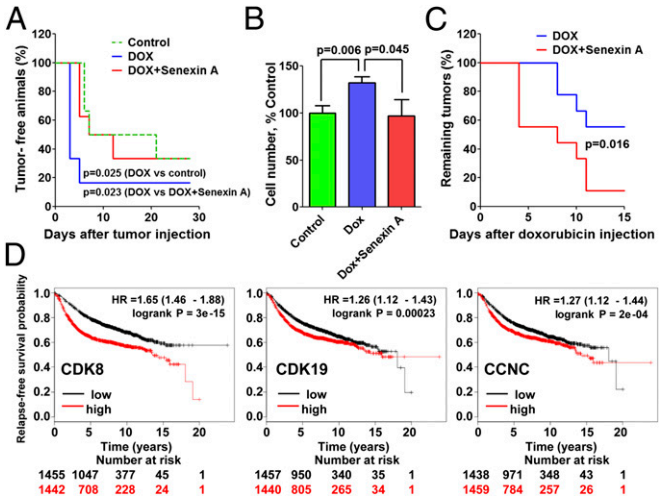

Fig. 4. Effects of CDK8 inhibitor and clinical correlations of CDK8 expression in vivo. (*A*) Effect of pretreatment with doxorubicin, with or without Senexin A, on A549 xenograft tumor engraftment in SCID mice. Mice were either untreated (n = 6) or treated with a single i.p. injection of 4 mg/kg doxorubicin, followed by five daily i.p. injections of either carrier (n = 6) or 20 mg/kg Senexin A (n = 8). A total of 2×10^6 A549 cells were injected s.c. 5 d after doxorubicin injection. The time when tumors became detectable by palpation was recorded. (*B*) Effects of sera from mice that were untreated (n = 9) or pretreated i.p. with doxorubicin, with (n = 8) or without (n = 5) 5-d treatment with Senexin A, on the growth of A549 cells in culture. Serum was isolated 5 d after the initiation of therapy. Cell number was assessed 48 h after the addition of mouse sera (each sample assayed in triplicate). (*C*) Survival of xenograft tumors formed in SCID mice by A549 mixed with MEF (1:1). Once tumors became palpable, mice were treated with a single i.p. injection of 4 mg/kg doxorubicin, followed by five daily i.p. injections of either carrier or 20 mg/kg Senexin A. The time until tumors became undetectable by palpation was recorded. (*D*) Correlations of CDK8, CDK19, and *CCNC* expression with relapse-free patient survival in microarray data from 2,897 breast cancers, determined using an online survival analysis tool. Kaplan–Meier correlations with relapse-free survival are plotted for high (above-median) and low (below-median) expression of each gene.

were also observed for CDK19 and *CCNC* (cyclin C; Fig. 4*D*). This correlation was especially drastic because CDK8, CDK19, and *CCNC*, unlike all of the genes high expression of which showed comparable correlation with bad prognosis in this analysis (36), are not markers of proliferation. CDK8 expression also strongly correlated with poor survival among ovarian cancer patients, and this correlation became even stronger among ovarian cancer patients treated with DNA-damaging platinum compounds (Fig. S8). Remarkably, the correlation of gene expression with poor survival was lost among 529 patients treated with the antimicrotubule drug Taxol (Fig. S8). Similar correlations in platinum-treated ovarian cancers were observed for *CCNC* and CDK19 (Fig. S8). Hence, CDK8/CDK19/cyclin C expression is strongly associated with poor survival and failure of DNA-damaging chemotherapy in clinical cancers.

Discussion

The growing evidence for chemotherapy-induced tumor-promoting paracrine activities is extended by our present findings that pretreatment of tumor-free mice with a DNA-damaging chemotherapeutic drug stimulates tumor engraftment and elevates mitogenic activity in the serum from treated animals. This secretory response, induced by damage in both normal and tumor tissues and perpetuated by the apoptosis-resistant senescent cells that arise upon chemotherapy (11, 14), is expected to decrease treatment efficacy and possibly increase chemotherapy-induced inflammation and fatigue due to the induction of proinflammatory cytokines (6). Several proteins up-regulated in damaged and senescent cells have been implicated in age-related diseases other than cancer (10, 14), and ablation of senescent cells was recently shown to alleviate aging-associated pathologies in mice (37). The results of the present study reveal an essential, druggable mediator of disease-promoting paracrine activities associated with DNA damage and senescence, offering a pharmacological approach to inhibiting these activities in cancer and other aging-associated diseases.

The present finding that p21 is required for damage-induced paracrine antiapoptotic activity (Fig. 3*A*) extends our prior observations that ectopic expression of p21 from an inducible promoter in HT1080 cells mimics transcriptional and paracrine effects of DNA damage (10, 11). We have used the latter cellular system as a tool to develop small-molecule inhibitors of damage-induced transcription downstream of p21 and to identify their druggable targets. Senexin A and related molecules developed in the present study inhibited p21-induced transcription, with no effects on p21 expression, p21-mediated cell-cycle arrest, senescent phenotype, inhibition of genes involved in cell cycle progression, or basal transcription from NF-κB–dependent or FOPflash promoters. These results indicate that p21 induces transcription through a mechanism distinct from its effects on the cell cycle. Senexin A inhibited not only p21-stimulated transcription but also cytokine production by damaged cells and all of the tested paracrine activities of chemotherapy-damaged tumor and normal cells in vitro and in vivo.

SNX2-class compounds were identified as highly selective inhibitors of CDK8 and its isoform CDK19 (Fig. 2*B*), CDK family members that function in the regulation of transcription but not cell-cycle progression (18). The role of CDK8 as a mediator of the induction of transcription by p21 and the target of Senexin A responsible for its transcriptional activity has been demonstrated by shRNA knockdown assays. CDK8 and its binding partner cyclin C form a part of a regulatory module of the Mediator complex that connects transcriptional regulators with RNA polymerase II to initiate transcription of the regulated genes, but CDK8/cyclin C complex also functions outside of the Mediator (23, 24). Senexin A, which inhibits CDK8 kinase activity by binding at the ATP pocket, also inhibits known cellular functions of CDK8, including the potentiation of β-catenin–dependent transcription and induction of gene expression upon serum stimulation. shRNA analysis confirmed the role of CDK8 as a mediator of p21-induced transcription and the target of Senexin A. Surprisingly, we have found that p21 stimulates CDK8 kinase activity. p21 activation of CDK8, leading to transcriptional stimulation, stands in striking contrast to its inhibition of CDK2, the cell-cycle regulator primarily responsible for the ability of p21 to stop cell-cycle progression. p21 also altered the subcellular localization of CDK8, through forming INoBs, where p21 and CDK8 coaccumulated. The CDK8 inhibitor prevented the appearance of INoBs and p21/CDK8 localization to nucleoli, indicating that p21 and CDK8 cooperate in INoB formation. Time-lapse analysis (Movie S1) indicates that the INoBs appear in the first 20 h of p21 induction by IPTG, before the onset of p21-induced transcription in this cellular system (10), suggesting that the INoBs could be mechanistically related to the induction of CDK8-mediated transcription.

CDK8 has been identified as an oncogene amplified in ∼50% of colon cancers where it potentiates Wnt/β-catenin (28, 29), and as a melanoma oncogene associated with the loss of a histone variant macroH2A (38). CDK8 has also been implicated in Notch signaling (39) and Smad activation in BMP and TGF-β pathways (40). CDK8 was shown to potentiate transcriptional effects of p53, including p21 induction (41). However, the effects of CDK8 inhibition observed in the present study occurred downstream of p21 and were not due to diminished p21 induction. In fact, Senexin A had no effect on p21 expression from the IPTG-inducible

promoter or in irradiated WI38 fibroblasts. Although the CDK8 inhibitor partially decreased p21 induction by doxorubicin in HCT116 cells, this decrease was minor relative to the overall magnitude of p21 induction and to the effect of Senexin A on the antiapoptotic activity. Importantly, CDK8 inhibitor blocked the stimulating effect of p21 on transcription factor NF-κB (Fig. 1*D*), which plays a major role in damage- and p21-induced transcription (16, 17). The mechanism of the effect of CDK8 on NF-κB is under investigation.

CDK8 is required for embryonic development at the preimplantation stage (42), probably because of its role in the pluripotency of embryonic stem cells (24), and CDK19 haploinsufficiency has been linked to a congenital neurological defect (43). CDK8 knockdown did not, however, affect normal cell growth (28, 42). In the present study, the CDK8/19 inhibitor Senexin A did not inhibit reporter cell growth and showed no detectable toxicity in a mouse study. These observations suggest that pharmacological inhibition of CDK8/19 will likely have an acceptable toxicity profile.

The appeal of CDK8 inhibition in cancer has been suggested by the role of CDK8 in the Wnt/β-catenin pathway (28, 29) and its recently found association with the cancer stem-cell phenotype (24). The present study demonstrates the role of CDK8 in damage-induced tumor-promoting paracrine activities and the striking correlations of CDK8 and cyclin C expression with poor survival in breast cancer and with platinum treatment failure in ovarian cancer. The generation of selective and nontoxic CDK8/19 inhibitors suitable for in vitro and in vivo applications makes it possible now to investigate CDK8 inhibition as a unique approach to improving the treatment of cancer and other aging-associated diseases.

Materials and Methods

SI Materials and Methods provide further details for all of the experiments. CMV-GFP and NF-κB-GFP reporter cell lines were derived from HT1080 p21-9 with IPTG-inducible p21 (25). HTS was conducted on ChemBridge Corp. Microformat 04 and DiverSet collections (50,000 compounds each, screened at 20 μM) and on 2,080 compounds with known activities from the MicroSource SpectrumPlus collection. GFP fluorescence was normalized by Hoechst 33342 staining of cellular DNA. Kinase activity was measured using ProQinase assay kits. Kinase ATP binding-site competition assays (27) were conducted by KinomeScan. Cytokine expression was analyzed using R&D Systems Human Angiogenesis Antibody Array.

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