Inhibitors of Ras/Raf-1 interaction identified by two-hybrid screening revert Ras-dependent transformation phenotypes in human cancer cells

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The interaction of activated Ras with Raf initiates signaling cascades that contribute to a significant percentage of human tumors, suggesting that agents that specifically disrupt this interaction might have desirable chemotherapeutic properties. We used a subtractive forward two-hybrid approach to identify small molecule compounds that block the interaction of Ras with Raf. These compounds (MCP1 and its derivatives, 53 and 110) reduced serum-induced transcriptional activation of serum response element as well as Ras-induced transcription by way of the AP-1 site. They also inhibited Ras-induced Raf-1 activation in human embryonic kidney 293 cells, Raf-1 and mitogen-activated protein kinase kinase 1 activities in HT1080 fibrosarcoma cells, and epidermal growth factor-induced Raf-1 activation in A549 lung carcinoma cells. The MCP compounds caused reversion of *ras*-transformed phenotypes including morphology, *in vitro* invasiveness, and anchorage-independent growth of HT1080 cells. Decreased level of matrix metalloproteinases was also observed. Further characterization showed that MCP compounds restore actin stress fibers and cause flat reversion in NIH 3T3 cells transformed with H-Ras (V12) but not in NIH 3T3 cells transformed with constitutively active Raf-1 (RafΔN). Finally, we show that MCP compounds inhibit anchorage-independent growth of A549 and PANC-1 cells harboring K-*ras* mutation. Furthermore, MCP110 caused G_1 enrichment of A549 cells with the decrease of cyclin D level. These results highlight potent and specific effects of MCP compounds on cancer cells with intrinsic Ras activation.

The Ras family GTPases play a central role in the growth factor signaling controlling cell proliferation, differentiation, and survival (1, 2). The significance of the Ras signaling pathway in human cancer has been underscored by the detection of mutations targeting a *ras* gene (H-, N-, or K-*ras*) in ≈30% of human tumors, with high incidence of K-*ras* mutation found in pancreatic, colon, and lung carcinomas (3). Additionally, Ras overexpression and accumulation of the active GTP-bound form of Ras, have been detected in colorectal cancers and astrocytomas (4, 5) lacking mutations within the *ras* gene. Overexpression of several receptor tyrosine kinases also leads to Ras activation (6). Downstream effectors of Ras involved in transformation include Raf, phosphatidylinositol 3-kinase, and RalGDS. The Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway is necessary for Ras-regulated cellular proliferation (7). For these reasons, identification of agents that modulate the activity of Ras has been of particular interest for anticancer drug development, as well as for further characterization of the Ras signaling pathway. A variety of approaches including antisense oligonucleotides, kinase-directed inhibitors of receptor tyrosine kinases, Raf-1 and MEK, and inhibitors of farnesyltransferase have been attempted (6, 8, 9). Recently, short peptides derived from Ras-binding domain of Raf (10) and anti-Raf-1 RNA aptamers (11) have been developed; however, their efficacy *in vivo* has not been reported.

Our approach has been to identify small molecule compounds that inhibit the interaction between H-Ras and Raf-1. We have previously developed a dual-bait two-hybrid system as a means to probe selectivity of protein–protein interactions among small GTPases (12, 13). By using permeability-enhanced versions of these tools in a forward screening strategy incorporating subtractive elimination of false-positive compounds, we obtained compounds that inhibit the H-Ras/Raf-1 interaction, and Ras-mediated Raf-1 activation. We show that MCP compounds are able to reverse Ras-transformed phenotypes such as morphology, *in vitro* invasiveness, cell cycle, and anchorage-independent growth.

Materials and Methods

Materials. pcDNA3-H-Ras (V12) was constructed by using the H-Ras cDNA fragment (14). pLNC-RafFH6 was provided by M. McMahon (University of California, San Francisco). pET16b-His-MEK and an expression vector for GST-MAPK (D57) were provided by E. Nishida (Kyoto University). His-MEK and GST-MAPK (D57) were purified as described (15). Antibodies against H-Ras (SC-520), Raf-1 (SC-7267), MEK1, mitogen-activated protein kinase 1/2, phospho-mitogen-activated protein kinase 1/2, cyclin D1/2, HDJ-2, FLAG (M2) were purchased from Santa Cruz Biotechnology, Transduction Laboratories (Lexington, KY), Cell Signaling (Beverly, MA), Upstate Biotechnology (Lake Placid, NY), Neo Markers (Freemont, CA), and Sigma. Epidermal growth factor (EGF) (Upstate Biotechnology), platelet-derived growth factor-BB (Sigma), and phorbol 12-myristate 13-acetate (Sigma) were purchased. PD98059 and U0126 were purchased from Cell Signaling. MCP1 was resynthesized multiple times and purified by different methods. In a process to be described in detail elsewhere, MCP53, 110 and 122,

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Abbreviations: AD, activation domain; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; β-gal, β-galactosidase; CHO, Chinese hamster ovary; DBD, DNA-binding domain; EGF, epidermal growth factor; HEK, human embryonic kidney; MMP, matrix metalloproteinase; SRE, serum response element; ECM, extracellular matrix; FTIs, farnesyltransferase inhibitors.

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were generated as MCP1-related compounds. Each compound was confirmed by HPLC to be >80% pure.

Yeast Strains and Expression Vectors. Yeast reagents used are derivatives of Dual Bait system components described in ref. 12. Strains SKY54 (*MAT**a** ura3 his3 trp1 3LexA-operator-Leu-2 λcI-*operator-*Lys-2 pdr1*::*GAL1pro-HXT9 pdr3*::*GAL1pro-HXT11*) and SKY197 (*MAT**a** ura3 his3 trp1 1LexA-operator-Leu-2 λcI-operator-Lys-2 pdr1*::*GAL1pro-HXT9 pdr3*::*GAL1pro-HXT11*) were derived from strains SKY48 and SKY191 (12). To assess permeability, exponentially growing cells were harvested, mixed with low-melt agarose, poured on YP media with glucose or galactose (gal), and treated with a dilution series of test compounds. DNA-binding domain (DBD) and activation domain (AD) fusion plasmids have been described (12, 16), or are listed at www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html.

Screening Strategy and Yeast Manipulations. Standard protocols were used for manipulation of yeast (17). Exponentially growing SKY54 containing cI-DBD-H-Ras (G186) and AD-Raf-1 (full length), or LexA-DBD-hsRPB7 and AD-hsRPB4, were harvested. Cells were mixed with low-melt agarose, BU-salts, and 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-Gal), and poured as a monolayer. A library of 73,400 chemically diverse compounds (2.5 mM stock) was applied in 96-well-array format, and growth/*lacZ* inhibition was assessed after 24–48 h. For β-galactosidase (β-gal) assay, strains were cultured in UHW gal/raffinose media for 19 h in the presence of 30 μM compounds or DMSO, and β-gal activity was measured by using chlorophenored-β-D-galactopyranoside as substrate after lysing cells with chloroform.

Mammalian Cell Growth. Chinese hamster ovary (CHO), human embryonic kidney (HEK293), human fibrosarcoma (HT1080), human pulmonary carcinoma (A549), human pancreatic adenocarcinoma (PANC-1), and human melanoma A2058 cells were maintained in DMEM supplemented with 10% (vol/vol) FBS. For serum starvation, cells were maintained in DMEM supplemented with 1 mg/ml BSA for 20 h. NIH 3T3 stably transfected with H-Ras (V12) and/or N-terminally truncated and constitutively active Raf-1 (Raf22w) (18) (provided by C. Der, University of North Carolina, Chapel Hill) were maintained in DMEM supplemented with 10% (vol/vol) calf serum. Plasmids were introduced into subconfluent CHO cells by Lipofectamine (GIBCO), and HEK293 cells by the standard calcium phosphate-DNA precipitation. Flow cytometry of A549 cells was performed as described (19) after fixing cells with ethanol. Soft agar assays were performed as described (20).

Transcriptional Reporter Assays. For the serum response element (SRE)-luciferase assay, CHO cells were transfected with pSRE-luciferase (containing four repeats of SRE from the c-*fos* promoter) and serum-starved for 16 h. After 1 h of preincubation with various compounds, cells were stimulated with 10% (vol/vol) FBS for 5 h. For the AP-1 luciferase assay, HEK293 cells were cotransfected with pAP-1-Luc (Stratagene), pCMV-lacZ (provided by D. Chang, University of California, Los Angeles), and pcDNA3-H-Ras (V12). Cells were serum-starved in the presence of various compounds. Promega luciferase assay kit was used according to the manufacturer's protocol. Values were normalized either to the protein concentration or the β-gal activity of samples. HEK293 cellular lysates were subjected to SDS/PAGE, followed by immunoblotting with anti-H-Ras antibody.

***In Vitro* Kinase Assays.** Raf-1 activity was measured as described (21), with the following modifications. For ectopically expressed Raf-1, Flag-tagged Raf-1 was immunoprecipitated with an anti-FLAG antibody conjugated to protein G Sepharose 4FF. Endogenous Raf-1 was immunoprecipitated with anti-Raf-1 antibody. For the MEK1 assay, endogenous MEK1 was immunoprecipitated with anti-MEK1, and rabbit anti-mouse IgG antibodies. Washed immunoprecipitates were incubated in kinase reaction buffer containing His-MEK (for the Raf-1 assay), GST-MAPK (D57), and [γ-^{32}P]ATP. The samples were analyzed by a PhosphorImager (Molecular Dynamics). Expression level of Ras and immunoprecipitated kinases were confirmed by immunoblotting with corresponding antibodies.

Immunofluorescence Analysis. Immunofluorescence analysis was performed as described (22), with the following modifications. HT1080 and NIH 3T3 cells were cultured in uncoated chamber slides and treated with various compounds for 24 h. Cells were stained with tetramethylrhodamine isothiocyanate-conjugated phalloidin and 4′,6-diamidino-2-phenylindole and mounted with Fluoromount-G (Southern Biotechnology Associates). The samples were analyzed by using an Olympus PROVIS AX70 fluorescent microscope mounted with a digital camera (Hamamatsu Photonics, Hamamatsu City, Japan).

***In Vitro* Invasion Assay and Gelatin Zymography.** *In vitro* invasion of HT1080 cells across membranes coated on the top with 85 μg/cm^2 extracellular matrix (ECM, Sigma) was assessed in a transwell assay. Compounds were added to the culture inserts in DMEM with BSA and to the bottom well in DMEM with 10% (vol/vol) FBS, and cells were allowed to invade for 24 h. The results were quantitated by counting the number of invaded cells in five microscopic fields for each membrane. The amounts and activities of cytosolic and secreted matrix metalloproteinases (MMPs) were analyzed by gelatin zymography as described (23).

Results

Developing Robust Screening Tools for Protein Interaction Disrupting Agents. To identify small molecules that disrupt protein–protein interactions, we created several modifications to the yeast two-hybrid system (12). An important consideration was to increase the chance of identifying small molecule inhibitors by enhancing the permeability of screening strains to small molecules. The yeast Pdr1p and Pdr3p transcriptional regulatory proteins positively control the expression of *PDR5*, *SNQ2*, and *YOR1*. These encode ABC transporters which, when overexpressed, contribute to pleiotropic drug resistance (24). Pdr1p and Pdr3p also positively control the transcription of the hexose transporters *HXT11* and *HXT9*. Overexpression of Hxt11p and Hxt9p enhances the sensitivity of yeast to drugs (25). We have modified previously constructed strains SKY48 and SKY191 such that the *PDR1* and *PDR3* genes were disrupted, whereas the *HXT11* and *HXT9* genes were simultaneously induced under the control of the *GAL1* promoter. As shown in Fig. 1*A Upper*, the modified strains, SKY197 and SKY54, were significantly more sensitive than SKY191 and SKY48 to tester small molecules such as cycloheximide and 4-nitroquinoline *N*-oxide. The sensitivity was further increased by growing on media containing galactose (Fig. 1*A Lower*). Sensitivity to sulfomethuron methyl and zeocin was not significantly altered by the modifications, indicating that the uptake of some but not all small molecules was enhanced. The modifications did not affect yeast viability under nonselective conditions (data not shown).

Library Screening. To screen for chemical compounds that would effectively block or diminish the interaction between DBD-H-Ras and AD-Raf-1 (full length), these proteins were expressed in SKY54 and the cells were spread as a monolayer in a soft agar top layer on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) plates. Yeast cells containing DBD-hsRPB7 and AD-hsRPB4 were similarly plated, as a negative control to detect and subtract

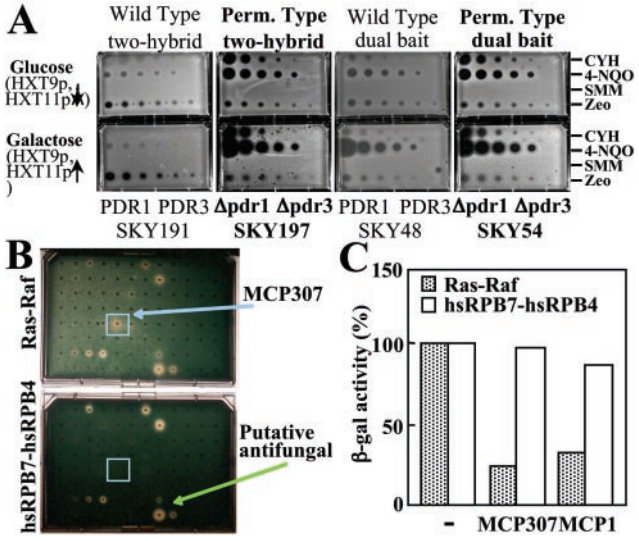

Fig. 1. Identification of compounds that inhibit Ras/Raf interaction by subtractive two-hybrid screening. (*A*) Improved sensitivity of the modified two-hybrid strains to drugs. SKY191 (wild-type two-hybrid) and SKY48 (wild-type dual bait), modified SKY197 (permeability-enhanced two-hybrid), and SKY54 (permeability-enhanced dual bait) strains were grown, and sensitivity to cycloheximide (CYH), 4-nitroquinoline *N*-oxide (NQO), sulfomethuron methyl (SMM), and zeocin (Zeo) was analyzed by spotting chemicals onto the lawn of yeast after serial 4-fold dilution. (*B*) Screening of compounds that inhibit Ras/Raf interaction. SKY54 expressing cI-DBD-H-Ras and AD-Raf-1 (*Upper*) and SKY54 expressing LexA-DBD-hsRPB7 and AD-hsRPB4 (*Lower*) were plated. Compounds were spotted and the appearance of white zones due to decreased expression of β-gal was examined. A specific compound (MCP307) identified in this way and studied further herein is shown. (*C*) Inhibition of β-gal activity by 30 μM MCP307 and MCP1 was examined.

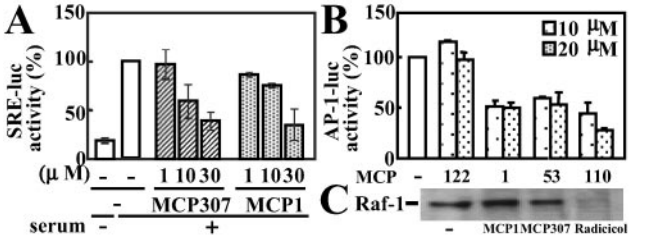

Fig. 2. Inhibition of transcriptional activation by MCP compounds. (*A*) CHO cells were transfected with pSRE-luc and serum-starved. Cells were preincubated with DMSO (−), MCP307 or 1 as indicated and stimulated with 10% FBS for 5 h. Lucifererase assay was performed. (*B*) HEK293 cells were cotransfected with pAP-1-luc and pcDNA3-H-Ras (V12). Cells were serum-starved in the presence of compounds as indicated and luciferase assay was performed. Mean ± SD ($n = 3$). (*C*) HT1080 cells were incubated with DMSO (−), 40 μM MCPs, or 20 μM radicicol for 40 h, and the level of Raf-1 was examined by immunoblotting.

compounds with general antifungal activity. A library of 73,400 compounds was screened in 96-well-plate format. A representative panel demonstrating specific vs. nonspecific inhibition of growth is shown in Fig. 1*B*. 1,959 compounds (2.7%) produced a growth-inhibitory effect in SKY48, vs. 3,009 compounds (4.1%) in SKY54 with DBD-H-Ras and AD-Raf-1, indicating modification of PDR and HXT expression had increased the range of detectable compounds by 50%. Of the 3,009 compounds, 708 induced a phenotype of reduced growth and β-gal activity with some degree of selectivity for H-Ras/Raf-1 versus hsRPB7/hsRPB4 on plates. Assessment of selectivity by liquid β-gal assay identified 38 compounds which produced a clear reduction (with a final range of 3–45% of starting values) in β-gal activity in SKY54 expressing DBD-H-Ras and AD-Raf-1, but not DBD-hsRPB7 and AD-hsRPB4 at 30 μM. Fig. 1*C* shows such results for two compounds MCP307 and MCP1. Similar inhibition of β-gal activity in cells expressing H-Ras and Raf-1 was observed with MCP53 (data not shown). (See below for chemical formula.) These 38 compounds, reflecting a yield of ≈0.05% from the starting library, were used for subsequent functional analyses in mammalian cells.

SRE- and AP-1-Luciferase Secondary Screening for Compound Ranking and Validation. To determine whether the identified compounds inhibit Ras-dependent signaling, serum- or Ras-induced transcriptional activation through SRE and AP-1 sites from the c-*fos* promoter (26) was measured. Both stable and transient expression systems with CHO or NIH 3T3 cells were used to examine the ability of the 38 compounds identified in the interactor screen to inhibit SRE-luciferase expression. Thirteen of the 38 compounds induced significant (2- to 4-fold) reductions of luciferase activity versus DMSO (data not shown). Of these 13 compounds, two compounds (MCP1 and 307) that had excellent profiles in the two-hybrid primary assay (Fig. 1*C*) showed the most robust dose response in CHO cells (Fig. 2*A*). MCP1, 53, and 110 also inhibited H-Ras (V12)-induced AP-1 activation in HEK293 cells (Fig. 2*B*). Based on a combination of cytotoxicity analyses, two-hybrid-based specificity testing, and other cell-based assays (see below), we focused on MCP1 for further characterization and optimization. A detailed description of this process will appear elsewhere; for the immediate studies described here, we compare the originally derived MCP1 ($C_{29}H_{27}ClN_2O_3$, M_r 487) with a similarly active analogue, MCP53 ($C_{29}H_{27}FN_2O_3$, M_r 471); an improved analogue, MCP110 ($C_{33}H_{36}N_2O_3$, M_r 508); and a less active analogue, MCP122 ($C_{22}H_{24}N_2O_2$, M_r 348).

MCP Compounds Do Not Destabilize Raf-1. To exclude the possibility that MCP compounds act in a manner similar to radicicol, which indirectly disrupts Ras/Raf interaction by inhibiting Hsp90 and destabilizing the structurally fragile Raf-1 (27), HT1080 cells were incubated with various compounds for 40 h, and the level of Raf-1 was assessed by immunoblotting. Incubation with MCP1 or 307 did not reduce the level of endogenous Raf-1, whereas Raf-1 was absent after radicicol treatment (Fig. 2*C*).

MCP Compounds Inhibit Ras-Induced Raf/ERK Kinase Cascade. To determine whether MCP110 inhibits Ras-induced Raf-1 kinase activation, HEK293 cells were cotransfected with Flag-tagged Raf-1 and H-Ras (V12). After treatment with MCP compounds, an *in vitro* Raf-1 kinase assay was performed with ectopically expressed Raf-1. Ras stimulation of Raf-1 activity (an 8-fold increase) was significantly decreased by MCP110 (Fig. 3*A*). In fibrosarcoma HT1080 cells, endogenous Raf-1 activity is constitutively up-regulated due to an activated N-*ras* allele (28). MCP110 showed a dose-dependent inhibition of this elevated Raf-1 activity, whereas MCP122 did not (Fig. 3*B*). Also, endogenous MEK1 activity (Fig. 3*C*) and ERK phosphorylation (Fig. 3*D*) in HT1080 cells were inhibited by MCP110 to an extent similar to that seen with MEK inhibitors (PD98059 and U0126), whereas MCP122 had no effect. Furthermore, active MCP compounds (MCP1 or 110) were capable of inhibiting EGF-induced Raf-1 activation in lung carcinoma A549 cells that overexpress the EGF receptor (29), whereas, again, MCP122 showed no detectable effect (Fig. 3*E*). These results demonstrate the potency of MCP1 and derivatives to inhibit Ras-mediated Raf-1 kinase activation. In contrast, MCP110 does not inhibit Raf-1 catalytic activity, as examined by the incubation of active MCP compounds versus Raf kinase inhibitor (Calbiochem) with purified preactivated Raf-1 kinase (data not shown). These

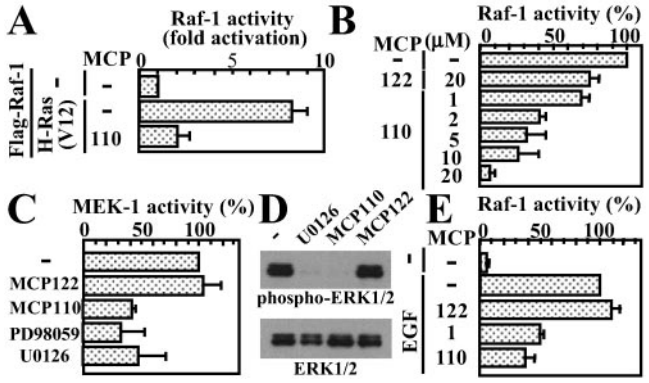

Fig. 3. Inhibition of Raf/MEK/ERK activation by MCP compounds. (*A*) HEK293 cells were cotransfected with expression vectors for Flag-tagged Raf-1 and H-Ras (V12). Cells were serum-starved in the presence of DMSO (−) or 20 μM MCP110. Raf-1 assay was performed by immunoprecipitating Flag-tagged Raf-1. Mean ± SEM ($n = 3$). (*B*) HT1080 cells were cultured for 20 h in the presence of DMSO (−) or MCP compounds as indicated. Endogenous Raf-1 activity was measured. Mean ± SEM ($n = 4$). (*C*) HT1080 cells were cultured in the presence of DMSO (−), 20 μM MCP compounds or PD98059, or 10 μM U0126. Endogenous MEK-1 activity was measured. Mean ± SEM ($n = 3$ or 4). (*D*) HT1080 cells were serum-starved for 20 h in the presence of DMSO (−), 10 μM U0126, or 20 μM MCP compounds. Total as well as phosphorylated ERK1/2 level was examined by immunoblotting. (*E*) A549 cells were serum-starved and stimulated with 100 ng/ml EGF for 5 min. Two hours before EGF stimulation, cells were incubated with DMSO (−) or 20 μM MCP. Endogenous Raf-1 activity was measured. Mean ± SEM ($n = 6$).

compounds did not inhibit Ras-induced Akt phosphorylation in COS-7 cells cotransfected with activated H-Ras and Myc-tagged Akt (data not shown).

MCP Compounds Reverse *ras*-Transformed Phenotypes of HT1080 Cells. Effects of MCP1 and derivatives on *ras*-transformed phenotypes have been examined by using HT1080 cells. We first

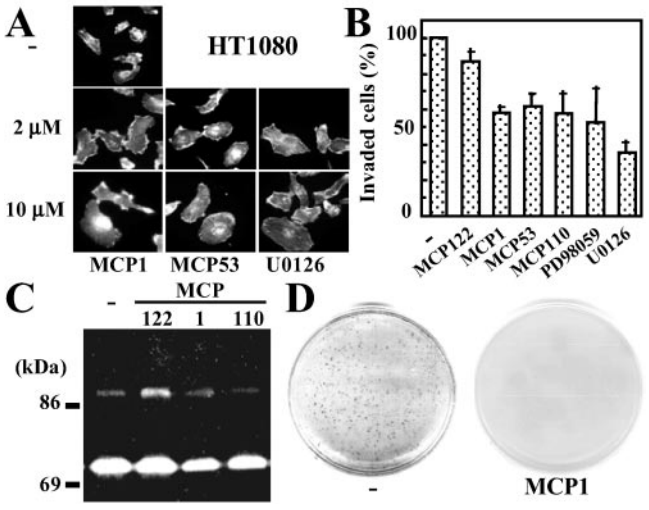

Fig. 4. Reversion of *ras*-transformed phenotypes by MCP compounds in HT1080. (*A*) Cells were treated with DMSO (−) or 2 or 10 μM various compounds for 24 h as indicated and stained with phalloidin. Images are representative of three independent experiments (×400 magnification). (*B*) The *in vitro* invasion assay was performed in the presence of various compounds (10 μM). Mean ± SEM ($n = 3$). (*C*) Inhibition of MMPs by MCPs. Cells were cultured in serum-free medium in the presence of DMSO (−) or 20 μM MCP compounds for 24 h. A gelatin zymogram with conditioned media is shown. Results are representative of three independent experiments. (*D*) Cells were cultured in soft agar in the presence of DMSO (−) or 10 μM MCP1. Results are representative of three independent experiments.

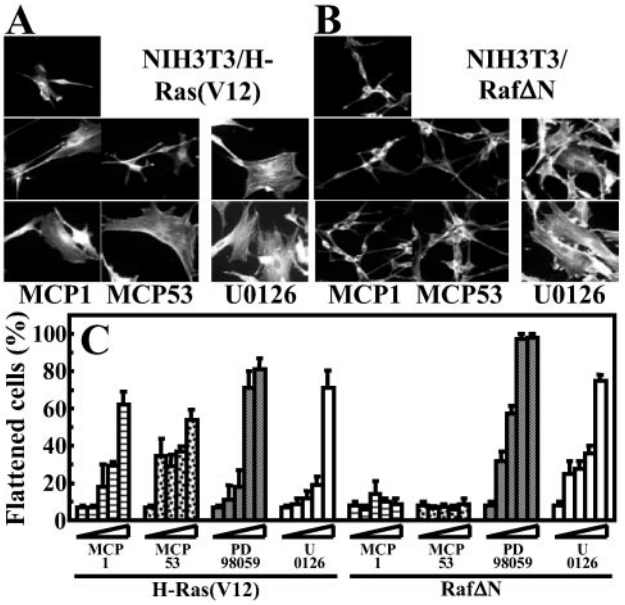

Fig. 5. Morphological reversion of Ras-transformed but not Raf-transformed NIH 3T3 cells by MCP compounds. Cell morphology and actin cytoskeleton of Ras-transformed (*A*) and Raf-transformed (*B*) cells were examined after the treatment with MCPs or U0126. (*Top*) DMSO; (*Middle*) 2 μM; (*Bottom*) 10 μM. Images are representative of three independent experiments. (*C*) Percentage of enlarged cells with actin filaments in *A* and *B* is shown. A total of 0, 1, 2, 5, and 10 μM of each compound was used. Mean ± SEM ($n = 3$).

examined whether MCP compounds are capable of causing morphological changes, because it has been reported that activation of the Ras/Raf/ERK pathway leads to morphological changes (30, 31) and the loss of the mutated N-*ras* allele in HT1080 cells results in reversion to flat morphology (32). As shown in Fig. 4*A*, incubation of HT1080 cells with MCP1, MCP53, or U0126 caused these cells to revert from a characteristic rounded phenotype to a more flattened phenotype. Next, we focused on invasive properties of HT1080 cells. The ability of MCP compounds to inhibit the invasive properties of HT1080 cells was evaluated in transwell assays by using ECM-coated membranes. Incubation with active MCP compounds (MCP1, 53, or 110) led to ≈40% inhibition of the number of cells that invaded through the ECM compared with control treatments, whereas MCP122 showed only a minor effect (Fig. 4*B*). The extent of inhibition of HT1080 invasion by the MCP compounds was similar to that obtained with PD98059. Effects of MCP compounds on the production of MMPs that degrade the ECM were examined by gelatin zymography with the conditioned media (Fig. 4*C*) from cells treated with MCP compounds. MCP1 and 110 treatment decreased gelatinolytic activities detected at the position corresponding to the apparent molecular weight of MMP-9 (92,000) and -2 (72,000), whereas no significant changes were observed with MCP122. These results are consistent with the report that the Ras/Raf/ERK pathway up-regulates MMPs including MMP-9 (33). Finally, as shown in Fig. 4*D*, MCP1 inhibited anchorage-independent growth of HT1080 cells.

MCP Compounds Morphologically Revert *ras*-Transformed but Not Activated *raf-1*-Transformed NIH 3T3 Cells. We used two different transformed NIH 3T3 cells to characterize further the mode of action of MCP compounds. Fibroblasts transformed with *ras* lose actin stress fibers and exhibit spindle-like cell morphology with a small round cell body. Incubation with MCP1, 53, or U0126 caused increased cell spreading and restoration of actin filaments

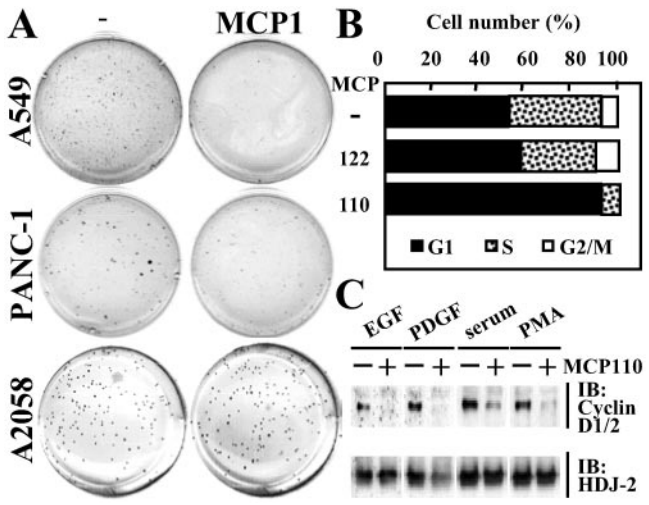

Fig. 6. Induction of G_1 accumulation by MCP compounds. (*A*) Inhibition of anchorage-independent growth of A549, PANC-1, and A2058 cells by MCP compounds. Cells were cultured in soft agar in the presence of DMSO (−) or 10 μM MCP1. Results are representative of three independent experiments. (*B*) The cell cycle profile of A549 cells after treatment with DMSO (−) or MCP compounds (20 μM) for 72 h. The percentage of cells in G_1, S, and G_2/M was determined by flow cytometry with CELL QUEST software. A representative profile from three independent experiments is shown. (*C*) Inhibition of cyclin D expression by MCP110. A549 cells were serum-starved in the presence of DMSO (−) or 20 μM MCP110. Cells were stimulated with 100 ng/ml EGF, 20 ng/ml platelet-derived growth factor, 10% FBS, or 100 nM phorbol 12-myristate 13-acetate for 4 h. The level of cyclin D was examined by immunoblotting. Heat shock protein HDJ-2 was immunoblotted as a loading control. Results are representative of three independent experiments.

in NIH 3T3 cells transformed with H-Ras (V12) (Fig. 5*A*). In contrast, MCP compounds did not affect the morphology of NIH 3T3 cells transformed with catalytically active Raf22w (RafΔN), whereas U0126 induced morphological reversion in these cells (Fig. 5*B*). The percentage of enlarged cells with actin filaments as a factor of the concentration of the different compounds tested was plotted in Fig. 5*C*. These results clearly place the mode of action of MCP compounds as affecting Ras-initiated events upstream of Raf.

MCP Compounds Are Active Against Human Cancer Cells with K-*ras* Activation. Because *ras* mutations are found primarily in K-*ras* in human cancer (3), it was of interest to examine the effect of MCP1 on human cancer cell lines harboring K-*ras* mutation. We first examined the ability of MCP1 to inhibit soft agar growth of lung carcinoma A549 and pancreatic adenocarcinoma PANC-1 cells both of which harbor activated K-*ras*. As shown in Fig. 6*A*, significant inhibition was observed with MCP1. Similar results were obtained with MCP53 and 110 (data not shown). In contrast, MCP1 did not inhibit soft agar growth of A2058 melanoma cells harboring activated *B-raf* mutation (E599) (ref. 34; Fig. 6*A*), even though it could be inhibited by U0126 (data not shown). Similar results were obtained with MCP53 (data not shown). Next, we focused on A549 and examined effects of MCP on cell cycle progression. A significant increase in the percentage of G_1 phase cells and simultaneous decrease in S and G_2/M phase cells were observed after the treatment of A549 cells with MCP110 (Fig. 6*B*). No sub-G_1 peaks, indicative of apoptotic cells, were observed. No significant change of cell cycle profile was detected with MCP122 treatment. Furthermore, MCP110 decreased cyclin D level stimulated by the treatment with EGF, platelet-derived growth factor, serum, or phorbol 12-myristate 13-acetate (Fig. 6*C*). These results are consistent with the report that activation of the Ras/Raf/ERK pathway increases the level of cyclin D (35). In addition, these results contrast with the effects of farnesyltransferase inhibitors (FTIs) on A549; FTIs cause accumulation of G_2/M phase cells (see *Discussion*).

Discussion

We report here the identification of small molecular weight compounds (MCP1 and its derivatives) that effectively inhibit the activation by Ras of one of its downstream effectors, Raf-1. This work demonstrates the feasibility of using a forward two-hybrid format to achieve robust protein interaction-based primary drug screening. We used a two-hybrid subtractive approach, where the differential ability of compounds to disrupt H-Ras/Raf-1 interaction versus the interaction of a negative control allowed the facile elimination of nonspecific compounds. Standard microplate-based drug screening formats readily support such replicate analysis, and the approach can be potentially adapted to high-throughput computational image analysis. Coupled with the application of a moderate-throughput mammalian cell-based secondary assay using reporter genes, we rapidly resolved a large starting set of compounds to a smaller group whose mode of activity could be analyzed in detail.

We have established that the MCP compounds inhibit activation of Raf-1 by Ras in human cancer cells. MEK1 and ERK activation downstream of Raf-1 is also inhibited by the treatment with these compounds. MCP compounds reverse *ras*-transformed phenotypes. First, they induced morphological reversion of HT1080 and *ras*-transformed NIH 3T3 cells. However, they were incapable of causing flat reversion of activated *raf*-transformed NIH 3T3 cells, consistent with the idea that these compounds act between Ras and Raf. Second, MCP compounds decrease the activity of MMPs, and inhibit the *in vitro* invasiveness of cells with N-*ras* activation. Third, MCP compounds cause a decrease in cyclin D levels and enrichment of G_1 phase cells. Finally, MCP compounds inhibit anchorage-independent growth of cancer cells with K-*ras* activation. It has been reported that the inhibition of the Raf/MEK/ERK pathway has significant consequences on *ras*-transformed phenotypes in these cell lines (36, 37). MCP compounds do not destabilize Raf-1 nor inhibit kinase activity of preactivated Raf-1. It is the Ras-induced Raf-1 activation that is inhibited by these compounds. The activation of Raf by Ras involves multiple events that include membrane recruitment, phosphorylation, and conformational changes of Raf-1, involving protein–protein interaction (38). It will be of interest to determine which of these processes is affected by these MCPs. In our two-hybrid assay (Fig. 1*C*), we used H-Ras and full-length Raf-1 to demonstrate effects of MCP. However, no significant inhibition was observed when Ras-binding domain (56–134 aa) of Raf-1 was used (unpublished data). Although further experiments are needed, one possibility we envision is that MCP may bind to a complex interface between Ras and Raf-1, involving determinants beyond the Ras-binding domain of Raf-1 in a manner that induces an allosteric effect, negatively impacting its activation by Ras. Elucidation of this point requires biochemical analysis to examine the inhibitory mechanism of MCP1 on H-Ras/Raf-1 interaction.

Our studies to date highlight significant biological differences between the MCP compounds and FTIs. FTIs inhibit farnesylation and the membrane association of Ras proteins, and hence interrupt Ras-dependent signaling (9). However, FTIs are incapable of inhibiting prenylation of K-Ras, because K-Ras has been found to be geranylgeranylated in the presence of FTIs (39). In fact, soft agar growth of PANC-1 and A549 cells harboring K-*ras* mutation is reported to be resistant to FTIs (40, 41). In contrast, MCP1, 53, and 110 caused significant inhibition of anchorage-independent growth of PANC-1 and A-549 cells. These compounds may also be effective on geranylgranylated TC21/R-Ras2, which is mutated in some human tumor cell lines (42). Furthermore, MCP compounds induced enrichment of G_1 phase

cells with a lung cancer cell line, A549, an effect expected from the inhibition of Ras-dependent signaling. In contrast, FTIs induce enrichment of G_2/M phase cells with A549 (43, 44), possibly reflecting their lack of specificity for Ras as a target. On the basis of these properties, MCP1 and its derivatives may prove to be valuable alternatives to FTIs against K-*ras*-activated tumors, which constitute the major class of Ras-initiated human cancers.

The MCP compounds reported here are capable of reversing the *ras*-transformed phenotypes because of the activation of the Raf/MEK/ERK pathway. Modification of these MCP lead compounds is currently ongoing to obtain more potent compounds. It is also important to point out that these MCP compounds may have a broader spectrum of activity than MEK inhibitors, because Raf-1 initiates MEK-independent signaling pathways. For instance, Raf-1 binds and phosphorylates Rb and reverses Rb-mediated E2F activity (45). It also binds and phosphorylates Bcl-2 and cooperates in regulating the apoptotic regulatory function of Bcl-2 in mitochondria (46). Raf-1 is reported to phosphorylate IkB and NF-κB, leading to the activation of the NF-κB survival pathway (47). Finally, Raf-1 interacts with the proapoptotic, stress-activated protein kinase ASK1 and inhibits ASK1-induced apoptosis (48). It will be interesting to examine what effects MCP compounds have on these MEK-independent activities of Raf-1. A comprehensive analysis of MCP effects on other signaling pathways is ongoing, and the results should provide further insight into cellular activities of these compounds.

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