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Synthetic Inhibitors of Proline-Rich Ligand-Mediated Protein-Protein Interaction: Potent Analogs of UCS15A

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Summary

The proline-rich motif in proteins is known to function as a ligand sequence that binds to protein modules such as SH3, WW, and several other protein interaction domains. These proline-rich ligand-mediated protein-protein interactions (abbreviated PLPI) are important in many signaling pathways that are involved in various diseases. Our previous studies showed that UCS15A, produced by *Streptomyces* species, inhibited PLPI. Here we report on synthetic analogs of UCS15A that show more potent activity than UCS15A in inhibiting PLPI. A synthetic analog, compound 2c, blocked in vitro PLPI of Sam68-Fyn-SH3 as well as in vivo PLPI of Grb2-Sam68 and Grb2-Sos1. Activation of MEK was also inhibited by compound 2c. Unlike UCS15A, compound 2c was an order of magnitude less cytotoxic and did not cause morphological changes in treated cells.

Introduction

Signal transduction is carried out by networks of signaling molecules that carry signals from the cell surface to the nucleus and cause diverse cellular responses including proliferation, differentiation, migration, metabolism, cytoskeletal reorganization, and transcriptional regulation [1, 2]. These signaling proteins are connected spatiotemporally by sequential protein-protein interactions between protein modules that determine the specificity of these interactions in the transduction of signals [1, 3]. Since deregulated signal transduction has been implicated in several human diseases, drugs that regulate cellular signaling are actively being sought [4]. Such drugs would have the potential to not only regulate the local function of signaling proteins but could conceivably trigger dynamic changes in signaling pathways downstream of the target proteins. In this regard, protein-kinase inhibitors represent one of the best examples of signaling proteins against which small molecule inhibitors have been successfully developed [5].

In the mid-1980s, the discovery of potent protein kinase inhibitors staurosporine and K-252, derived from microbial products [5–7], attracted much attention from the pharmaceutical industry and provided the impetus to initiate studies to develop selective inhibitors of protein kinases [8, 9]. Our screens of microbial products to identify anatagonists targeting signal transduction by protein tyrosine kinases resulted in the rediscovery of radicicol [10, 11] and UCS15A (SI-4228A/Luminacin) [12], which was previously identified as having antifungal and angiogenesis inhibitory activities [13–16]. UCS15A was identified as a small molecule inhibitor of Src-signal transduction using a yeast-based assay, in which inhibitors were detected based on their ability to rescue the growth arrest of yeast, resulting from the overexpression of activated v-src [12, 17]. Early investigations into the mode of action of UCS15A revealed that UCS15A differed from conventional Src inhibitors in that it did not inhibit the tyrosine kinase activity of v-src, nor did it alter the stability of v-src by antagonizing the molecular chaperone hsp90 [12]. Further studies revealed that UCS15A represented the first example of a nonpeptide, small molecule agent capable of disrupting SH3-mediated protein-protein interactions by interacting directly with proline-rich ligands in target proteins [18]. Src, the first tyrosine kinase to be identified [19], contains protein domains that were later identified in a host of other proteins, including many nontyrosine kinases, and were therefore designated Src homology (SH) domains. In addition to the catalytic tyrosine kinase domain (designated the SH1 domain), Src also contains two noncatalytic domains, designated SH2 and SH3 domains. These noncatalytic domains were subsequently shown to be responsible for critical protein-protein interactions in signal transduction [20, 21], and many subsequent studies highlighted their biological importance. Our studies on UCS15A have shown that the growth arrest-rescue assay using Src-overexpressing yeast is a productive method to discover drugs that inhibit protein-protein interactions [12].

Proline-rich ligands recognize and bind to SH3, WW, and several protein interaction domains [22]. Proline-rich ligand-mediated protein-protein interactions (PLPI) have been implicated in various human diseases such as cancer, allergy, asthma, osteoporosis, and several virus-induced pathologies [22–24]. Given the importance of PLPI, there is a great deal of interest in finding inhibitors of this interaction. Since UCS15A is the first and only small molecule inhibitor of PLPI thus far identified, it represents a promising lead compound for the development of drugs that inhibit PLPI. Given UCS15A's unique ability to disrupt PLPI, we synthesized compounds that were structurally related to UCS15A and

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Table 1. Inhibitory Activity of UCS15A Analogs on PLPI In Vitro

UCS15A analog	Inhibitory Activity (%)		
	20 μM	100 μM	300 μM
UCS15A	8	39	100
1a		85	100
1b		95	100
1c	15	100	100
1d		0	0
1e		80	100
1f		39	100
2a	45	100	100
2b		0	0
2c	72	100	100
2d		32	100
2e		13	35

Percentage of inhibitory activity of UCS15A analogs on the association of Sam68ΔC with Fyn-SH3 in vitro. Values shown are averages of data obtained in three independent experiments.

examined their potential inhibitory activities toward PLPI both in vitro and in vivo. These UCS15A derivatives were synthesized from UCS15A that was prepared by fermentation. In addition, the recently reported total synthesis and establishment of the absolute structure of UCS15A [25, 26] provided us access to a number of synthetic small molecules that were related to UCS15A. Our current studies identify essential regions of the chemical backbone of UCS15A as well as chemical groups that are important for its ability to disrupt PLPI. In addition, our studies have identified compound **2c**, a "simple analog" of UCS15A, as the minimal chemical entity responsible for this activity. Compound **2c** exhibited enhanced potency and reduced cytotoxicity compared to the native drug.

Results

Effect of UCS15A Analogs on PLPI by In Vitro Assays

We previously showed that UCS15A-mediated disruption of PLPI could be reproduced in an in vitro assay system consisting of two recombinant proteins, Fyn-SH3 and Sam68ΔC [18]. Thus, this highly pure in vitro system was used to assay for other inhibitors of PLPI, since it allowed the direct examination of the interaction. To this end, Sam68ΔC protein was incubated with increasing concentrations of each compound (Figure 2 and Table 1), as detailed in the Experimental Procedures section. Subsequently, the AC-Fyn-SH3 domain beads were added to the reaction. Sample processing and quantitation of binding were determined as detailed in Experimental Procedures.

The dose dependency of UCS15A for disrupting the interaction between Sam68ΔC and Fyn-SH3 was examined in the in vitro system. As shown previously [18], in the absence of UCS15A, Sam68ΔC associated with Fyn-SH3 and UCS15A inhibited this interaction in a dose-dependent manner (Figure 2). UCS15A-related compounds were classified into two categories: derivatives of natural UCS15A (Figure 1A) and synthetic compounds with partial structure(s) of UCS15A (Figure 1B). Based on these results, the inhibitory activity of all the UCS15A analogs was examined at 100 and 300 μM, and particularly potent inhibitors (which abolished this association completely, even at concentration of 100 μM) were evaluated at an even lower concentration of 20 μM (Table 1). In most of the derivatives of UCS15A, the C1 aldehyde moiety of UCS15A was modified (Figure 1A). Five of the six derivatives of UCS15A tested disrupted PLPI in a dose-dependent manner, albeit with different efficacies (Table 1, compounds **1a–1f**). Compound **1c**, a peracetate derivative of UCS15A, was slightly more efficient that UCS15A in inhibiting PLPI, while compound **1d**, a methoxime derivative of UCS15A, showed no inhibition of PLPI even at concentration as high as 300 μM (Table 1). Synthetic compounds with partial structures of UCS15A, shown in Figure 1B, were examined to discover the importance of chemical groups neighboring the benzene ring of UCS15A. Among five synthetic compounds with partial structures of UCS15A, compounds **2a** and **2c** showed more potent inhibition of PLPI in this system than UCS15A, while compound **2b** did not show any inhibitory activity even at a concentration as high as 300 μM (Figure 1B and Table 1). Compound **2c** was the most potent of the UCS15A analogs analyzed in this study. The only structural difference between compounds **2c** and **2b** was the presence of an acyl group at the C3 position. Modified compounds with partial structures of UCS15A, compounds **2d** and **2e**, were examined to better understand the contribution of the methoxy group at the C1″ position. Interestingly, C1″ hydroxy compound **2d** was similar to UCS15A, while compound **2e**, which has a sugar-like moiety at the C1″ position, showed reduced activity toward disrupting PLPI. Taken together, these findings indicate that some UCS15A analogs have potent inhibitory activities on PLPI as assayed by the in vitro system and that chemical groups surrounding the benzene ring modulate this activity.

Compound 2c Disrupts PLPI between Grb2-Sam68 and Grb2-Sos1 In Vivo

In vitro results suggested that compound **2c** was the most potent inhibitor of PLPI in the in vitro system (Table 1). We therefore focused our attention on the activity of compound **2c** in vivo. Previous studies have shown that UCS15A disrupted several combinations of proline-rich mediated interactions [18]. Among them, the association of Grb2 with Sam68 was chosen to study the effect of UCS15A analogs on PLPI in vivo. In the experiment shown in Figure 3A, Grb2 protein was immunoprecipitated from UCS15A- or compound **2c**-treated HCT116 cells, and the associated Sam68 protein was examined by immunoblot analyses with Sam68 antibody. As shown previously [18], Sam68 was found to associate with Grb2 in untreated cells (Figure 3A, lanes 3 and 4), and UCS15A effectively blocked the Grb2-Sam68 complex in HCT116 cells (Figure 3A, lanes 4–6). Consistent with the in vitro data, compound **2c** had a potent effect on the in vivo Grb2-Sam68 complex, even at concentrations as low as 2 μM (Figure 3A, lanes 1–3). Since the physiological relevance of the Grb2-Sam68 complex is at present unclear, we also examined the effect of the drugs on a more established SH3 domain-mediated interaction, namely the Grb2-Sos1 complex, in HeLa cells (Figure 3B). To this end, Grb2 protein was immuno-

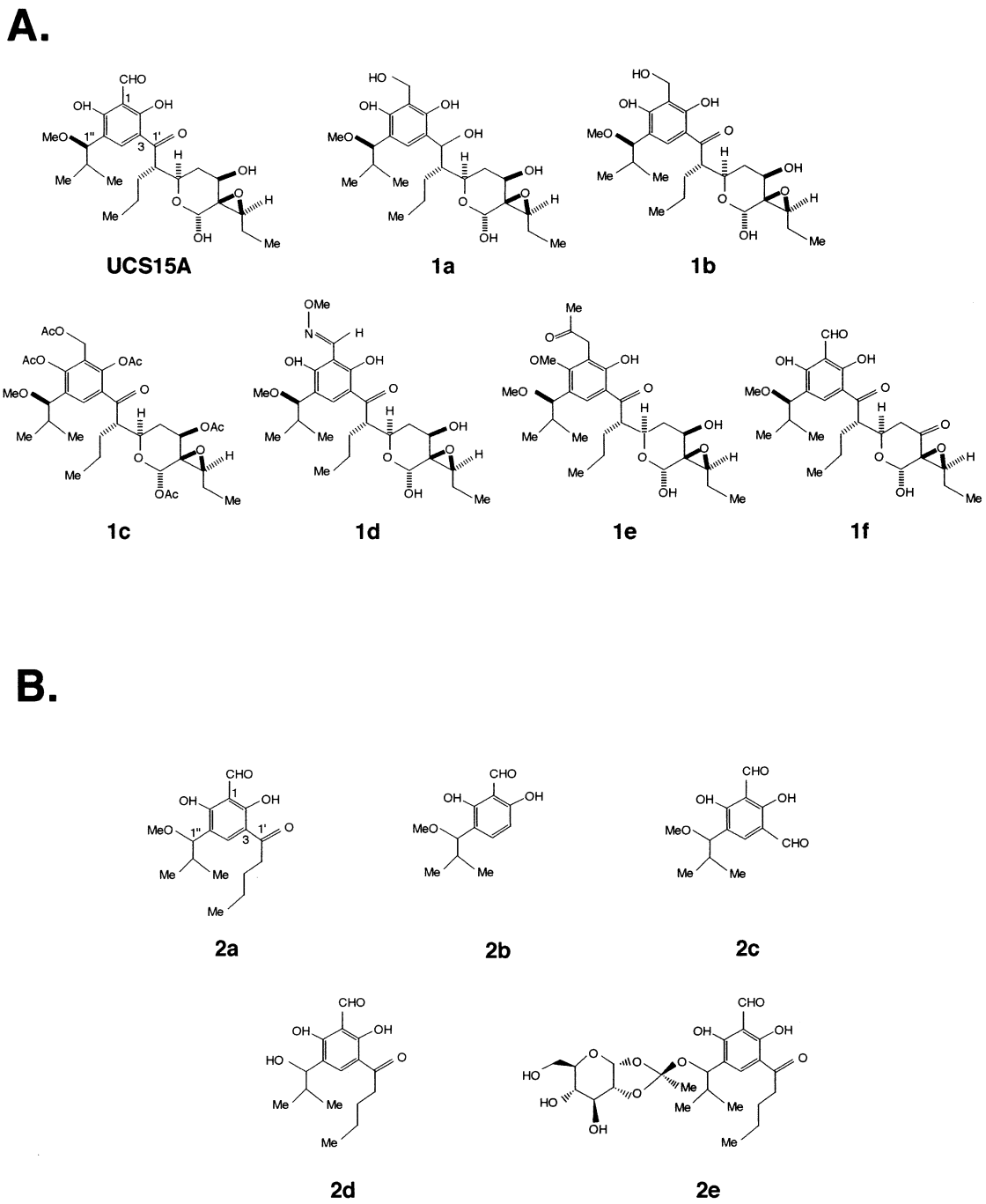

Figure 1. Chemical Structure of UCS15A and Analogs

(A) Structures of UCS15A and its derivatives (**1a–1f**).

(B) Structures of synthetic analogs of UCS15A (**2a–2e**).

precipitated from UCS15A-, compound **2c**-, or compound **2b**-treated HeLa cells, and the associated Sos1 protein was examined by immunoblot analyses with Sos1 antibody. As shown previously in HCT116 cells, Sos1 was found to associate with Grb2 in untreated HeLa cells (Figure 3B, lane 1), and both UCS15A and compound **2c** very effectively disrupted the Grb2-Sos1 complex (Figure 3B, lanes 2–4 and 5–7, respectively). By contrast, compound **2b** had no effect on the Grb2-Sos1 complex even at concentration as high as 10 μM (Figure 3B, lanes 8–10). These results demonstrated that compound **2c**, the most potent inhibitor of PLPI in vitro, was also effective in vivo. In addition, the fact that compound **2c** was able to disrupt PLPI in vivo indicated that the drug was capable of permeating cells.

Compound 2c Inhibits the Activation of MAPK Kinase

The finding that compound **2c** could inhibit the PLPI between Grb2-Sam68 and Grb2-Sos1 in cells suggested

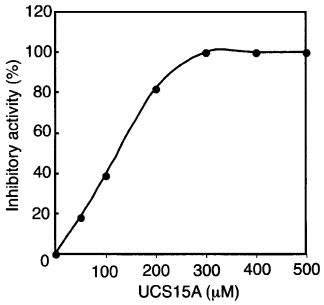

Figure 2. Inhibitory Activity of UCS15A on PLPI In Vitro

GST-Sam68ΔC was added in the absence or presence of the indicated concentrations of UCS15A and incubated for 6 hr at 37°C. Subsequently, Fyn-SH3 AC beads were added to the reactions and incubated for 12 hr at 4°C. Following the incubation, affinity precipitates with Fyn-SH3 AC beads were probed with anti-GST antibody. To quantitate the inhibitory activities of UCS15A on PLPI, the densities of both the Sam68ΔC and Fyn-SH3 bands were scanned from the autoradiogram of the Western blot. Subsequently, the ratio of the density of the Sam68ΔC and Fyn-SH3 bands was calculated and compared to the ratio of the two bands in the absence of the drug (for details, see Experimental Procedures). Each experiment was repeated three times, and results were expressed as averages of the data.

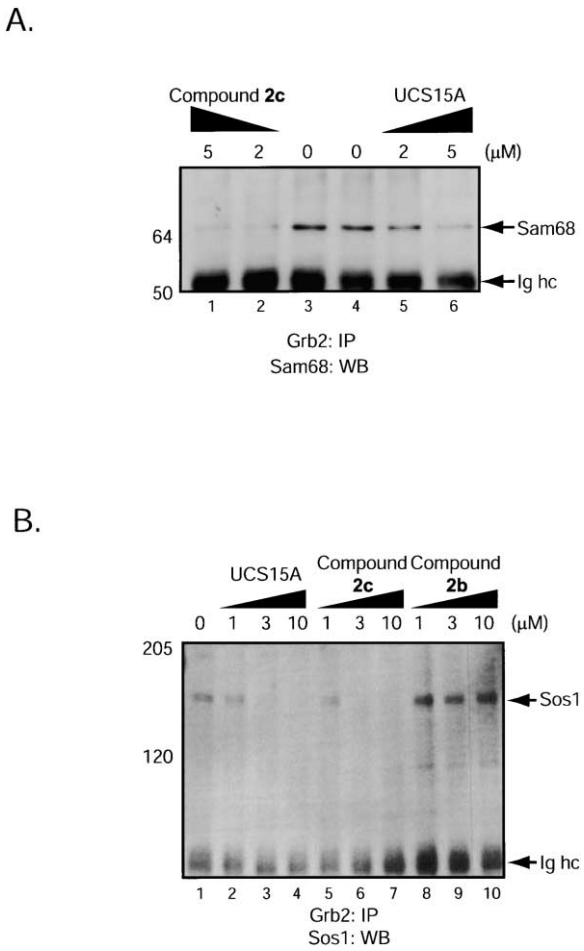

Figure 3. Effect of UCS15A, Compound **2c**, and Compound **2b** on PLPI In Vivo

(A) HCT116 cells were treated for 2 hr with the concentrations of UCS15A and compound **2c** indicated at the top of each lane. Anti-Grb2 immunoprecipitates (IP) were immunoblotted and probed (WB) with anti-Sam68 antibody.

(B) HeLa cells were treated for 2 hr with the concentration of UCS15A, compound **2c**, and compound **2b** indicated at the top of each lane. Anti-Grb2 immunoprecipitates (IP) were immunoblotted and probed (WB) with anti-Sos1 antibody. Positions of migration of Sam68, Sos1, and the heavy chain of immunoglobulin (Ig hc) are indicated to the right of the autoradiograms. Approximate positions of migration of prestained molecular weight markers are indicated in kilodaltons to the left of the autoradiograms.

the possibility that the drug may exert an effect on signaling pathways downstream of Grb2. Therefore, activation of MEK, which is regulated in EGF-induced signaling, was chosen as an example of an interaction that functions downstream of the PLPI of the Grb2-Sos1 complex. In addition, the effect of the drug on MEK activation was also tested in HCT116 cells that harbor an activated K-ras gene; therefore, activation of MAP kinase should be independent of Grb2-Sos1. Previous studies have shown that phosphorylation of serines 217 and 221 in the MEK protein were essential for its kinase activity [27, 28]. The effect of compound **2c** was tested on the in vivo activation state of MEK in drug-treated HeLa cells stimulated with EGF, or HCT116 cells without serum stimulation. Activation status of MEK was assayed using a phosphospecific antibody for MEK (Ser217/221) (Figures 4A and 4B). Serine 217/221 was not phosphorylated in serum-starved HeLa cells (Figure 4A, lane 1) and phosphorylated after stimulation with EGF (Figure 4A, lane 2). UCS15A treatment decreased the EGF-dependent phosphorylation of serine 217/221 in a dose-dependent manner (Figure 4A, lanes 3–5). Under the same conditions, compound **2c** was slightly more effective at inhibiting the activation of MEK (Figure 4A, lanes 6–8), while compound **2b** did not alter the status of MEK phosphorylation under the same conditions (Figure 4A, lanes 9–11). In untreated HCT116 cells, as expected, MEK was phosphorylated on Ser 217/221 (Figure 4B, lanes 1 and 2). However, surprisingly, UCS15A treatment also decreased the phosphorylation of MEK on serine 217/221 in a dose-dependent manner (Figure 4B, lanes 3–5). Similarly to HeLa cells, compound **2c** more effectively decreased the phosphorylation of MEK at concentrations as low as 1 μM. Taken together, these data suggested that compound **2c** prevented activation of MEK. The surprising fact that it did so even in the context of an activated ras gene (in HCT116 cells) suggested that it may have additional point(s) of action downstream of ras.

Compound 2c Is Distinct from UCS15A in Inducing Morphological Change in Cells

Previous studies have shown that UCS15A-treated v-src 3T3 cells exhibited a very characteristic and reversible morphological change, resulting in more refractile cells with almost complete disappearance of filopodia [12]. Similarly, treatment of HCT116 cells for 6 hr with UCS15A resulted in the characteristic refractile morphology at 1 μM and 5 μM without any effect on the viability of the cells (Figure 5, panels B1 and B2). UCS15A inhibited the growth of HCT116 cells with an IC_{50} of 5.8 μM after 72

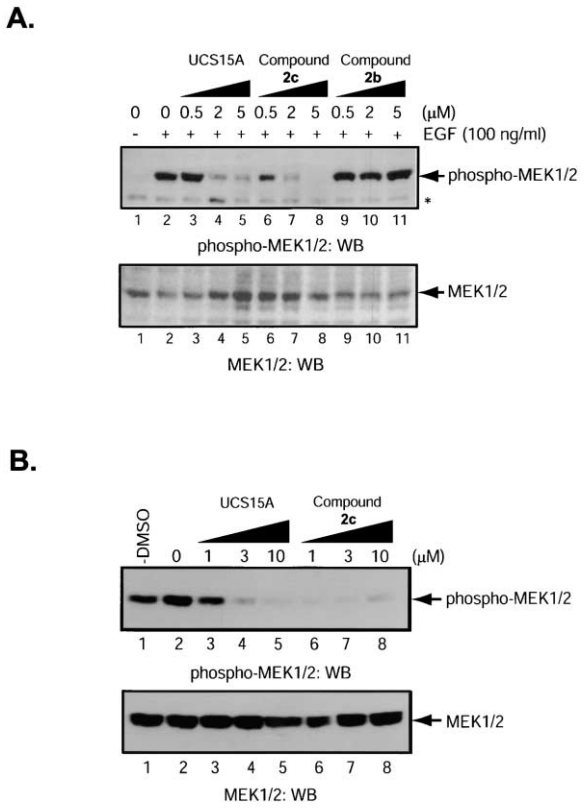

Figure 4. Effect of UCS15A, Compound **2c**, and Compound **2b** on the Activation of MEK in HeLa Cells Stimulated with Epidermal Growth Factor and in HCT116 Cells

(A) Serum-starved HeLa cells were treated for 4 hr with the concentrations of UCS15A, compound **2c** and compound **2b** indicated at the top of each lane. Subsequently, the cells were stimulated with 100 ng/ml of EGF for 15 min as indicated at the top of each lane.

(B) HCT116 cells were treated for 2 hr with the concentrations of UCS15A and compound **2c** indicated at the top of each lane. Cells were lysed and 10 μg of total cellular proteins was analyzed by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phosphospecific anti-MEK1/2 antibody (upper panel). The membrane from the upper panel was stripped of the antibody probe and reprobed with anti-MEK1/2 antibody (lower panel). The position of migration of phospho-MEK1/2 and MEK1/2 is indicated to the right of the autoradiograms.

hr incubation (data not shown). In contrast, HCT116 cells treated with compound **2c** for 6 hr appeared almost identical to untreated control cells with typical epithelial appearance, even at much higher concentrations of 5 or even 20 μM (Figure 5, panels C1 and C2). Compound **2c** inhibited the growth of HCT116 cells with an IC_{50} of 87 μM after 72 hr incubation (data not shown). Unlike UCS15A, compound **2c** had no effect on the morphology of v-src-transformed NIH3T3 cells (data not shown). Taken together, these data indicated that compound **2c** was more selective than UCS15A and did not cause the morphological changes induced by UCS15A.

Discussion

In the present study, several analogs of UCS15A were synthesized and shown to inhibit PLPI. Of these, the synthetic compound **2c**, with a partial structure of UCS15A, was the most potent inhibitor of PLPI in both in vitro and in vivo assays. Both UCS15A and compound **2c**, were capable of inhibiting the activation of MAPK in cells harboring wild-type or activated ras genes. However, unlike UCS15A, compound **2c** did not induce drastic morphological changes in treated cells and was less cytotoxic to cells.

Since UCS15A contains a reactive epoxide group in the molecule, it might be expected to bind covalently to proteins through their amino or thiol group [29–31]. Surprisingly, the present study revealed that the epoxide group of UCS15A did not appear to play a role in disrupting PLPI, since compounds lacking the epoxide group retained the ability to inhibit PLPI (Figure 1B and Table 1). It is interesting that compound **1d**, the methoxime derivative of UCS15A, did not show inhibitory activity for PLPI, while other derivatives disrupted PLPI with varying efficiencies (Figure 1A and Table 1).

Interestingly, even compounds with partial structure of UCS15A effectively inhibited PLPI, with the exception of compound **2b** (Figure 1B and Table 1). This suggested that the substitution at the C3 position could be important for inhibition of PLPI. Moreover, compound **1a**, in which a hydroxy group was substituted for a carbonyl group, retained blocking activity, suggesting that the presence of either a carbonyl or hydroxy group at the C3 position is sufficient for PLPI inhibitory activity. In addition, compound **2e**, in which the methoxy group at the C1″ position was substituted for a glucose unit, drastically reduced the inhibitory activity of PLPI (Figure 1B and Table 1). This result suggested that the methoxy group at the C1″ position is also important for blocking PLPI, and the presence of bulky groups may interfere with this function. Consistent with this hypothesis, several synthetic compounds lacking the bulky sugar-like unit of compound **2e** were potent inhibitors of PLPI (Figure 1B and Table 1). These results suggested that the sugar-like structure of UCS15A does not contribute to its PLPI blocking activity, but may in fact interfere with this activity.

Compound **2c** was the smallest synthetic analog of UCS15A capable of blocking PLPI both in vitro as well as in vivo (Table 1 and Figure 3, respectively). This suggested that compound **2c** permeated through cell membranes and exerted its inhibitory effects on the proline-rich-mediated protein-protein complex in vivo. Further evidence for the cell permeability of compound **2c** emerged from the observation that it inhibited MEK activity in treated cells (Figure 4). While the in vitro system consisted of only two recombinant proteins, and it was both simple and direct to examine the potential of a compound for disrupting PLPI, it is necessary to examine the effect of the drug on PLPI in vivo to know its biological efficacy. In this regard, these results suggest that compound **2c** is promising for its potential therapeutic activity in animal models of disease in which PLPI have been implicated. Surprisingly, compound **2c** was relatively nontoxic (IC_{50} = 87 μM) despite its ability to disrupt PLPI. In this regard, it differed from the parent compound UCS15A that also disrupted PLPI but was quite toxic (IC_{50} = 5.8 μM).

An intriguing observation was the fact that **2c** required

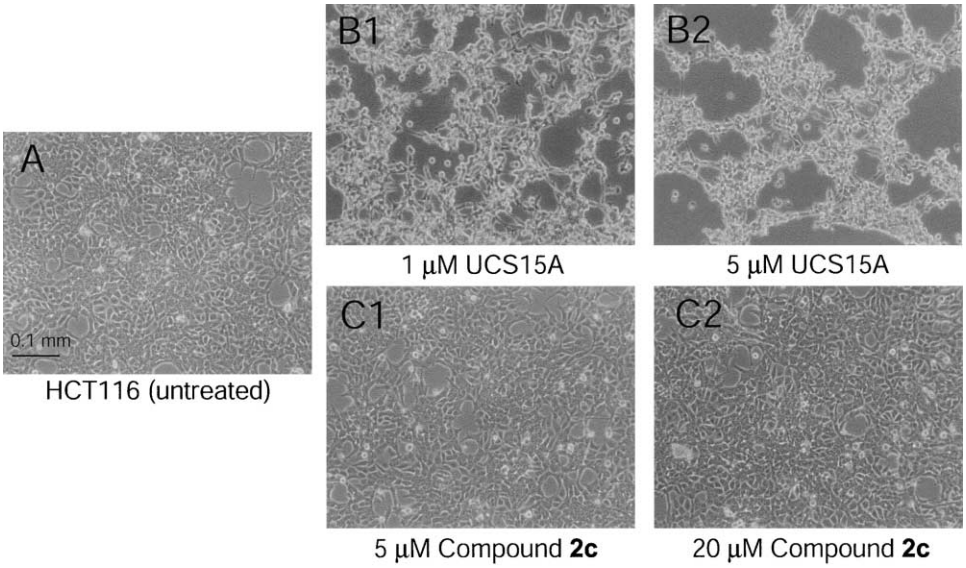

Figure 5. Effect of UCS15A and Compound **2c** on the Morphology of HCT116 Cells

HCT116 cells were treated for 6 hr with the concentration of each drug shown at the bottom of each panel. (A), untreated; (B), UCS15A treated; (C), compound **2c** treated. Cells were photographed under the microscope at a magnification of 100×.

an order of magnitude more drug to exert its effects in vitro instead of in vivo (mM versus μM range). This is identical to the observation with the parental drug UCS15A [18]. In addition to the numerous explanations that were advanced to account for these differences (for details please see discussion in [18]), an additional possibility that we can neither confirm or exclude at this point is that, in vivo, the drugs (UCS15A and **2c**) are metabolized into a more potent form. Furthermore, our previous results strongly suggested that UCS15A inhibited proline rich-SH3 interaction but did not disrupt preexisting proline rich-SH3 complexes [18]. It is therefore possible that, in vivo, the number of proteins with free proline-rich domains may be far fewer than in the in vitro situation, which might also account for the differences observed.

Consistent with the ability of compound **2c** to inhibit PLPI between Grb2 and Sos1, the compound also inhibited the downstream activation of MEK in EGF-stimulated HeLa cells (Figure 4A). However, simply because **2c** reduced Grb2/Sos1 interaction in vivo does not suggest direct causality. That the situation is more complex than heretofore appreciated is highlighted by the fact that **2c** also inhibited the activation of MEK in HCT116 cells (Figure 4B), a cell line that harbors an activated K-ras gene [32]. This suggests that additional points of action of **2c** may, in fact, be present downstream of ras. One possibility is that **2c** may be acting directly on the Raf/MEK complex, which previous studies have shown is mediated in part by a proline-rich sequence in MEK1/2, albeit not through SH3 domains [33]. Furthermore, numerous recent studies have suggested that the linear representation of the Ras-Raf-MEK pathway may be an oversimplification and that there are additional levels of regulation of this pathway that are controlled by protein-protein interactions. One such regulatory molecule is the KSR protein (kinase suppressor of ras), which seems to be a scaffolding molecule that interacts with various components of the MAP kinase cascade, including Raf-1, MEK1/2, and ERK1/2, and is involved in coordinating the assembly of a multiprotein MAP kinase complex at the cell membrane (reviewed in [34, 35]). Interestingly, KSR has a proline-rich domain of as yet unknown function. Another MAP kinase system that is regulated by a scaffolding complex is the TAK1-TAB1-TAB2 scaffolding complex that regulates activation of p38 MAP kinase (reviewed in [36]). It is therefore possible that UCS15A and **2c** might work at a point downstream of ras to disrupt MAPK activation, in spite of the cells having an activated ras gene. While none of the systems described above involve SH3 domains and are hence unlikely targets of UCS15A/**2c**, one cannot exclude the possibility that an as yet undiscovered SH3-PLPI interaction downstream of ras is the critical point of action of **2c**. Clearly, this issue is far from resolved and needs further investigation.

It is interesting that, as opposed to UCS15A, compound **2c** did not induce morphological changes in treated HCT116 cells (Figure 5) or v-src 3T3 cells (data not shown). Previous studies suggested a relationship between PLPI and cytoskeletal organization [37, 38]. Our previous studies also showed that UCS15A disrupted the PLPI between the cytoskeletal proteins cortactin and ZO1 [18, 39]. While the reason for the difference between UCS15A and compound **2c** with regard to their effects on cell morphology is presently unclear, two hypotheses can be extended to account for these differences. One possibility is that compound **2c** disrupts a subset of PLPI involved in signal transduction, whereas UCS15A is a more generalized disruptor of PLPI that are involved in signal transduction as well as cytoskeletal organization. The second possibility is that while both UCS15A and compound **2c** disrupt PLPI, UCS15A may have additional as yet unknown activities

that allow it to cause morphological changes in cells. The latter possibility would argue that disrupting PLPI is unrelated to morphological change-inducing activity. At present, our results cannot distinguish between these two hypotheses.

These studies suggest the feasibility of performing structure-function analysis with UCS15A. Until recently, these studies were extremely difficult to conduct, given the difficulty in synthesizing derivatives of UCS15A and the very large and complex interaction surface between SH3 domains and their proline-rich domain ligands. These studies represent the first steps in this regard and only became possible following the total synthesis of UCS15A [25]. Clearly, more detailed structural studies (NMR as well as X-ray crystallographic) will be necessary to completely elucidate the mechanism of action of this drug. Nonetheless, the results of this study highlight structural components of UCS15A that are important for disrupting PLPI and suggest the possibility that analogs of UCS15A might be promising leads for the development of novel drugs for the treatment of various human diseases in which PLPI are involved.

Significance

Proline-rich ligand-mediated protein-protein interactions (PLPI) play important roles in signaling pathways. These interactions occur between several critical intracellular proteins involved in various pathologies. Therefore, PLPI can be therapeutic targets, and a search for inhibitors to disrupt these interactions is ongoing. So far, only peptide-based inhibitors of these interactions have been reported, and the possibility for the development of small molecule, nonpeptide-based inhibitors of PLPI is believed to be remote. Our previous studies demonstrated that UCS15A, a natural compound produced by *Streptomyces* species, is a novel small molecule with SH3-mediated interaction-blocking activity. As a first step toward further development, we have tried to investigate the structural moiety of UCS15A that is both necessary and sufficient for its inhibitory activity toward PLPI. The present studies led to the discovery of compound 2c, a "simple analog" of UCS15A that retained PLPI blocking activity. In addition, these studies identified critical regions of UCS15A that are responsible for its PLPI disrupting activity. These findings will contribute to our ability to build agents to block specific PLPI. This is, however, presently difficult due to the ability of UCS15A and, perhaps, compound 2c as well to inhibit PLPI in a broadly specific manner. In an attempt to generate more specific agents, it would be worth analyzing the detailed costructure of proline-rich regions with compound 2c by X-ray crystallography and NMR analyses. Based on these analyses, it should be possible to design more potent agents that will enhance the affinity and selectivity for blocking specific PLPI. In addition, the structural information described here may be useful for further chemical modifications. The synthetic small molecule compound 2c represents a significant step toward development of second-generation drugs that may be clinically useful in the treatment of various human diseases in which PLPI are implicated.

Experimental Procedures

Chemicals

UCS15A was produced by *Streptomyces* species and purified in our laboratories. Derivatives of natural UCS15A (compounds **1a–1f**) and related synthetic compounds (compounds **2a–2e**) were prepared in our laboratories. Synthetic procedures and analytical data for all compounds are described in the Supplemental Data (see http://www.chembiol.com/cgi/content/full/10/5/443/DC1 or write to chembiol@cell.com for a PDF).

Reagents and Cell Lines

McCoy's 5A modified medium was obtained from GIBCO. EGF was purchased from Life Technologies. GST antibody (Santa Cruz) was purchased as a horseradish peroxidase (HRP) conjugate. Primary antibodies used were as follows: anti-Grb2, anti-Sam68, anti-Sos1 (rabbit polyclonal; Santa Cruz); anti-MEK, anti-phospho MEK [$pS^{217/221}$] (rabbit polyclonal; NEB). Secondary antibody consisted of anti-rabbit antibody conjugated to HRP (Amersham). Immunoprecipitates were collected on protein A/G beads (Santa Cruz). SH3 agarose conjugates of Fyn corresponding to amino acids 85–139 of mouse Fyn were generated as a GST fusion protein and conjugated to agarose (AC). Sam68ΔC consisted of GST fused to amino acids 331–443 of mouse Sam68. Both GST fusion proteins were purchased from Santa Cruz. The HCT116 cell line was derived from a human colon cancer, and the HeLa cell line was derived from a human cervical cancer (American Type Culture Collection).

Immunoprecipitation and Immunoblotting Analyses

For immunoprecipitation analyses, two-day-old, subconfluent cells (two 100 mm dishes per treatment) were treated for 2 hr with different concentrations (ranging from 0 to 10 μM) of UCS15A, compound **2c**, or compound **2b** in DMSO. It was ensured that all samples, including the untreated cell controls, received the same volume of DMSO. At the end of the treatment period, cells were placed on ice, scraped, and collected in their own medium. Cell pellets were lysed by the addition of 1 ml of ice-cold Triton X-100/NP40 lysis buffer as described previously [12]. Lysates were clarified by microcentrifugation (15,000 rpm for 30 min at 4°C). The protein concentration of clarified lysates was determined, and equal amounts of total cell protein were immunoprecipitated overnight with 3 μg of the Grb2 antibody. Immunoprecipitates were collected on protein A/G beads (30 μl per immunoprecipitation). Beads were washed three times in lysis buffer, resuspended in 35 μl of Laemml's sample buffer, and resolved on 7.5% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (Protran; Schleicher and Schuell), immunoblotted with the indicated antibody, and subjected to chemiluminescent detection.

For the detection of GST fusion proteins, nitrocellulose membranes were incubated with HRP-conjugated GST antibody (1:1000 dilution) for 4 hr. For the detection of Sam68, Sos1, or MEK proteins, membranes were incubated with the relevant antibody (1:1000 dilution) for 2 hr, followed by secondary antibodies (1:4000 dilution).

The effect of UCS15A, compound **2c**, and compound **2b** on the activation of MEK in EGF-stimulated HeLa cells was examined as follows. Briefly, a subconfluent 100 mm dish of cells was trypsinized and plated into a 6 well multiplate. After the cells had adhered and spread on the dish (approximately 6 hr post-plating), cells were starved in serum-free medium overnight. Serum-starved HeLa cells were treated with different concentrations of UCS15A, compound **2c**, or compound **2b** (0, 0.5, 2, and 5 μM, respectively) for 4 hr. Subsequently, cells were stimulated with 100 ng/ml of EGF for 15 min. In the case of HCT116 cells, cells were neither serum starved nor serum stimulated. At the end of the treatment period, cells were scraped and collected by low-speed centrifugation. Cell pellets were lysed by the addition of 75 μl of ice-cold RIPA lysis buffer as described previously [12]. Lysates were clarified by microcentrifugation (15,000 rpm for 30 min at 4°C). A third of each clarified lysates (25 μl) was mixed with an appropriate volume of 4× Laemmli's sample buffer, boiled for 10 min, and proteins were separated by electrophoresis on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose membranes, which were then blocked in TBS-TB buffer (Tris-buffered saline containing 0.1% Tween-20 and

3% bovine serum albumin) for 1 hr. Membranes were then incubated with the primary antibody (rabbit polyclonal anti-MEK [pS$^{217/221}$] at a 1:1000 dilution) in TBS-TB overnight at 4°C. After briefly washing with TBS-T, the filter was incubated with the secondary antibody (HRP-conjugated rabbit IgG at a 1:4000 dilution) in TBS-TB for 1 hr, followed by chemiluminescent detection. The blot was stripped as described previously [12] and reprobed with an appropriate primary antibody (rabbit polyclonal anti-MEK) and secondary antibody, followed by chemiluminescent detection.

In Vitro Proline-Rich SH3 Domain Interaction Assay

To examine the effect of UCS15A-related compounds indicated in Figure 1 on PLPI, an in vitro system consisting of two proteins was used as described previously [18]. The two proteins used in this in vitro system consisted of the recombinant proteins, Sam68ΔC (0.5 μg per reaction) and Fyn-SH3 AC beads (5 μl of resuspended beads per reaction). Each reaction was done in 1 ml of binding buffer as described previously [18]. Binding of compounds to Sam68ΔC was carried out for 6 hr at 37°C. The concentrations of UCS15A ranged from 0 to 500 μM, and UCS15A-related compounds ranged from 0 to 300 μM as indicated in Figure 2 and Table 1. Following binding of the compound to Sam68ΔC, Fyn-SH3 AC slurry was added to the reaction, and protein-protein interaction was allowed to proceed overnight at 4°C. Subsequently, the beads were washed three times in Triton X-100/NP40 lysis buffer, resuspended in 30 μl of Laemmli's sample buffer, and the proteins were resolved by electrophoresis on 10% SDS-polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes, immunoblotted with the indicated antibody, and subjected to chemiluminescent detection.

Quantification of Inhibitory Activity of UCS15A Analogs on PLPI

To quantitate the inhibitory activities of UCS15A and related compounds on PLPI, the densities of both the Sam68ΔC and Fyn-SH3 bands were scanned (PDI scanner; TOYOBO). Subsequently, the ratio of the density of the Sam68ΔC and Fyn-SH3 bands was calculated and compared to the ratio of the two bands in the absence of the drug, which served as the baseline (0% of inhibitory activity). Each experiment was repeated three times, and results were expressed as averages of the data.

Photomicroscopy

HCT116 cells were plated in 6-well multiplates as described above. Approximately 8 hr after plating, cells were either left untreated or treated with UCS15A or compound **2c** for 6 hr. At the end of the treatment period, cell morphology was visualized by light microscopy (100× magnification).

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References

1. Pawson, T. (1995). Protein modules and signaling networks. Nature *373*, 573–580.
2. Hunter, T. (2000). Signaling: 2000 and beyond. Cell *100*, 113–127.
3. Cohen, G.B., Ren, R., and Baltimore, D. (1995). Modular binding domains in signal transduction. Cell *80*, 237–248.
4. Persidis, A. (1998). Signal transduction as a drug-discovery platform. Nat. Biotechnol. *16*, 1082–1083.
5. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{++}dependent protein kinase. Biochem. Biophys. Res. Commun. *135*, 397–402.
6. Kase, H., Iwahashi, K., Nakanishi, S., Matsuda, Y., Yamada, K., Takahashi, M., Murukata, C., Sato, A., and Kaneko, M. (1987). K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem. Biophys. Res. Commun. *142*, 426–440.
7. Nakano, H., Kobayashi, E., Takahashi, I., Tamaoki, T., Kuzuu, Y., and Iba, H. (1987). Staurosporine inhibits tyrosine-specific protein kinase activity of Rous sarcoma virus transforming protein p60. J. Antibiot. (Tokyo) *40*, 706–708.
8. Tamaoki, T., and Nakano, H. (1990). Potent and specific inhibitors of protein kinase C from microbial origin. Biotechnology *8*, 732–735.
9. Cohen, P. (2002). Protein-kinases-the major drug targets of the twenty-first century? Nat. Rev. Drug Discov. *1*, 309–315.
10. Pillay, I., Nakano, H., and Sharma, S.V. (1996). Radicicol inhibits tyrosine phosphorylation of the mitotic substrate SAM68 and retards subsequent exit from mitosis of src-transformed cells. Cell Growth Differ. *7*, 1487–1499.
11. Sharma, S.V., Agatsuma, T., and Nakano, H. (1998). Targeting of the protein chaperone HSP90, by the transformation-suppressing agent, Radicicol. Oncogene *16*, 2639–2645.
12. Sharma, S.V., Oneyama, C., Yamashita, Y., Nakano, H., Sugawara, K., Hamada, M., Kosaka, N., and Tamaoki, T. (2001). UCS15A, a non-kinase inhibitor of Src signal transduction. Oncogene *20*, 2068–2079.
13. Suzuki, M., Kobayashi, I., and Mitsutake, K. (1983). A new antibiotic SI-4228, method for the production and agricultural microbicide containing the same as an active constituent. Jpn. Kokai Tokkyo Koho *116*, 686.
14. Wakabayashi, T., Kageyama-Kawase, R., Naruse, N., Funahashi, Y., and Yoshimatsu, K. (2000). Luminascins: a family of capillary tube formation inhibitors from Streptomyces sp. II. Biological activities. J Antibiot. (Tokyo) *53*, 591–596.
15. Naruse, N., Kageyama-Kawase, R., Funahashi, Y., and Wakabayashi, T. (2000). Lumimacins: a family of capillary tube formation inhibitors from Streptomyces sp. I. Taxonomy, fermentation, isolation, physico-chemical properties and structure elucidation. J Antibiot. (Tokyo) *53*, 579–590.
16. Hata-Sugi, N., Kawase-Kageyama, R., and Wakabayashi, T. (2002). Characterization of rat aortic fragment within collagen gel as an angiogenesis model; capillary morphology may reflect the action mechanism of angiogenesis inhibitors. Biol. Pharm. Bull. *25*, 446–451.
17. Kornbluth, S., Jove, R., and Hanafusa, H. (1987). Characterization of avian and viral p60src proteins expressed in yeast. Proc. Natl. Acad. Sci. USA *84*, 4455–4459.
18. Oneyama, C., Nakano, H., and Sharma, S.V. (2002). UCS15A, a novel small molecule, SH3 domain-mediated protein-protein interaction blocking drug. Oncogene *21*, 2037–2050.
19. Hunter, T., and Sefton, B.M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA *77*, 1311–1315.
20. Mayer, B.J., Hamaguchi, M., and Hanafusa, H. (1988). A novel viral oncogene with structural similarity to phospholipase C. Nature *332*, 272–275.
21. Koch, C.A., Anderson, D., Moran, M.F., Ellis, C., and Pawson, T. (1991). SH2 and SH3 domain: elements that control interactions of cytoplasmic signaling proteins. Science *252*, 668–674.
22. Kay, B., Williamson, M., and Sudol, M. (2000). The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. FASEB J. *14*, 231–241.
23. Dalgarno, D., Botfield, M., and Rickles, R. (1997). SH3 domains and drug design: ligands, structure, and biological function. Biopolymers *43*, 383–400.
24. Saksela, K., Cheng, G., and Baitimore, D. (1995). Proline-rich (PxxP) motifs in HIV-1 Nef bind to SH3 domains of a subset of Src kinases and are required for the enhanced growth of Nef^{+} viruses but not for down-regulation of CD4. EMBO J. *14*, 484–491.
25. Tatsuta, K., Nakano, S., Narazaki, F., and Nakamura, Y. (2001). The first total synthesis and establishment of absolute structure of Lumimacins C1 and C2. Tetrahedron Lett. *42*, 7625–7628.
26. Shotwell, J.B., Krygowski, E.S., Hines, J., Koh, B., Huntsman, E.W., Choi, H.W., Schneekloth, J.S., Jr., Wood, J.L., and Crews,

C.M. (2002). Total synthesis of Lumimacin d. Org. Lett. *4*, 3087–3089.

27. Alessi, D., Saito, Y., Cambell, D., Cohen, P., Sithanandam, G., Rapp, U., Ashworth, A., Marshall, C., and Cowley, S. (1994). Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. EMBO J. *13*, 1610–1619.
28. Kolch, W. (2000). Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. Biochem. J. *351*, 289–305.
29. Greene, J., Zheng, J., Grant, D., and Hammock, B. (2000). Cytotoxicity of *1*,2-epoxynaphthalene is correlated with protein binding and in situ glutathione depletion in cytochrome P4501A1 expressing Sf-21 cells. Toxicol. Sci. *53*, 352–360.
30. Davies, A., Martin, E., Jones, R., Lin, C., Smith, L., and White, I. (1995). Peroxidase activation of tamoxifen and toremifene resulting in DNA damage and covalently bound protein adducts. Carcinogenesis *16*, 539–545.
31. Slaughter, D., and Hanzlik, R.P. (1991). Identification of epoxide- and quinone-derived bromobenzene adducts to protein sulfur nucleophiles. Chem. Res. Toxicol. *4*, 349–359.
32. Shirasawa, S., Furuse, M., Yokoyama, N., and Sasazuki, T. (1993). Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science *260*, 85–88.
33. Catling, A.D., Schaeffer, H.J., Reuter, C.W.M., Reddy, G.R., and Weber, M.J. (1995). A proline-rich sequence unique to MEK1 and MEK2 is required for Raf binding and regulates MEK function. Mol. Cell. Biol. *15*, 5214–5225.
34. Raabe, T., and Rapp, UR. (2002). KSR—a regulator and scaffolding protein of the MAP kinase pathway. Sci. STKE *136*, 1–3.
35. Morrison, D.K. (2002). KSR: a MAPK scaffold of the Ras pathway? J. Cell Sci. *114*, 1609–1612.
36. Johnson, G. (2002). Signal transduction scaffolding proteinsmore than meets the eye. Science *295*, 1249–1250.
37. Tsukita, S., Furuse, M., and Itoh, M. (1999). Structural and signaling molecules come together at tight junctions. Curr. Opin. Cell Biol. *11*, 628–633.
38. Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V., and Schlessinger, J. (1993). SH3 domains direct cellular localization of signaling molecules. Cell *74*, 83–91.
39. Katsube, T., Takahisa, M., Hashimoto, N., Kobayashi, M., and Togashi, S. (1998). Cortactin associates with the cell-cell junction protein ZO-1 in both *drosophila* and mouse. J. Biol. Chem. *273*, 29672–29677.