Small Molecule Protein Assembly Modulators with Pan-Cancer Therapeutic Efficacy

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Abstract

Two structurally-unrelated small molecule chemotypes, represented by compounds PAV-617 and PAV-951 with antiviral activity in cell culture against monkeypox virus (MPXV) and human immunodeficiency virus (HIV) respectively, were studied for anti-cancer efficacy. Each exhibited apparent pan-cancer cytotoxicity, reasonable pharmacokinetics, and non-toxicity in mice at active concentrations. Anti-tumor properties of both chemotypes, were validated in mouse xenografts against A549 human lung cancer and, for one of the chemotypes, against HT-29 colorectal cancer. The targets of these compounds are unconventional: each binds to a different transient, energy-dependent multi-protein complex containing the protein TRIM28/KAP1, an allosteric modulator known to regulate mechanisms underlying viral and nonviral disease states including cancer. Treatment with these compounds alters the target multi-protein complexes in a manner consistent with allosteric modulation as their mechanism of action. These compounds appear to remove a block, crucial for cancer survival and progression, on the homeostatic linkage of uncontrolled cellular proliferation to apoptosis. These compounds provide starting points for development of next-generation non-toxic, pan-cancer therapeutics.

Background

The similarity of the interactions of viral infection and cancer with the healthy host has often been noted (1). Both represent pathological processes of extremely diverse origin, that overcome the complex feedback controls of homeostasis, to the detriment of the host (2). Both exploit natural selection as a powerful weapon to overcome host defenses. Viruses have done so over deep evolutionary time through co-evolution with their hosts and through the emergency of resistance mutations in response to the selective pressure of treatments (3,4). Cancers regularly use the latter mechanism, resulting in clonal mutants that drive cancer progression (5).

46 At least seven different viruses-- Epstein-Barr virus (EBV), hepatitis B virus (HBV), hepatitis C
47 virus (HCV), human T-lymphotropic virus 1 (HTLV-1), human papillomavirus (HPV), Kaposi sarcoma-
48 associated herpesvirus (KHSV or HHV-8), and Merkel cell polyomavirus (MCPyV)—are known to be
49 directly oncogenic through their alteration of the cellular environment and/or impairment of the host's
50 innate immune system defenses (6,7). It has therefore been proposed that viruses could play a key role
51 in the discovery of new cancer treatments by identifying cellular targets that drive tumorigenesis (8).

52 The incompleteness in our current understanding of the dynamics of host homeostasis and its
53 myriad of feedback controls has been a disadvantage for efforts to design novel therapeutic
54 countermeasures against both viruses and cancer. However, a recent unconventional approach to
55 antiviral drug discovery has identified small molecules targeting host allosteric sites essential for
56 homeostasis, that are repurposed by viral infection (9). The antiviral compounds identified by this
57 approach appear to restore key features essential to host homeostasis (9). We wished to determine
58 whether those compounds might have therapeutic applicability against cancer, given the analogy in how
59 both viruses and cancers drive departure from homeostasis. The results to be presented here suggest
60 this is the case and shed light on molecular pathways relevant for both viral and neoplastic disease,
61 providing a strategy for development of novel cancer cell therapeutics.

62 Utilizing a cell-free protein synthesis and assembly (CFPSA) system, compounds from a library of
63 approximately 150,000 drug-like small molecules were screened for hits which blocked the assembly of
64 viral capsids without inhibiting protein synthesis (9–12). Hit compounds identified have been termed
65 "protein assembly modulators". A collection of 300 structurally-diverse protein assembly modulators
66 were identified from hits demonstrating activity against capsid assembly in one or more viral family (9).
67 Antiviral protein assembly modulators from this collection have been validated against infectious virus
68 in cell culture for multiple viral families including *Retroviridae*, *Rhabdoviridae*, *Poxviridae*, *Adenoviridae*,
69 *Herpesviridae*, *Paramyxoviridae*, *Coronaviridae*, *Orthomyxoviridae*, and *Picornaviridae* (9,11–13). In two

70 cases, *Coronaviridae* and *Paramyxoviridae*, cellular antiviral activity has been confirmed in animal
71 disease models (9).

72 These antiviral protein assembly modulators appear to target host-viral protein-protein
73 interactions via allosteric sites that control repurposing of host assembly machinery for viral capsid
74 formation and which also allow disengagement of host innate immune defenses such as autophagy (9).
75 In an earlier study, a class of protein assembly modulators was shown to change the composition of a
76 transient, energy-dependent multi-protein complex whose components include p62/SQSTM1, a key
77 regulator of autophagy (9). This multi-protein complex is co-opted upon viral infection and modified in
78 composition. p62/SQSTM1 is lost from the complex, and the viral nucleoprotein added (9). Upon
79 treatment with an antiviral protein assembly modulator, the target multi-protein complex is restored to
80 its composition in uninfected cells, with loss of the viral nucleoprotein component and restoration of
81 p62/SQSTM1 (9).

82 The discovery of dynamic multi-protein complexes whose composition changes with drug
83 treatment offers a new means of parsing out post translational protein heterogeneity and its relevance
84 for diseased states. The amount of a given protein that has been observed in the multi-protein
85 complexes targeted by antiviral assembly modulators comprises only a very small fraction of the total
86 amount of the component proteins present in a cell (9). The role played by the subset of the protein that
87 is part of a particular multi-protein complex may reflect a “moonlighting” function, as observed for a
88 growing number of cellular and viral proteins (14–16).

89 We hypothesized that if an overlap between viral and oncogenic pathways exists, some antiviral
90 assembly modulators might be capable of disrupting a multi-protein complex associated with a hallmark
91 of cancer (17,18). To test the hypothesis, we established a cancer-relevant counter screen and applied it
92 to previously-identified antiviral protein assembly modulator compounds. In this paper, we will describe

93 two protein assembly modulators which were originally characterized for their antiviral properties but
94 are now shown to have potential as cancer therapeutics based on *in vitro* screening and *in vivo*
95 validation studies. Just as the previously cited study discovered a pan-respiratory antiviral chemotype
96 that appears to target a host-viral interface, so also we hypothesized that it should be possible to find a
97 comparable anti-cancer chemotype that reverses changes selected by the cancer for allowing it to
98 escape from feedback constraints that normally prevent uncontrolled proliferation.

99

100 Results

101 *Uncontrolled cellular proliferation: a hallmark of cancer inhibited by protein assembly modulators*

102 No hallmark of cancer is more fundamental than uncontrolled proliferation (18). Uncontrolled
103 proliferation normally triggers cell death mechanisms, including apoptosis (19). Therefore, to survive a
104 cancer must achieve a means of evading cell death long enough to complete cell division and reset the
105 cell death timer. This, in turn, allows further proliferation, during which time additional mutations can
106 occur and selection pressure will drive higher and higher grade malignancy and so on, ultimately
107 resulting in metastasis (20). The precise regulatory feedback loops that detect uncontrolled proliferation
108 and direct such cells to apoptosis are not well understood. If early cancers emerge where those
109 mechanisms are impaired, it seemed plausible to us that assembly modulators identified through CFPSA
110 phenotypic screens might include some chemotypes that restore the relevant feedback loops.
111 Compounds capable of arresting proliferation, either directly or indirectly, would make potent anti-
112 cancer agents especially if the delay in cancer progression would provide an opportunity for a patient's
113 innate immune system and other homeostatic feedback loops to re-establish themselves.

114 Abnormal signaling pathways triggered by aberrant protein-protein interactions is one way that
115 neoplastic cells are able to achieve uncontrolled proliferation (20). In order to characterize whether

116 assembly modulators could arrest the proliferation of neoplastic cells by redirecting key protein-protein
117 interactions, we first sought to identify a cell line in which endogenous apoptosis was substantially
118 lacking. While a successful anti-cancer compound would likely exhibit both anti-proliferative and
119 cytotoxic efficacy, we wanted to conduct our screen under conditions where a readout measuring the
120 arrest of proliferation would not be obscured by downstream activation of the normal cascade of events
121 comprising cell death pathways.

122 We assessed caspase-3/7 activity in multiple tumor cell lines with an Apo-ONE assay (see **Figure**
123 **1A**). The expected correlation between endogenous triggers of apoptotic death and cancer progression
124 was demonstrated in the LNCaP prostate cancer progression cell model, where LNCaP-C33 early
125 (hormone sensitive) cancer cells displayed substantially more markers of apoptosis than LNCaP-C81 late
126 (hormone resistant) cancer cells (21). In the Apo-ONE assay, CHO K1 cells show very little endogenous
127 apoptosis (see **Figure 1A**). Hennes-20 a CHO K1 derivative into which the human APP gene has been
128 transfected, show even less apoptosis than their parental line (see **Figure 1A**).

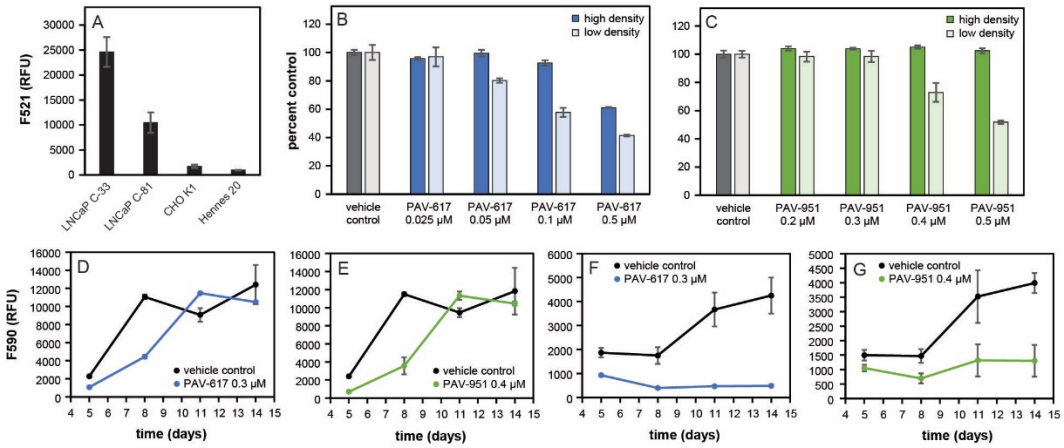
129 Our collection of anti-viral assembly modulator compounds was then counter screened in
130 Hennes-20 cells plated at low (500 cells/well) versus high (15,000 cells/well) densities and treated with
131 DMSO (vehicle) or dose-titration of compounds. The rationale for this screen is than an intrinsically toxic
132 compound should kill cells regardless of density, including in Hennes-20 cells. However, a compound
133 that selectively triggers the arrest of proliferation will appear cytotoxic due to inhibition of cell growth
134 when plated at a low density, but will appear non-toxic to cells plated at a high density where cells are
135 approaching confluence and the readout detected by a cell viability assay is already close to the
136 maximum.

137 Two structurally-unrelated small molecules (Tanimoto score of 41%), PAV-617 (see synthetic
138 **Supplemental Figure 1** for chemical structure and synthetic scheme) and PAV-951 (structure not

139 disclosed) displayed the desired phenotype (see **Figures 1B** and **1C**). The Hennes results at low versus
140 high density comparison suggested that the effect of these compounds in cells lacking endogenous
141 apoptosis was due to inhibition of proliferation.

142 To confirm that the inhibition observed in low-density Hennes 20 cells resulted from temporary
143 arrest of proliferation and not cell death, compound was removed after a period of treatment and cell
144 growth was measured over the subsequent two weeks for recovery potential. The cells treated with
145 PAV-617 and PAV-951 initially showed reduced cell density relative to the control, but once compound
146 was removed, growth was restored over time (see **Figures 1D** and **1E**). By day 11, cell density in
147 compound-treated cells caught up to the DMSO-treated cells (see **Figures 1D** and **1E**).

148 Since Hennes 20 cells do not appear to have endogenous apoptosis, we sought to use a different
149 cancer cell line to assess whether that inhibition of proliferation would be accompanied by cell death.
150 The recovery experiment was repeated in LNCaP C-33 cells which appear to have substantial
151 endogenous apoptosis and treatment with PAV-617 and PAV-951 did inhibit cancer cell growth relative
152 to the DMSO-treated control but the cells did not recover or grow significantly once compound was
153 removed (see **Figures 1A**, **1F**, and **1G**).



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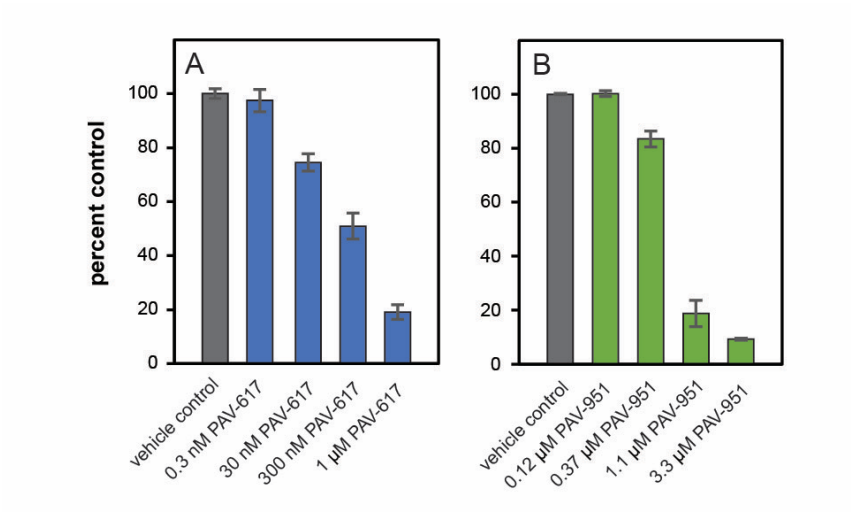
155 **Figure 1. Assay development and activity of hit compounds PAV-617 and PAV-951. Figure 1A** shows
156 assessment of the endogenous apoptosis response in multiple cell lines. Plates were seeded with LNCaP
157 C-33, LNCaP C-81, CHO K-1, and Hennes 20 cells. After three days of growth Apo-ONE reagent was added
158 and caspase-3/7 activity was determined by fluorescent readout. Averages and standard deviation of
159 observed activity in triplicate-repeated samples were calculated and graphed in Microsoft Excel. The
160 Hennes 20 cell line was chosen for the counterscreen based on its low levels of caspase activity. Figures
161 **1B** and **1C** show activity of PAV-617 and PAV-951 in the "arrest of proliferation" assay, where parallel
162 plates of Hennes 20 cells were seeded at a high density of 15,000 cells per well and a low density of 500
163 cells per well and treated with DMSO, PAV-617, or PAV-951 in triplicate-repeated dose titrations.
164 Fluorescent reading (RFU) corresponding to cell viability was calculated using an AlamarBlue™ assay and
165 the averages and standard deviations for triplicate-repeated samples were calculated and graphed on
166 Microsoft Excel as a percentage of the DMSO-treated cells. PAV-617 exhibited dose-dependent inhibition
167 of cell growth in the low density plate, indicating an EC50 between 0.1uM and 0.5uM. PAV-617 exhibited
168 some toxicity to cells at the higher doses tested, indicating a CC50 slightly greater than 0.5uM. PAV-951
169 exhibited dose-dependent inhibition of cell growth in the low-density plate, indicating an EC50 around
170 0.5uM. PAV-951 exhibited no significant inhibition of cell growth at the tested doses in the high-density
171 plate, indicating a CC50 greater than 0.5uM. **Figures 1D-1G** show recovery of cancer cell growth following
172 removal of compound. Hennes 20 or LNCaP C-33 cells were seeded at a low density then incubated with
173 DMSO, PAV-617, or PAV-951. After a period of treatment, the medium containing compound was
174 removed and replaced with fresh media. Plates were assessed for cell viability by AlamarBlue™ on days 5,
175 8, 11, and 14 and the averages and standard deviations of triplicate-repeat samples were calculated and
176 graphed over time on Microsoft Excel. PAV-617 and PAV-951 treated Hennes 20 and LNCaP C-33 cells all
177 showed reduced viability compared to matched DMSO-treated cells on day 5. However, cell growth in the
178 Hennes 20 cells which had been treated with compound recovered with time (**Figure 1D, E**), while the
179 LNCaP C-33 cells which had been treated with compound did not recover (**Figure 1F, G**).

180

181 *Investigating the Activities of PAV-617 and PAV-951: from modulators of viral capsid assembly to pan-*
182 *cancer therapeutics*

183 The anti-proliferative compounds PAV-617 and PAV-951 had originally emerged from our CFPSA
184 screen as inhibitors of viral capsid formation. The CFPSA model had been validated by demonstrating
185 that antiviral assembly modulator hits display activity against infectious viruses in cell culture (9,11–13).
186 PAV-617 is active against pox viruses in cell culture (13). The effective concentration for half maximal
187 activity (EC50) of PAV-617 against monkeypox (MPXV) is approximately 300 nM (see **Figure 2A**). PAV-
188 951 is active against HIV in cell culture with an EC50 between approximately 300 nM and 1uM (see
189 **Figure 2B**).



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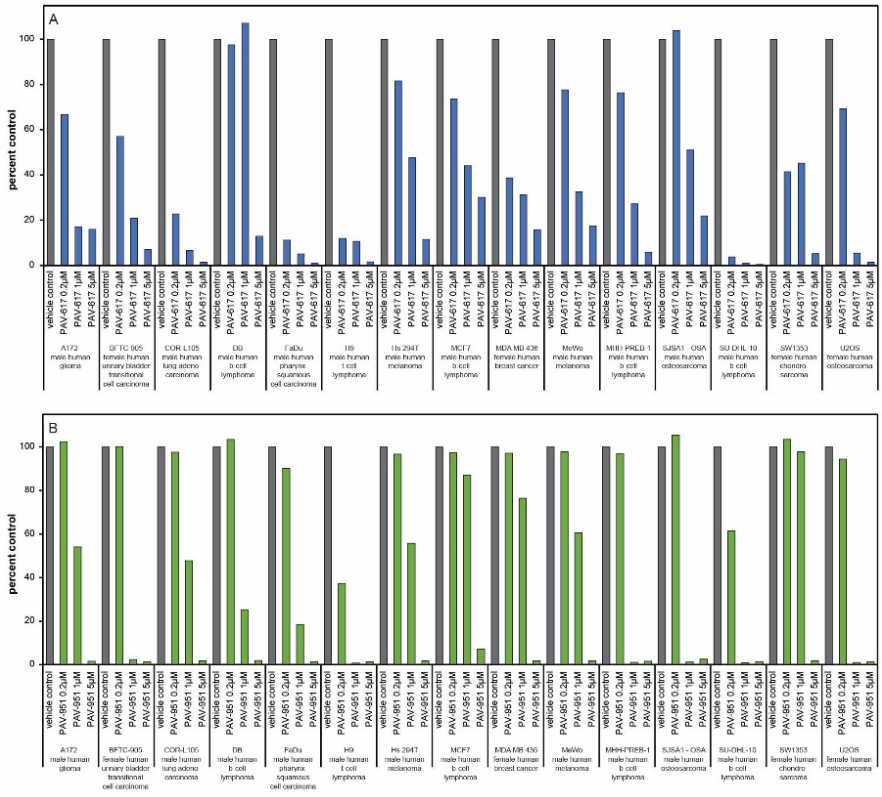
191 **Figure 2. Anti-viral properties of PAV-617 and PAV-951. Figure 2A** shows activity of PAV-617 against
192 MPXV. BSC-40 cells were infected with 100 plaque forming units of MPXV Zaire 79 and treated with PAV-
193 617 for three days. Averages and standard deviation for plaques observed with triplicate-repeated dose-
194 titration of PAV-617 are shown as a percentage of the plaques observed in untreated cells with an EC50 of
195 approximately 300 nM. **Figure 2B** shows activity of PAV-951 against HIV. MT-2 cells were infected with
196 NL4-3 Rluc HIV and treated with PAV-951 for four days. Averages and standard deviation of viral titer

observed with triplicate-repeated dose-titration of PAV-951 are shown as a percentage of the titer observed in DMSO-treated cells, with an EC50 between 0.37 uM and 1.1 uM.

When PAV-617 and PAV-951 were identified as having anti-cancer activity in addition to anti-viral properties, we suspected that the compounds might correct cancer-induced defects in protein assembly that were related to the aberrant assembly induced by viruses to promote capsid assembly. To get a better understanding of how the defects present themselves across diverse cancers, we assessed the anti-cancer activity of these two compound chemotypes on a panel of 15 cancer cell lines from the Eurofins OncoPanel™. The cell lines were derived from a variety of tissues and were representative of male and female patients of different ages from pediatric to senior. Both PAV-617 and PAV-951 showed pan-cancer activity with dose-dependent tumor growth inhibition in all 15 cell lines (see **Figures 3A** and **3B**).



210 **Figure 3. Pan-cancer activity of PAV-617 and PAV-951**. **Figures. 3A** and **3B** show results from a panel of
211 human tumor cell lines— A172 (male human glioma), BFTC-905 (female human urinary bladder
212 transitional cell carcinoma), COR-L105 (male human lung adenocarcinoma), DB (male human b-cell
213 lymphoma), FaDu (male human pharynx squamous cell carcinoma), H9 (male human t-cell lymphoma), Hs
214 294T (male human melanoma), MCF7 (female human breast cancer), MDA MB 436 (female human breast
215 cancer), MeWo (male human melanoma), MHH-PREB-1 (male human b-cell lymphoma), SJSA1-OSA (male
216 human osteosarcoma), SU-DHL-10 (male human b-cell lymphoma), SW1353 (female human
217 chondrosarcoma), and U-2 OS (female human osteosarcoma). Cells were grown for 24 hours then treated
218 with either vehicle, PAV-617, or PAV-951. Cell viability after 3 days of treatment was measured as
219 bioluminescence intensity and averages of triplicate-repeat dose-titrations with PAV-617 and PAV-951
220 were graphed on Microsoft Excel as percent of bioluminescence observed in DMSO-treated cells. Both
221 compounds showed inhibitory effects in all 15 cell lines.

222

223 Confirmation of the pan-cancer activity of these compounds was received through a sixty cancer line screen
224 carried out by the National Cancer Institute (see **Supplemental Figure 2**)

225

226 *Animal validation of PAV-617 and PAV-951 anti-cancer efficacy*

227 With demonstrated anti-viral activity, demonstrated anti-cancer activity, and data supporting a
228 proliferation-based mechanism of action, we assessed mouse toxicology and pharmacokinetic (PK)
229 properties in order to determine suitability for efficacy studies in an animal model.

230 The maximum tolerated dose (MTD) estimates how much compound can be administered to an
231 animal without adverse effects (22). When administered orally to mice, both PAV-617 and PAV-951
232 displayed MTDs greater than 20 mg/kg, which was the highest dose tested, as no clinical symptoms or
233 significant differences were observed between vehicle and treatment groups. When administered by

234 intraperitoneal (IP) injection, the MTD of PAV-617 was safe at 10 mg/kg. When administered by IP
235 injection, the MTD of PAV-951 was determined to be 2.5 mg/kg. Subsequently, chemical analogs of PAV-
236 951 have been identified with MTDs of 15 mg/kg when dosed IP. Some of those analogs, including
237 compound PAV-442, displayed comparable activity as PAV-951 against A549 lung cancer and PANC-1
238 pancreatic cancer tumor lines in cell culture (see **Supplemental Figures 3A** and **B** for activity of PAV-
239 442). PAV-181, a chemical analog of PAV-617, was found to be safe in mice at 20 mg/kg (higher doses
240 were not tested). PAV-181 retained the pan-cancer activity of PAV-617 in the Eurofins OncoPanel™ (see
241 **Supplemental Figure 3C** for the disclosed chemical structure of PAV-181 and **3D** for anti-cancer activity).

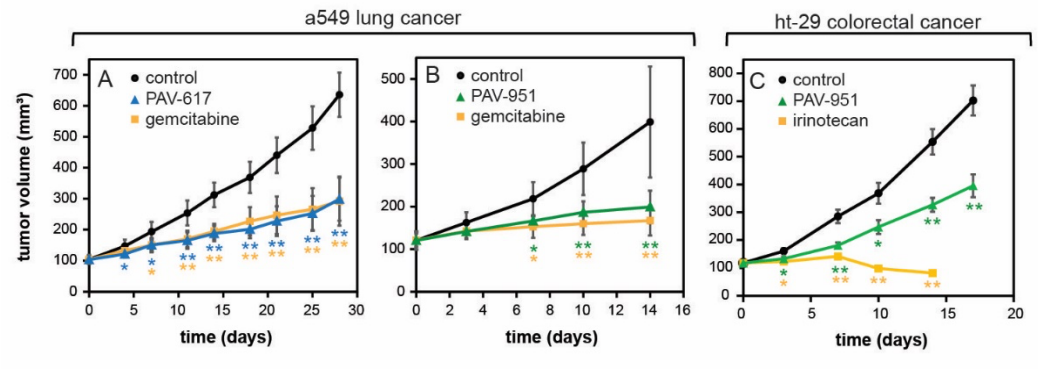
242 The PK properties are based on the absorption, distribution, metabolism, and excretion of a
243 compound in a living organism and are necessary to determine dosing parameters because any
244 compound designed for clinical use needs to achieve an efficacious concentration in a target organ
245 (23,24). As an early measure of PK, we determined the concentration of compound in the plasma of rats
246 or mice over time following one intravenous (IV), one IP, or one oral dose. Both compounds were
247 detectable in the animals through all administration routes, though the maximum concentration
248 achieved (Cmax) and rate of elimination were variable across conditions (See **Supplemental Figure 3**).

249 We determined that, while both chemical series would need optimization on the PK and
250 toxicological properties before being named as clinical drug-candidates, PAV-617 and PAV-951 would be
251 adequate for a preliminary animal efficacy study in order to validate whether or not the anti-
252 proliferative properties of the compounds observed in cell culture translates to anti-proliferative
253 properties in animals.

254 In the first set of animal efficacy studies, human A549 non-small cell lung cancer cells were
255 grafted subcutaneously onto mice. After 30 days of tumor establishment, the animals received daily
256 treatment with PAV-617 or PAV-951 and tumor volume was measured over time. The doses and routes

257 of administration for PAV-617 (10 mg/kg IP injection) and PAV-951 (1.5 mg/kg IV injection) were
258 determined based on their MTD and Pk properties. The PAV-617 study was conducted for 28 days and
259 the PAV-951 study was conducted for 14 days. As negative and positive controls, both studies included a
260 group treated with vehicle only and a group treated with Gemcitabine hydrochloride, an FDA approved
261 drug for non-small cell lung cancer, administered to animals in the same way as the test compound. The
262 Gemcitabine was administered at the standard dose of 100 mg/kg. Both PAV-617 and PAV-951 reduced
263 tumor growth significantly compared to the vehicle-only groups and performed comparably to
264 Gemcitabine despite being administered at substantially lower doses. PAV-617 displayed a tumor
265 growth inhibition (TGI) of 63% compared to Gemcitabine's TGI of 64% while PAV-951 had a TGI of 72%
266 compared to Gemcitabine's TGI of 84% (see **Figures 4A** and **4B**).

267 In the second animal efficacy study, human HT-29 colorectal adenocarcinoma cells were grafted
268 subcutaneously onto mice. After tumor establishment, the animals received treatment with vehicle, 3
269 mg/kg PAV-951, or 60 mg/kg of a positive control drug Irinotecan. After 17 days of treatment, PAV-951
270 had significantly reduced tumor growth relative to the vehicle-only group (TGI of 52%), though the
271 impact was lower than Irinotecan (TGI of 108%) (See **Figure 4C**).



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273 **Figure 4. Antiproliferative activities of PAV-617 and PAV-951 in animal cancer xenografts.** Tumors were
274 grafted onto mice via a subcutaneous injection and grown until they reached a volume of 100mm^3.
275 Animals were divided into randomized groups and treated with vehicle, PAV-617, PAV-951, or an FDA

276 approved cancer drug as a positive control. **Figure 4A** shows tumor volume across time for an A549 lung
277 cancer treated with vehicle, PAV-617, or Gemcitabine hydrocholoride. **Figure 4B** shows tumor volume
278 across time for a A549 lung cancer treated with vehicle, PAV-951, or Gemcitabine hydrochloride. **Figure**
279 **4C** shows tumor volume across time for a HT-29 colorectal cancer treated with vehicle, PAV-951, or
280 Irinotecan.

281 *Characterizing the targets of PAV-617 and PAV-951*

282 As PAV-617 and PAV-951 were identified by a phenotypic screen, their actual targets were
283 unknown during the early stages of compound advancement. To identify their targets, each molecule
284 was coupled to Affi-gel resins from a position on the molecule unrelated to proliferation arrest activity
285 based on structure-activity-relationship (SAR) exploration (see **Supplemental Figure 1B** for the synthetic
286 scheme of the PAV-617 resin used). In that way, they could serve as target-binding ligands for drug resin
287 affinity chromatography (DRAC) (25). LNCaP C-33 cells were chosen for DRAC starting material because
288 we knew that PAV-617 and PAV-951 displayed efficacy against them and we wanted a model for our
289 target engagement studies that would account for mechanisms of endogenous apoptosis responses.
290 Extracts were prepared from LNCaP C-33 cells that were treated for 22 hours with either DMSO, PAV-
291 617, or PAV-951. The extracts were applied to the PAV-617, PAV-951, or control drug resins, washed
292 with 100 bed volumes of buffer, and eluted with either 100 uM PAV-617 or 100 uM PAV-951.

293 Samples of the DRAC eluate were sent for analysis by tandem mass spectrometry (MS-MS). The
294 DRAC eluate from the PAV-617 and PAV-951 resins contained large sets of proteins missing from the
295 control resin eluate. This included cancer-implicated proteins from the literature. 92 proteins from the
296 DMSO-treated cell extracts were identified in the PAV-617 resin eluate that were not present in the
297 control resin eluate. 116 proteins from the DMSO-treated cell extracts were identified in the PAV-951
298 resin eluate which were not present in the control resin eluate. Of the proteins identified by MS-MS as
299 unique or greatly enriched in the drug resin eluates, 38 proteins in the DMSO-treated cell extracts were

unique to PAV-617, 62 proteins in the DMSO-treated cell extracts were unique to PAV-951, and 54 proteins from the DMSO-treated cell extracts were found in both the PAV-617 and the PAV-951 resin eluates (see **Figures 5A-C**). When the proteins detected in the eluates were searched in a database for cancer-implicated proteins, 23 of the proteins from the PAV-617 resin eluate and 29 proteins from the PAV-951 resin eluate were known to be part of cancer-relevant interactomes (See **Figures 5A-C**).

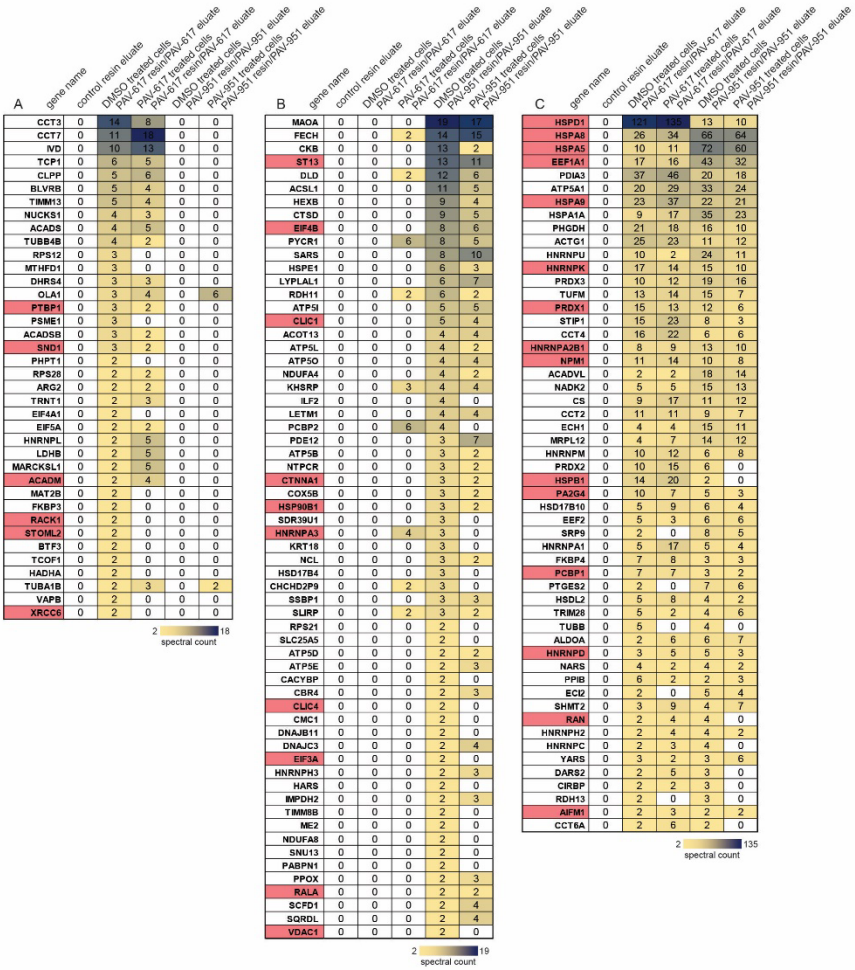

Figure 5. Identification of proteins in PAV-617 and PAV-951 resin eluates by MS-MS. DRAC experiments were performed where 30 ul of DMSO or compound-treated LNCaP cell extract, adjusted to a protein concentration of approximately 10 mg/ml in column buffer, was incubated on a column containing 30ul of affigel resin coupled to either PAV-617, PAV-951, or a 4% agarose matrix (control) for one hour at 4 degrees Celsius. The input material flow-through was collected and the resin was washed with 3 mL

311 column buffer then eluted overnight at 4 degrees Celsius in 100 ul of either 100uM PAV-617 or 100uM
312 PAV-951 in column buffer. **Figures 5A-5C** show spectral counts of proteins detected by MSMS in single-
313 point DRAC eluates. **Figure 5A** shows the set of proteins only detected in PAV-617 resin eluate. Figure 5B
314 shows the set of proteins only detected in the PAV-951 resin eluate. **Figure 5C** shows the set of proteins
315 detected in both the PAV-617 and the PAV-951 resin eluates. Conditional formatting has been applied
316 where relative abundance (by spectral count) of a given protein in a sample is visualized on a yellow-to-
317 black scale. Proteins implicated with cancer in the Bushman labs oncogene database
318 (http://www.bushmanlab.org/links/genelists) have been indicated in red.

319 The MS-MS indicated that when LNCaP C-33 cells were treated with compound, composition of
320 the eluate was subsequently affected. For both the PAV-617 and the PAV-951 resin eluates, the spectral
321 counts of some particular proteins detected by MS-MS increased or decreased in treatment conditions
322 (See **Figures 5A-C).** However, for other proteins, the number of spectral counts detected in the eluates
323 remained unchanged upon treatment. When we entered the proteins identified from the DRAC eluate
324 into a database of protein-protein interactions, we discovered that many of the proteins eluted by
325 either or both PAV-617 or PAV-951 were known to be involved in networks that involved frequent
326 interactions and associations with each other (see **Supplemental Figure 5**) and relevance for cancer (see
327 **Supplemental Figure 6**).

328 DRAC experiments were conducted side-by-side in triplicate with and without addition of
329 metabolic energy by running the experiment at 4°C versus 22°C and supplementing with an "energy
330 cocktail" of ribonucleotide triphosphates (1mM rATP, 1mM rGTP, 1mM rCTP, 1mM UTP) and 5ug/mL
331 creatine kinase. Changes in the amounts of proteins that bound and eluted with PAV-617 or PAV-951
332 were observed by western blot in the presence of these metabolic energy substrates (see **Figures 6A**
333 and **6B**). For the PAV-617 resin eluate, B-tubulin (TUBB), KRAB-associated protein 1 (KAP1 or TRIM28),
334 Methylenetetrahydrofolate dehydrogenase 1 (MTFGD1), and protein disulfide isomerase (PDI)—

335 proteins which had previously been identified in the eluate by MS-MS—all showed up by western blot in
336 larger amounts under energy-supplemented conditions (See **Figure 6A**). For the PAV-951 resin eluate,
337 MTHFD1, PDI and heterogenous ribonucleoprotein K (hnRNPK) were observed to be enhanced under
338 energy-supplemented conditions (see **Figure 6B**). By contrast, the amount of KAP1 identified in the PAV-
339 951 resin eluate decreased in the presence of metabolic energy substrates (see **Figure 6B**).

340 To confirm that the enrichment required the combination of metabolic energy substrates and
341 compound rather than the presence of energy substrates alone, resins were eluted side-by-side in
342 triplicate with either PAV-617 or PAV-951, or 1% DMSO all containing the energy cocktail. By western
343 blot, eluates from both PAV-617 and PAV-951 resins were found to contain significant amounts of
344 TRIM28/KAP1, MTHFD1, and PDI relative to the control resin or DMSO elution. The eluate of the PAV-
345 617 resin contained TUBB which the PAV-951 resin eluate did not have in any greater amount than the
346 control. The eluate of the PAV-951 resin contained hnRNPK, not present in the PAV-617 resin eluate in
347 greater amount than the control.

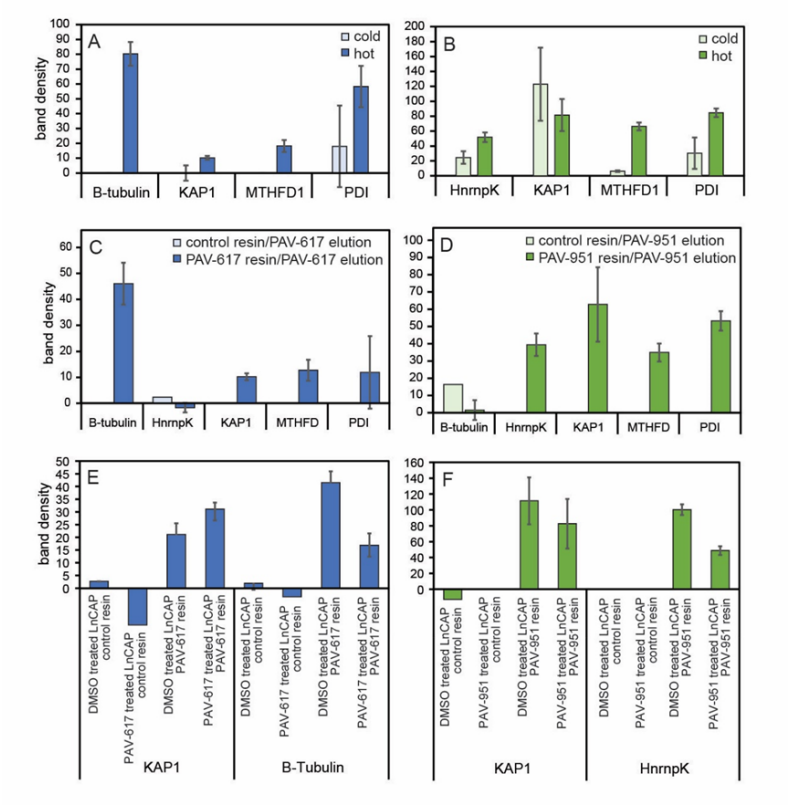

Figure 6. Exploration of changes to PAV-617 and PAV-951 resin eluates under different conditions. DRAC experiments using LnCAP C-33 starting extract as described in Fig. 5 were conducted under conditions which included supplementing the starting material and eluate with an energy cocktail to a final concentration of 1mM rATP, 1mM rGTP, 1mM rCTP, 1mM UTP, and 0.05 ug/mL creatine kinase and running the experiment at room temperature versus cold conditions at 4 degrees Celsius and without the energy cocktail. **Figures 6A** and **6B** show quantitation of average integrated density of protein band detected by western blot in triplicate-repeat samples eluted with PAV-617 from the PAV-617 or eluted with PAV-951 from the PAV-951 resin when DRAC was conducted side-by-side under energy-supplemented warm and non-energy-supplemented cold conditions. **Figures 6C** and **6D** show quantitation of average integrated density of protein band detected by western blot for TUBB, hnRNPK, KAP1, MTHFD1, and PDI in triplicate-repeat eluates under energy-supplemented conditions from the PAV-617 resin or PAV-951 resin and a single point elution with each compound from the control resin. Resins were eluted with either compound or 1% DMSO and the amount of protein detected in the 1% DMSO elution is

362 subtracted from the compound elution in the figure. **Figure 6E** shows quantitation of average integrated
363 density of protein band detected by western blot for KAP1 and B-tubulin in DRAC eluates generated under
364 energy-supplemented warm conditions of DMSO versus PAV-617 treated cells eluted with PAV-617 in
365 triplicate from the PAV-617 resin and in single-point from the control resin. **Figure 6F** shows quantitation
366 of average integrated density of protein band detected by western blot for KAP1 and hnRNPK in DRAC
367 eluates generated under energy-supplemented warm conditions for DMSO versus PAV-951 treated cells
368 eluted with PAV-951 in triplicate from the PAV-951 resin or in single-point from the control resin.

369 DRAC experiments were also carried out under conditions where the starting extract was
370 prepared from LNCaP C-33 cells that had been treated with DMSO, 100 uM PAV-617, or 100 uM PAV-
371 951 for 24 hours, in order to see if the changes to the eluate observed by MS-MS would repeat with
372 energy supplementation. Western blots of triplicate-repeated samples showed the PAV-617 resin eluate
373 contained increased amounts of KAP1 and decreased amounts of TUBB when cells were treated with
374 PAV-617, meanwhile PAV-951 resin eluate showed decreased amounts of both KAP1 and hnRNPK when
375 cells were treated with PAV-951 (See **Figures 6E-F**).

376 One explanation for why the DRAC eluates contain large numbers of proteins is that the targets
377 for PAV-617 and PAV-951 are themselves multi-protein complexes, as had been observed for analogous
378 studies on structurally unrelated protein assembly modulator chemical series effective in other
379 therapeutic areas. To test this hypothesis and determine which proteins directly bind the compounds
380 and which are indirectly associated with the compounds via protein-protein interactions involving the
381 direct drug-binding protein(s), we modified the compounds into photocrosslinker analogs by attachment
382 of diazirine and biotin functional groups at the same position to where the resin had previously been
383 attached. The photocrosslinker analogs were designed so that after an incubation with cell extract that
384 would allow the compound to bind its target, with subsequent exposure to ultraviolet light forming a
385 covalent bond between the diazirine moiety of the compound and the nearest protein (26). The sample

386 could then be solubilized and precipitated with streptavidin beads (which bind biotin) to identify the
387 drug-binding protein(s). The streptavidin precipitation (SAP) could be done using a native sample, which
388 would pick up the direct drug-binding protein(s) and with it any co-associated proteins. Or the SAP could
389 be done using a denatured sample which would, by virtue of the covalent bond to biotin, identify only
390 the direct drug binding protein(s), with all other associated proteins of the target multi-protein complex
391 lost upon denaturation.

392 A549 cell extract was incubated with either 1% DMSO or the photocrosslinker analogs of PAV-
393 617 or PAV-951, then exposed to ultraviolet light. The samples were then divided into two equal parts,
394 where one part was left native and the other denatured, then both were adjusted to non-denaturing
395 conditions and incubated with streptavidin beads. Blots of the SAP samples for KAP1 indicate KAP1 is
396 only a component of the PAV-617 target under native conditions and is completely lost upon
397 denaturation (See **Figures 7A-B**). However, KAP1 is present to a significant extent in both native and
398 denatured conditions for the SAP with the PAV-951 crosslinker (see **Figures 7A** and **7C**). The SAP samples
399 were sent for MS-MS analysis, however high background in the samples with no crosslinker added
400 rendered the data uninformative for definitive binding partner identification (data not shown).

401 Conventional methods of drug discovery typically involve the use of recombinant proteins to
402 measure affinity between a drug and its target (23). However, we have previously observed that protein
403 assembly modulating compounds are specific to their target proteins only when that protein is found in
404 particular cellular contexts/co-associations and we were concerned that if the protein-protein
405 interactions between the direct drug-binding protein and other proteins comprises an important
406 dimension of PAV-617 and PAV-951's targets, isolated recombinant proteins would not be an
407 appropriate surrogate for the protein-protein interactions occurring *in vivo*. To measure target
408 engagement for PAV-617 and PAV-951, we returned to DRAC and determined whether passing cell
409 extract over the PAV-617 or PAV-951 resins would deplete the extracts of bindable target.

410 We applied the flow-through of PAV-617, PAV-951, and control resins to a second copy of the
411 drug resin for these reasons and demonstrated that the drug resins deplete the extract of essentially all
412 KAP1 capable of binding to the resins (see **Figures 7D-E**). Western blot of the resin flow-through showed
413 that a comparable amount of KAP1 was flowing through the PAV-617, PAV-951, and control resins
414 without binding. However, when the flow-through that had been depleted on the PAV-951 resin was
415 applied on to a new PAV-951 resin, very little additional KAP1 bound to the second resin (see **Figure 7E**).
416 By contrast, application of the control resin flow-through (which was not specifically depleted of
417 anything) to a second PAV-951 resin, showed significant KAP1 binding to the second resin. Application of
418 the PAV-617 resin flow-through to the PAV-951 resin showed significantly more KAP1 binding to the
419 second resin than from the PAV-951 flow through, but significantly less KAP1 binding than from the
420 control flow through (see **Figure 7E**).

421 One notable observation was that, even though the five-fold depletion by the PAV-951 resin of
422 its target compared to the control resin was statistically significant and reproducible, it accounted for a
423 tiny amount of the total KAP1 which had been detected by western blot in the starting extract. Only
424 0.7% of the total amount of KAP1 detected in the starting material bound to the PAV-951 resin after
425 passing over a control column (see **Figure 7E**). As a reference point to account for non-specific loss of
426 material of the course of the experiment (due to denaturation over time or nonspecific sticking removed
427 during the washing phase)—of the original PAV-951 resin with which the extract was depleted, serial
428 elution with PAV-951 and 1% SDS showed approximately 4% of the total KAP1 detected in the original
429 extract had bound to the resin (data not shown). We conclude that the compounds selectively target
430 this small subfraction of KAP1 (less than 5% of the total KAP1 in the extract) because once the
431 subfraction of KAP1 capable of binding to the resins is removed, the remaining extract will not bind
432 anymore, even though it still contains ample KAP1.

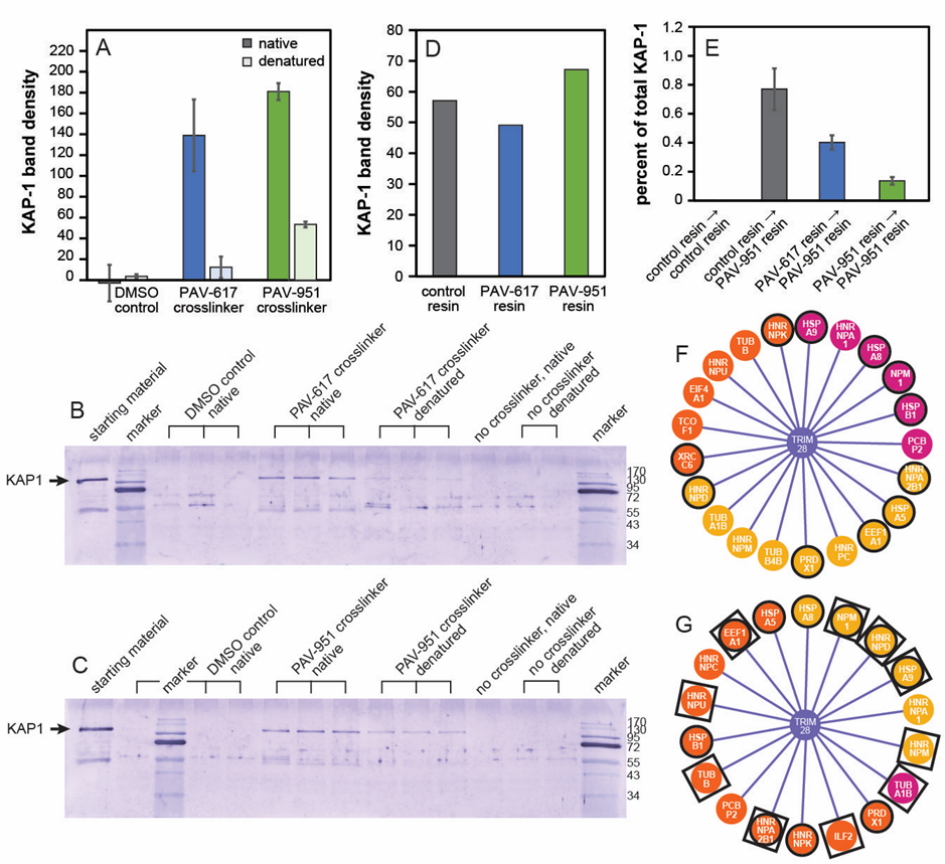

Figure 7. A subfraction of KAP-1/TRIM28 is a component of the PAV-617 and PAV-951 target complexes. **Figure 7A** shows quantitation of average integrated density of KAP1 protein band detected by western blot while **Figures 7B** and **7C** show the western blots themselves, in triplicate-run native and denatured streptavidin precipitations of photo crosslinked samples. Crosslinking experiments were performed where 65 uL of A549 cell extract was adjusted to a protein concentration of approximately 1 mg/ml in column buffer and supplemented with the energy cocktail, with either 1% DMSO or 1uM modified photo crosslinker analogs of PAV-617 and PAV-951 for one hour at room temperature then 20 minutes on ice. The extracts were exposed to UV light then divided into two aliquots. One aliquot was left native and one was denatured by adding DTT, SDS, and boiling. 800 uL of column buffer with 0.1% triton was added to both aliquots and then they were incubated with 2.5ul magnetic streptavidin beads for one hour at room temperature before being denatured in loading buffer containing SDS and heated to 100°C for 3 minutes. **Figures 7D** and **7E** show the show quantitation of integrated density of KAP1 protein band detected by

446 western blot in LNCaP extract depleted on the PAV-617, PAV-951, and control resins. 230 uL of LNCaP
447 extract was incubated with 230uL of PAV-617, PAV-951, or control resins in single point for one hour in
448 energy-supplemented conditions. Depleted flow-throughs were divided and put onto a subsequent PAV-
449 951 column in triplicate or onto a subsequent control column in single-point under energy-supplemented
450 conditions. Columns were eluted three times- a first overnight elution with PAV-951, a second overnight
451 elution with PAV-951, and a third elution with 1% SDS. Eluates were diluted 3:1 in loading buffer and
452 analyzed by western blot. Every western blot for the eluate included a sample of the original, un-depleted
453 starting LNCaP extract diluted 1:100 in loading buffer. **Figure 7D** shows quantitation from when the flow-
454 throughs from each columns were blotted for KAP1 to determine how much KAP1 had been depleted
455 from each resin. **Figure 7E** shows the amount of protein detected in the eluate normalized as percent of
456 their corresponding total sample, where the amount detected by eluate samples were divided by the
457 amount detected in the starting material sample, then multiplied by 0.013 to match the concentrations.
458 The percent detected by western blot from the two overnight elutions and the SDS elutions were added
459 together to determine the total percentage of cellular KAP1 was binding to and eluting from the resins).
460 **Figures 7F** and **7G** show diagrams of proteins identified by MS-MS in Figs. 5A-5C as comprising the PAV-
461 617 resin/eluate and PAV-951 resin/eluate found in the NURSA database of protein-protein interactions
462 as interacting with KAP1 (https://dknet.org/about/NURSA_Archive)(27). Orange indicates KAP1 implicated
463 proteins detected in the eluate which decreased with treatment. Magenta indicates KAP1 implicated
464 proteins detected in the eluate which increased with treatment. Yellow shows KAP1 implicated proteins
465 detected in the eluate which were unchanged with drug treatment. Circles indicate proteins from the
466 PAV-617 and PAV-951 resin eluates implicated in cancer from the Bushman lab oncogene database
467 (http://www.bushmanlab.org/links/genelists). Squares indicate proteins from the PAV-951 resin eluates
468 implicated in HIV from the virus mentha database (https://virusmentha.uniroma2.it/) (28).

469

470 Several of the proteins found by MS-MS in the PAV-617 and PAV-951 resin eluates are known to
471 interact directly with KAP1 (see **Figures 7F** and **7G**). These KAP1 implicated proteins include some whose

472 relative amount in the eluate increased and/or decreased with drug treatment and are part of cancer
473 associated interactomes identified from the literature (see **Figures 7F** and **7G**). The KAP1 implicated
474 proteins from the PAV-951 resin/eluate also contained several proteins that are associated with HIV in
475 the literature (see **Figure 7G**). These associations may shed light on the cellular role played by the
476 subfractions of KAP1 that are targeted by PAV-617 and PAV-951.

477

478 Discussion

479 Our data indicate that PAV-617 and PAV-951, two protein assembly modulator anti-viral
480 compounds selected for their ability to arrest proliferation in a distinctive way by a novel screen, are
481 cytotoxic to a wide range of neoplastic cell lines representing both rare and common cancers. These
482 compounds, while early in their drug optimization, performed comparably to the commercial anti-
483 cancer drug Gemcitabine, at 10 and 60 fold lower doses, to inhibit the growth of an A549 tumor in
484 immunodeficient mice. PAV-951 was also able to reduce growth of a HT-29 colorectal cancer in a mouse
485 xenograft study, though to a lesser degree than the positive control Irinotecan. These data suggest that
486 these two compounds are directed to two different targets common to a wide range of cancers, and
487 may provide a starting point for the development of novel cancer therapeutics.

488 DRAC and photocrosslinking experiments indicate that PAV-617 and PAV-951 interact with
489 proteins that are part of a multi-protein complex. These complexes are dynamic, as demonstrated by
490 changes in the eluate when DRAC is carried out in the presence versus absence of metabolic energy
491 substrates or from untreated versus compound-treated cell starting extract. Together, these findings
492 suggest a new model for disease pathogenesis in which previously unappreciated transient multi-protein
493 complexes plays an important role in the dynamics linking, in this case, cellular proliferation to
494 apoptosis. We hypothesize that cancer progression is facilitated by aberrant versions of these

495 multiprotein complexes in which the linkage of inappropriate proliferation to apoptosis is attenuated or
496 slowed. The effect of protein assembly modulating drugs is to restore the original version of the multi-
497 protein complex, possibly through an allosteric mechanism-of-action (29).

498 KAP1/TRIM28, an identified protein component of the PAV-617 and PAV-951target complexes,
499 is worthy of specific mention. KAP1 was identified by both MS-MS and western blot as being part of the
500 PAV-617 and PAV-951 targets (see **Figures 5-7**). Crosslinking experiments showed that for PAV-617 KAP1
501 is present in a complex targeted by the compound under native conditions, but is lost upon
502 denaturation, indicating it is not the direct drug-binding protein, but rather more likely a distal
503 component of the target multi-protein complex (see **Figure 7**). For PAV-951, KAP1 is also part of the
504 complex under native conditions. However the data indicates that a portion of KAP1 is a direct drug-
505 binding protein as well. This suggests more than one copy of KAP1 per multi-protein complex (see **Figure**
506 **7**), and is consistent with the hypothesis that KAP1 has multiple functions.

507 KAP1 stood out as being of particular interest because it is a known allosteric modulator,
508 implicated in both infectious and noninfectious disease (30). KAP1 is involved in a variety of protein-
509 protein interactions and an array of functions including transcriptional activation of HIV, T-cell
510 development, DNA damage repair, as a transcriptional co-repressor for many genes, and as a ligase for
511 post-translational modifications such as ubiquitination and SUMOlyation (30,31). Studies have shown
512 increased levels of KAP1 in many types of cancer and high levels of KAP-1 correlate with aggressive
513 clinical phenotype and progression to metastasis (32–34). However, other described functions of KAP1
514 are tumor suppressive and promote autophagy (30,35,36). KAP1 directly binds with other cancer-
515 implicated proteins including MDM2, TRIM14, Fructose-1,6-biphosphatase (FBP1), MAGE-A3, MAGE-C2,
516 Heat shock protein 70, and TWIST1 (36–40). The diversity of functions that KAP1 displays appears to be,
517 at least in part, through its assembly into different multi-protein complexes that carry out different
518 objectives.

519 The literature describes that KAP1 is utilized by HIV to the host's detriment but also functions as
520 a key component of the host's immune response in repressing HIV (30,41). KAP1 is implicated as a key
521 part of a pathway hijacked by the poxvirus p28 virulence factor (42). We show that PAV-951 is active
522 against HIV and PAV-617 is active against MPXV in addition to their anti-cancer phenotypes (see **Figure**
523 **2**). We hypothesize that there are shared alterations in protein assembly in neoplastic cells and virus-
524 infected cells. Advancing the SAR of PAV-617 and PAV-951 could be utilized to determine whether the
525 antiviral and anti-cancer targets are identical or merely similar. In the latter case, the activities would
526 separate with further SAR. Regardless, these findings suggest that the initial CFPSA capsid assembly
527 screen was successful in identifying novel targets relevant to both viruses and cancer.

528 We suggest that PAV-617 and PAV-951 redirect protein-protein interactions, whereby some
529 specific proteins are recruited to, and other specific proteins are expelled from, distinct multi-protein
530 complexes in the presence of compound (see **Supplemental Figure 7** for a diagram). KAP1 is known to
531 both promote and suppress tumorigenesis (30). Therefore, a compound which selectively targets some
532 forms of KAP1 and not others would be important regardless of the nature of the interaction with KAP1.
533 In some cases, as observed for PAV-951, a portion of KAP1 is a direct drug-binding protein, although
534 other copies of KAP1 appear not to be. In the case of PAV-617 resin, KAP1 is associated with the drug
535 only indirectly by virtue of being a protein present in the target multi-protein complex, but at a distance
536 from the drug-binding site, thus present by SAP under native but not denatured conditions.

537 We interpret the DRAC flow-through data to mean that, by virtue of conformation or other
538 differences among co-associated proteins in a transient multi-protein complex, subfractions of cellular
539 KAP1 are selectively targeted by PAV-617 and PAV-951. Results from the cross-depletion where the
540 flow-through of the PAV-617 resin was applied to a new PAV-951 resin further indicate that the PAV-
541 617-binding subfraction of KAP1 must be distinguishable from the PAV-951 binding subfraction of KAP1
542 because depletion of one does not fully deplete the other (see **Figure 7E**).

543 These findings about PAV-617 and PAV-951 mirror those made for PAV-431, a structurally-
544 unrelated protein assembly modulator with pan-respiratory antiviral activity (9). It appears as though
545 protein assembly modulating compounds, despite structural diversity, share characteristics including the
546 targeting of multi-protein complexes, selectivity for a small fraction of the total amount of a given
547 protein found in a cell, and allosteric mechanisms of action (9,11,29). Furthermore, they appear to have
548 remarkable activity across broader categories of pathogens (e.g. pan-viral family and pan-cancer) than is
549 generally observed for existing drugs. The antiviral assembly modulators appear to have a barrier to the
550 development of resistance (9,12). Further studies are needed to determine whether this property holds
551 true for the anti-cancer subset of protein assembly modulators.

552 Effective cancer drugs have been developed based on a number of mechanisms including
553 alkylating agents, antimetabolites, antimitotics, and monoclonal antibodies (43). However, as far as we
554 know, no one has attempted to treat cancer through modulation of protein assembly, giving our work
555 with PAV-617 and PAV-951 the potential to be both risky and rewarding. While the animal toxicity of
556 PAV-617 and PAV-951 is higher than would be ideal for the clinic, their anti-tumor activity is already on
557 par with existing cancer drugs and a handful of FDA approved cancer drugs have comparable toxicity
558 gauged by mouse MTD—cisplatin has a MTD of 6 mg/kg, Doxorubicin has a MTD of 10 mg/kg, and
559 Vinorelbine has a MTD of 10 mg/kg- and many other cancer drugs are administered to patients despite
560 adverse effects because of the urgency of their condition (44,45). Since PAV-617 and PAV-951 are early
561 compounds, further optimization will likely yield chemical analogs with substantially reduced toxicity
562 and further increased activity. Indeed, we have already identified chemical analogs that demonstrate
563 substantially increased safety in mice without losing anti-cancer activity in cells (see **Supplemental**
564 **Figure 3**). We hypothesize that driving SAR toward compounds that are selective for restoring feedback
565 loops of protein homeostasis will continue to improve the therapeutic indexes because the mechanism-
566 of-action does not inherently pose a risk of toxicity/collateral damage towards normal cells.

567 Molecular genetic tools such as CRISPR, siRNA knock down, and even use of recombinant
568 protein for protein-protein interaction studies, are unable to parse out the post-translational
569 heterogeneity introduced into proteins as part of normal and aberrant biochemical pathways. The
570 methods applied here are able to do so, as evidenced by the small fraction of the total of specific
571 proteins such as KAP-1 found in the target multi-protein complex. It is perhaps not surprising that new
572 tools, and the new targets they allow to be detected, make possible a path to drugs with novel
573 properties. Further work will clarify whether, as we hypothesize, the transience of our targets reflects
574 their involvement as a molecular basis for homeostasis. This conclusion, supported by the consequences
575 of protein assembly modulator treatment both here for cancer and previously for viruses, frames future
576 experiments to better understand this novel approach to more physiological disease therapeutics.

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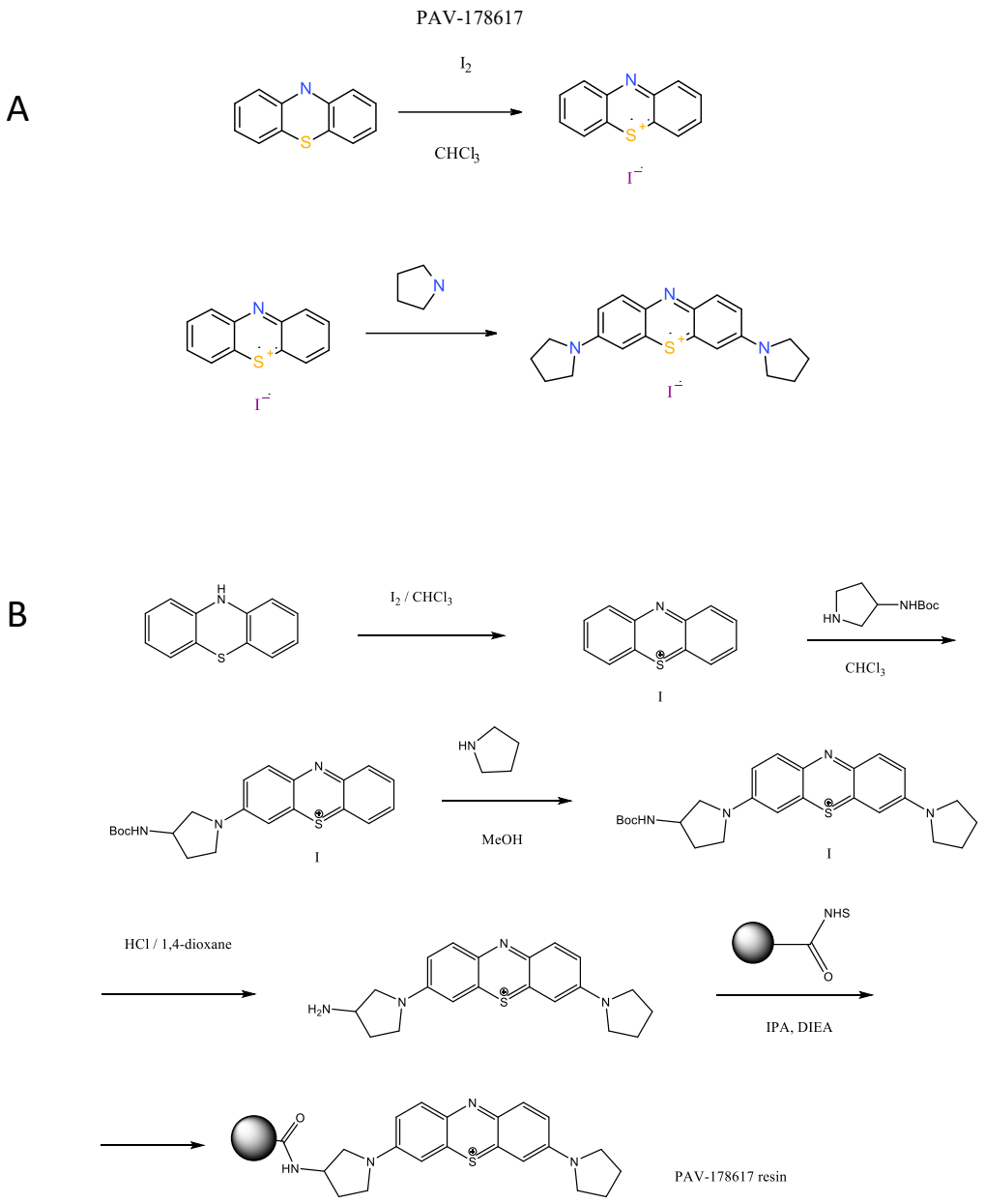
588 Supplemental Figures

589 Synthesis of A. PAV-617 and B. PAV-617 resin



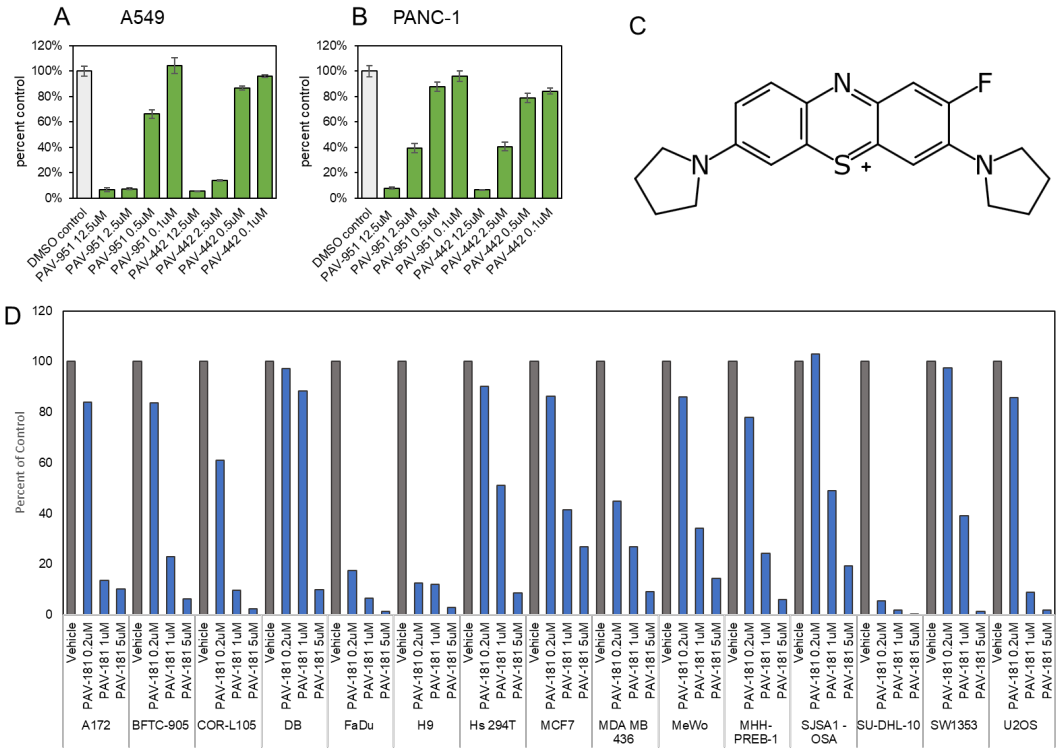
590

591 **Supplemental Figure 1. Structure and synthetic scheme of PAV-617 and its resin. Supplemental Figure 1A** shows

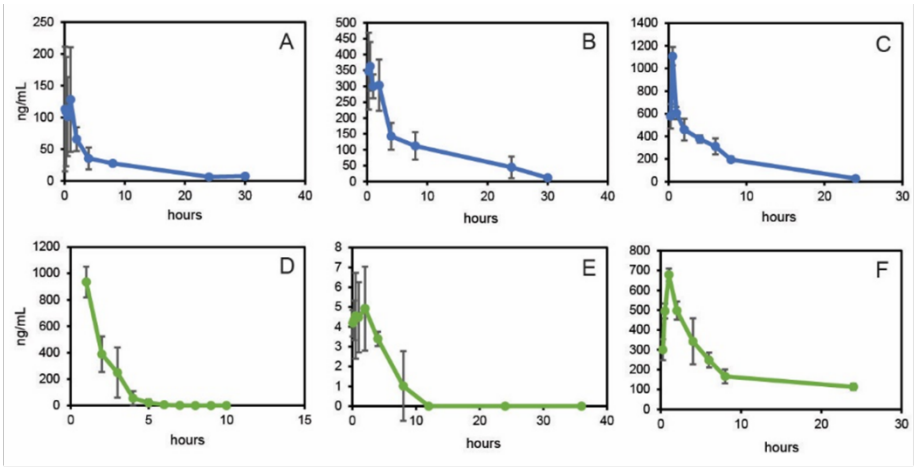
592 the chemical synthesis of PAV-617. **Supplemental Figure 1B** shows the chemical synthesis of PAV-617 resin.

593

Supplemental Figure 2. NCI-60 Cell screen with (A) PAV-617 and (B) PAV-951. Growth percent of each cell line after 48 hr treatment with 2.5 uM PAV-617 or 2.5 uM PAV-951 is relative to the vehicle treated control and the number of cells at time zero. Values between 0 and 100 represent percent growth inhibition and values between 0 and -100 represent percent cellular lethality. Figure A shows PAV-617 exhibiting greater than 50% growth inhibition on 37 cell lines and cellular lethality on 20 cell lines. Figure B shows PAV-951 exhibiting greater than 50% growth inhibition on 9 cell lines and cellular lethality on 45 cell lines. These findings corroborate and extend the initial findings.



Supplemental Figure 3. SAR optimization of the PAV-951 series. **Supplemental Figures 2A** and **2B** show comparable anticancer activity of PAV-951 and its less toxic (6x higher mouse MTD) chemical analog, PAV-442, on reducing growth of A549 lung cancer and PANC-1 pancreatic cancer tumor lines relative to a vehicle-only control. Supplemental Figure 2C shows the disclosed chemical structure of PAV-181, a chemical analog of PAV-617. Supplemental Figure 2D shows the activity of PAV-181 in at 5uM, 1uM, and 0.2uM against A172 (male human glioma), BFTC-905 (female human urinary bladder transitional cell carcinoma), COR-L105 (male human lung adenocarcinoma), DB (male human b-cell lymphoma), FaDu (male human pharynx squamous cell carcinoma), H9 (male human t-cell lymphoma), Hs 294T (male human melanoma), MCF7 (female human breast cancer), MDA MB 436 (female human breast cancer), MeWo (male human melanoma), MHH-PREB-1 (male human b-cell lymphoma), SJSA1-OSA (male human osteosarcoma), SU-DHL-10 (male human b-cell lymphoma), SW1353 (female human chondrosarcoma), and U-2 OS (female human osteosarcoma).



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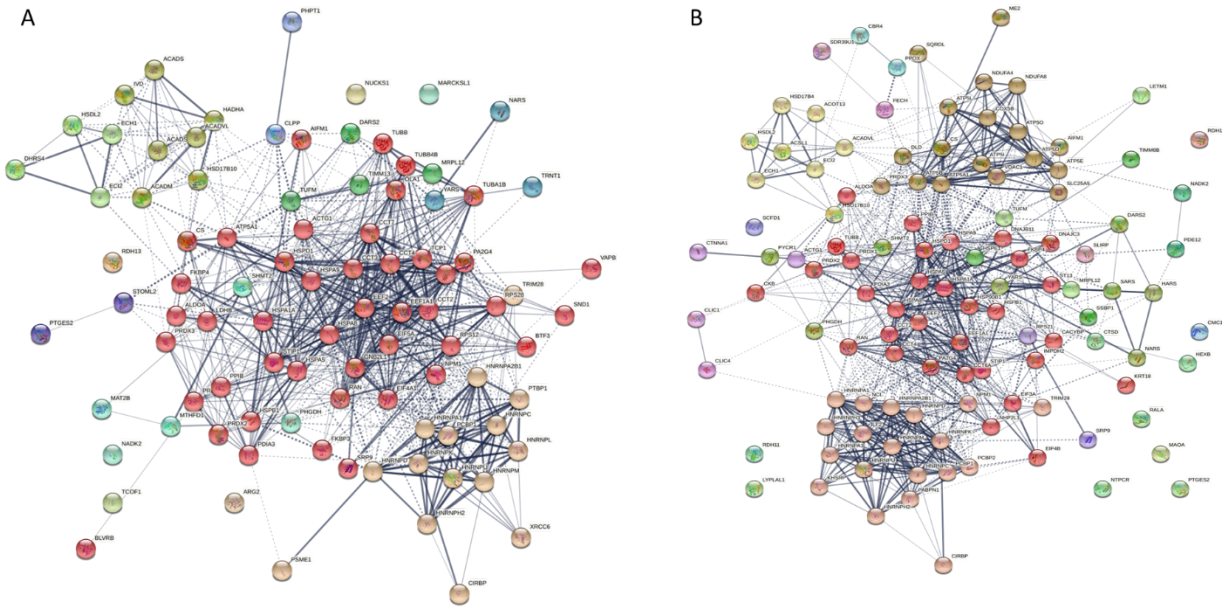
617 MW:336.5					
Animal	Route	Dose	Cmax (uM)	Half life (h)	MRT (h)
Rat	IV	1	0.38	7.84	7.03
Rat	IP	5	1.08	7.04	8.31
Mouse	Oral	10	3.3	5.56	7.66

H

951 MW:512.7					
Animal	Route	Dose	Cmax (uM)	Half life (h)	MRT (h)
Rat	IV	0.5	1.82	1.68	0.63
Rat	IP	2.5	0.0098	nd	2.92
Mouse	Oral	10	1.32	13.6	19.3

Supplemental Figure 4. Summary of PAV-617 and PAV-951 PK properties. **Supplemental Figures 3A-F** show plasma concentration over time for PAV-617 and PAV-951 when given to animals via three different routes of administration. Randomized treatment groups of four male Sprague Dawley rats or three CD1 mice were administered vehicle, PAV-617 (panel A-C), or PAV-951 (panel D-F) either IV (A, D), IP (B, E), or orally (C, F) and blood samples were collected before injection as well as at different time points after dosing. The concentration of compound in the plasma at different time points was measured by LC MS/MS. **Supplemental Figure 4G** summarizes PK properties observed for PAV-617 based on animal, administration route, and dose. **Supplemental Figure 4H** summarizes the PK properties observed for PAV-951 based on animal, administration route, and dose. The maximum concentration (Cmax) was determined to be the highest measured concentration within a dataset. The half-life and mean residence time (MRT) were calculated from the values determined over time.



Supplemental Figure 5. Known protein-protein interactions among PAV-617 and PAV-951 eluate components. **Supplemental Figures 5A** and **5B** show string-diagram analyses of the protein-protein interaction network of proteins identified in **Figures 5A-C** by tandem MSMS as comprising the PAV-617 (**Supplemental Figure 5A**) and PAV-951 (**Supplemental Figure 5B**) targets. Proteins were entered into the string database (string-db.org). The confidence mode view is shown where the confidence level was set to 0.4 (medium) and MCL clustering was applied with the inflation parameter of 3. The dotted lines represent edges between different clusters. The thickness of the lines represents the degree of confidence for each protein-protein interaction.

A

No change with treatment

Gene name	Control resin eluate	DMSO treated cells	PAV-617 resin / PAV-617 eluate	PAV-617 treated cells	PAV-617 resin / PAV-617 eluate
ACTG1	0	25	23
PRDX1	0	15	13
EEF2	0	5	3
NARS	0	4	2
TUBB4B	0	4	2
EEF1A1	0	17	16
TCP1	0	6	5
BLVRB	0	5	4
TIMM13	0	5	4
NUCKS1	0	4	3
YARS	0	3	2
ACADSB	0	3	2
PTBP1	0	3	2
SND1	0	3	2
CCT2	0	11	11
PCBP1	0	7	7
NADK2	0	5	5
ECH1	0	4	4
DHRS4	0	3	3
ACADVL	0	2	2
ARG2	0	2	2
CIRBP	0	2	2
EIF5A	0	2	2
RPS28	0	2	2
TUFM	0	13	14
HSPA5	0	10	11
HNRNPA2B1	0	8	9
FKBP4	0	7	8
CLPP	0	5	6
ACADS	0	4	5
OLA1	0	3	4
AIFM1	0	2	3
HNRNPC	0	2	3
TRNT1	0	2	3
TUBA1B	0	2	3
HNRNPM	0	10	12
PRDX3	0	10	12
HNRNPD	0	3	5
ACADM	0	2	4
HNRNPH2	0	2	4
RAN	0	2	4

Upregulated with treatment

Gene name	Control resin eluate	DMSO treated cells	PAV-617 resin / PAV-617 eluate	PAV-617 treated cells	PAV-617 resin / PAV-617 eluate
HSPD1	0	121	135
HSPA9	0	23	37
HNRNPA1	0	5	17
PDIA3	0	37	46
ATP5A1	0	20	29
HSPA8	0	26	34
STIP1	0	15	23
CS	0	9	17
HSPA1A	0	9	17
CCT7	0	11	18
HNRNPF	0	0	7
CCT4	0	16	22
HSPB1	0	14	20
SHMT2	0	3	9
PYCR1	0	0	6
LDHA	0	0	6
PCBP2	0	0	6
PRDX2	0	10	15
HSD17B10	0	5	9
ALDOA	0	2	6
CCT6A	0	2	6
HNRNPA3	0	0	4
KRTAP9-3	0	0	4
NPM1	0	11	14
IVD	0	10	13
HSDL2	0	5	8
MRPL12	0	4	7
DARS2	0	2	5
HNRNPL	0	2	5
LDHB	0	2	5
MARCKSL1	0	2	5
CTSA	0	0	3
DHRS7	0	0	3
GCDH	0	0	3
GRPEL1	0	0	3
HIST1H2BK	0	0	3
IARS2	0	0	3
KHSRP	0	0	3
DLD	0	0	2
FECH	0	0	2
RDH11	0	0	2
CHCHD2P9	0	0	2
COX6B1	0	0	2
DCXR	0	0	2
G6PD	0	0	2
GLUD1	0	0	2
KRTAP11-1	0	0	2
PDHA1	0	0	2
PSME2	0	0	2
RPL12	0	0	2
SLIRP	0	0	2
VAT1	0	0	2

Downregulated with treatment

Gene name	Control resin eluate	DMSO treated cells	PAV-617 resin / PAV-617 eluate	PAV-617 treated cells	PAV-617 resin / PAV-617 eluate
HNRNPU	0	10	2
CCT3	0	14	8
TUBB	0	5	0
PPIB	0	6	2
PHGDH	0	21	18
HNRNPK	0	17	14
PA2G4	0	10	7
TRIM28	0	5	2
MTHFD1	0	3	0
PSME1	0	3	0
RPS12	0	3	0
ECI2	0	2	0
PTGES2	0	2	0
SRP9	0	2	0
BTF3	0	2	0
EIF4A1	0	2	0
FKBP3	0	2	0
HADHA	0	2	0
MAT2B	0	2	0
PHPT1	0	2	0
RACK1	0	2	0
RDH13	0	2	0
STOML2	0	2	0
TCOF1	0	2	0
VAPB	0	2	0
XRCC6	0	2	0

2 135
spectral count

oncogene

Supplemental Figure 6A

B

No change with treatment

Gene name	Control resin eluate	DMSO treated cells PAV-951 resin / PAV-951 eluate	PAV-951 treated cells PAV-951 resin / PAV-951 eluate
PDIA3	0	20	18
HSPA8	0	66	64
NPM1	0	10	8
CCT2	0	9	7
PAZG4	0	5	3
HSD17B10	0	6	4
HSDL2	0	4	2
NADK2	0	15	13
MRPL12	0	14	12
HNRNPD	0	5	3
EIF4B	0	8	6
ST13	0	13	11
MAOA	0	19	17
ATP5L	0	4	2
HNRNPH2	0	4	2
NARS	0	4	2
NDUFA4	0	4	2
HSPA9	0	22	21
PCBP1	0	3	2
HNRNPA1	0	5	4
ECI2	0	5	4
PTGES2	0	7	6
CLIC1	0	5	4
ATP5B	0	3	2
COX5B	0	3	2
CTNNA1	0	3	2
HSP90B1	0	3	2
NCL	0	3	2
NTPCR	0	3	2
SLIRP	0	3	2
CCT4	0	6	6
FKBP4	0	3	3
EEF2	0	6	6
ATP5I	0	5	5
ACOT13	0	4	4
AIFM1	0	2	2
ATP5D	0	2	2
ATP5O	0	4	4
KHSRP	0	4	4
LETM1	0	4	4
RALA	0	2	2
SSBP1	0	3	3
ACTG1	0	11	12
CS	0	11	12
PPIB	0	2	3
ALDOA	0	6	7
FECH	0	14	15
LYPLAL1	0	6	7
ATP5E	0	2	3
CBR4	0	2	3
HNRNPH3	0	2	3
IMPDH2	0	2	3
PPOX	0	2	3
HNRNPM	0	6	8
TRIM28	0	4	6
SARS	0	8	10
DNAJC3	0	2	4
SCFD1	0	2	4
SQRDL	0	2	4

Upregulated with treatment

Gene name	Control resin eluate	DMSO treated cells PAV-951 resin / PAV-951 eluate	PAV-951 treated cells PAV-951 resin / PAV-951 eluate
HNRNPU	0	24	11
HSPA5	0	72	60
HSPA1A	0	35	23
EEF1A1	0	43	32
CKB	0	13	2
ATP5A1	0	33	24
TUFM	0	15	7
PHGDH	0	16	10
PRDX1	0	12	6
PRDX2	0	6	0
ACSL1	0	11	5
DLD	0	12	6
HNRNPK	0	15	10
STIP1	0	8	3
HEXB	0	9	4
TUBB	0	4	0
ECH1	0	15	11
ACADVL	0	18	14
PCBP2	0	4	0
CTSD	0	9	5
RDH11	0	6	2
HNRNPC	0	4	0
ILF2	0	4	0
RAN	0	4	0
HSPD1	0	13	10
PRDX3	0	19	16
HNRNPA2B1	0	13	10
DARS2	0	3	0
SRP9	0	8	5
HSPE1	0	6	3
PYCR1	0	8	5
CHCHD2P9	0	3	0
CIRBP	0	3	0
HNRNPA3	0	3	0
HSD17B4	0	3	0
KRT18	0	3	0
RDH13	0	3	0
SDR39U1	0	3	0
HSPB1	0	2	0
CCT6A	0	2	0
CACYBP	0	2	0
CLIC4	0	2	0
CMC1	0	2	0
DNAJB11	0	2	0
EIF3A	0	2	0
HARS	0	2	0
ME2	0	2	0
NDUFA8	0	2	0
PABPN1	0	2	0
RPS21	0	2	0
SLC25A5	0	2	0
SNU13	0	2	0
TIMM8B	0	2	0
VDAC1	0	2	0

Downregulated with treatment

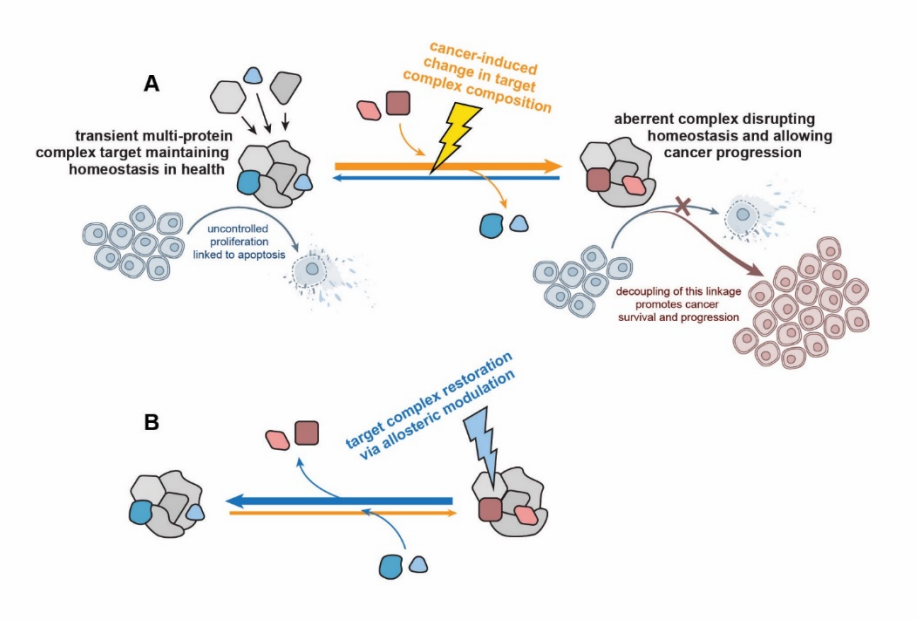
Gene name	Control resin eluate	DMSO treated cells PAV-951 resin / PAV-951 eluate	PAV-951 treated cells PAV-951 resin / PAV-951 eluate
CISD3	0	0	2
COX4I1	0	0	2
GLB1	0	0	2
HSD17B8	0	0	2
MDH2	0	0	2
NIPSNAP1	0	0	2
PABPC1	0	0	2
RMDN1	0	0	2
TIMM44	0	0	2
TUBA1B	0	0	2
TXN	0	0	2
SHMT2	0	4	7
YARS	0	3	6
CNPY2	0	0	3
ERP29	0	0	3
NSUN2	0	0	3
PDE12	0	3	7
DCXR	0	0	4
MTDH	0	0	4
OLA1	0	0	6

2 135
spectral count

oncogene

Supplemental Figure 6B

Supplemental Figure 6. The same data analyzed in terms of selective vs shared proteins between the drug resin in Figure 5 is here sorted by upregulation, downregulation or no change, with drug treatment for PAV-617 (A) and PAV-951 (B). The oncogenes and other cancer-related proteins are highlighted here in blue.



Supplemental Figure 7. Cartoon diagram of proposed mechanism of action of assembly modulating compounds. Supplemental Fig. 7A The proposed model where a normal multi-protein complex that plays a role mediating the linkage between uncontrolled proliferation and apoptosis is modified into an aberrant multi-protein complex at an early, precancerous stage allowing cancer progression rather than homeostatic elimination. **Supplemental Figure 7B** shows the proposed mechanism where treatment with a protein assembly modulating compound restores the original multi-protein complex and its homeostatic functions, including elimination of the cancer. Allosteric modulation is indicated as a means by which these changes may be induced(46).

675 Materials and Methods

676 **Lead Contact and Materials Availability**

677 Further information and requests for resources and reagents should be directed to and will be fulfilled
678 by the Lead Contact Vishwanath R. Lingappa (vlingappa@prosetta.com).

679 Use of unique compounds PAV-617 and PAV-951 and their stable derivatives may be available upon
680 request by the Lead Contact if sought for experimental purposes under a valid completed Materials
681 Transfer Agreement.

682 The number of replicates carried out for each experiment is described in the figure/table legends.

683

684 **Chemical Synthesis**

685 All compounds synthesized were confirmed by LCMS with purity typically > 98%

686

687 **Synthesis of PAV-617** (see **Supplemental Fig. 4A**)

688 Phenothiazin-5-ium tetraiodide hydrate

689 Phenothiazin-5-ium tetraiodide hydrate: a solution of phenothiazine (4.98 g, 25 mmol) in anhydrous
690 chloroform (50 ml) was stirred at 50C and the solution of iodine (12.7 g, 50 mmol) in CHCl3 (250 ml) was
691 added dropwise over 4h. The resulting dark solution was stirred for an additional 3h at 50C, monitored
692 by TLC. After the disappearance of the starting material, the resulting precipitate was filtered, washed
693 with a copious amount of chloroform, dried overnight in vacuo to afford a dark solid (13.9 g, 74%).

694

695 3,7-Di(pyrrolidin-1-yl)phenothiazinium iodide

696 A solution of phenothiazin-5-ium tetraiodide hydrate (2.8 g, 3.6 mmol) in mixture acetonitrile/methanol
697 (50 ml) and pyrrolidine (710 mg, 10 mmol) was stirred for 4 h at room temperature. The resulting
698 mixture was concentrated to dryness and purified by flash chromatography using the methanol-
699 chloroform gradient to purify the desired compound.

700

701 **Synthesis of PAV-617 resin and crosslinker** (see **Supplemental Fig. 4B**)

702 To 6-(tert-butoxycarbonylamino)-2-(9H-fluoren-9-ylmethoxycarbonylamino)hexanoic acid

703 [468mg (1mmol)] was added 3ml of 4N HCl and the resulting solution was stirred for 15min at room
704 temperature. The mixture was rotary evaporated to a residue which was then sequentially treated with
705 DMF (4ml), Diazirine 10 [128mg (1mmol)], DIEA [347ul (2mmol)] and HATU [380mg (1mmol)]. After
706 stirring for 30min at room temperature the mixture was diluted with EtOAc (20ml) and washed with
707 water (3x). The organic layer was dried (Mg2SO4) and the solvent removed affording the Diazirine acid
708 11 which was used as is.

709 To the Diazirine acid 11 [24mg (0.05mmol)] was added tert-Butyl 4-aminobutanoate [8mg (0.05mmol)],
710 DMF (1ml), DIEA [35ul (0.2mmol)] and HATU [19mg (0.05mmol)]. After stirring for 30min at room
711 temperature the mixture was diluted with EtOAc (5ml) and washed with water (3x1ml). The organic
712 layer was dried (Mg2SO4) and the solvent removed affording the Diazirine amide XX which was used as
713 is.

714 The crude Diazirine amide XX was next treated with a 50% Diethylamine / DMF solution (1ml) for 1h at
715 room temperature with mild agitation. The solvent and Diethylamine were then removed using high
716 vacuum overnight. The residue was the treated with DMF (1ml), DIEA (one drop) and Biotin-PEG2-NHS

717 [25mg (0.05mmol)]. The mix was stirred for 12 at room temperature and again the solvent was removed
718 using high vacuum affording the crude Biotinylated Diazirine YY.

719 The crude Biotinylated Diazirine YY was next treated with 4N HCl in Dioxane (1ml) for 30min at room
720 temperature then the mixture was rotary evaporated to dryness. The residue was then dissolved in DMF
721 (1ml) and then treated with DIEA [35ul (0.05mmol)], the Phenothiazinium chloride salt ZZ [21mg
722 (0.05mmol)] and HATU [19mg (0.05mmol)]. After 30min the solvent was removed using high vacuum.
723 The residue was the purified via reverse phase chromatography (0.1% TFA in ACN / 0.1% TFA in water)
724 which after lyophilization afforded 24mg (47% overall yield) of the desired PAV-617 crosslinker (XYZ).

725

726 **Experimental Models and Subject Details**

727 **Animal models**

728 Maximum tolerated dose (MTD) studies were conducted using female Balb/C mice, aged 8-10 weeks or
729 female CD1 mice, aged 5-6 weeks. Treatment groups were made up of 3 animals each, unless otherwise
730 noted, and dosing regimens for disclosed data is provided. Animals were sacrificed at the end of the
731 study period using an overdose of CO2. MTD studies were conducted at Vipragen Biosciences Private
732 Limited or Radiant Research Services Private Limited in accordance with the Committee for the Purpose
733 of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and Animal Research:
734 Reporting of In Vivo Experiments (ARRIVE) guidelines.

735 Pharmacokinetic (PK) studies were conducted in male Sprague Dawley Rats aged 8-10 weeks or male
736 CD1 mice aged 5-6 weeks. Treatment groups were made up of 4 animals each and dosing regimens for
737 disclosed data is provided. Animals were sacrificed at the end of the study period using an overdose of
738 CO2. PK studies were conducted at Vipragen Biosciences Private Limited in accordance with the

739 Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines
740 and Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

741 Tumor xenograft studies were conducted using female Athymic Nude mice, strain CrTac: Ncr-Foxn1^{nu},
742 aged 6-8 weeks. Tumor transplantation occurred through subcutaneous injection of a 0.1mL cell
743 suspension containing 1 to 5x10^{6} A549 lung cancer cells obtained from ATCC in Matrigel in PBS into the
744 left flank region of the mice. Treatment groups were made up of 6 animals each and dosing regimens for
745 disclosed data is provided. Animals were sacrificed at the end of the study period using an overdose of
746 isoflurane anesthesia. Both xenograft studies were conducted at Anthem Biosciences Private Limited in
747 Bangalore, India and were approved by the Institutional Animal Ethics committee (IAEC) of Anthem
748 Biosciences in accordance with the Committee for the Purpose of Control and Supervision of
749 Experiments on Animals (CPCSEA) guidelines and Animal Research: Reporting of *in vivo* Experiments
750 (ARRIVE) guidelines.

751

752 **Cell lines**

753 The *in vivo* xenograft study utilized A549 (male human lung cancer) and HT-29 (female human colon
754 cancer) cell lines. Cells were grown under sterile conditions. These studies were conducted at Anthem
755 Biosciences Private Limited in Bangalore, India.

756 The Human tumor cell proliferation assay used A172 (male human glioma), BFTC-905 (female human
757 urinary bladder transitional cell carcinoma), COR-L105 (male human lung adenocarcinoma), DB (male
758 human b-cell lymphoma), FaDu (male human pharynx squamous cell carcinoma), H9 (male human t-cell
759 lymphoma), Hs 294T (male human melanoma), MCF7 (female human breast cancer), MDA MB 436
760 (female human breast cancer), MeWo (male human melanoma), MHH-PREB-1 (male human b-cell
761 lymphoma), SJSA1-OSA (male human osteosarcoma), SU-DHL-10 (male human b-cell lymphoma),

762 SW1353 (female human chondrosarcoma), and U-2 OS (female human osteosarcoma) cell lines. This
763 study was conducted by Eurofins Scientific as part of their OncoPanel™.

764 The monkey pox virus infectious virus assay used BSC-40 cells (nonhuman primate kidney) and MPXV
765 Zaire 79 strain. These studies were conducted by the United States Army Medical Research Institute of
766 Infectious Diseases on Fort Detrick, Maryland.

767 Human immunodeficiency virus infectious virus assay used MT-2 cells (female human t-cell leukemia)
768 and the NL4-3 Rluc reporter virus. These studies were conducted at the University of Washington in
769 Seattle, Washington.

770 The *in vitro* screens for apoptosis, high-density/ low-density activity, and cell growth recovery utilized
771 the LNCaP C-33 (male human prostate cancer), LNCaP C-81 (male human prostate cancer), CHO-K1
772 (Chinese Hamster ovary), and Hennes 20 (Chinese Hamster ovary) cell lines. Cells were grown under
773 sterile conditions. These studies were conducted at Prosetta Biosciences in San Francisco, California.

774 The *in vitro* drug resin affinity chromatography and photocrosslinking experiments utilized A549 (male
775 human lung cancer) or LNCaP C-33 (male human prostate cancer) cell line. Cells were grown under
776 sterile conditions. Sterile conditions were not maintained once cells were harvested for *in vitro*
777 experiments. These studies were conducted at Prosetta Biosciences in San Francisco, California.

778

779 **National Cancer Institute Sixty Cell Line Screen**

780 The human tumor cell lines of the cancer screening panel are grown in RPMI 1640 medium containing
781 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells are inoculated
782 into 96 well microtiter plates in 100 µL at plating densities ranging from 5,000 to 40,000 cells/well
783 depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates are

784 incubated at 37° C, 5 % CO_2, 95 % air and 100 % relative humidity for 24 h prior to addition of
785 experimental drugs.

786 After 24 h, two plates of each cell line are fixed *in situ* with TCA, to represent a measurement of
787 the cell population for each cell line at the time of drug addition (Tz). Experimental drugs are solubilized
788 in dimethyl sulfoxide at 400-fold the desired final maximum test concentration and stored frozen prior
789 to use. At the time of drug addition, an aliquot of frozen concentrate is thawed and diluted to twice the
790 desired final maximum test concentration with complete medium containing 50 µg/ml gentamicin

791 Following drug addition, the plates are incubated for an additional 48 h at 37°C, 5 % CO_2, 95 %
792 air, and 100 % relative humidity. For adherent cells, the assay is terminated by the addition of cold TCA.
793 Cells are fixed *in situ* by the gentle addition of 50 µl of cold 50 % (w/v) TCA (final concentration, 10 %
794 TCA) and incubated for 60 minutes at 4°C. The supernatant is discarded, and the plates are washed five
795 times with tap water and air dried. Sulforhodamine B (SRB) solution (100 µl) at 0.4 % (w/v) in 1 % acetic
796 acid is added to each well, and plates are incubated for 10 minutes at room temperature. After staining,
797 unbound dye is removed by washing five times with 1 % acetic acid and the plates are air dried. Bound
798 stain is subsequently solubilized with 10 mM trizma base, and the absorbance is read on an automated
799 plate reader at a wavelength of 515 nm. For suspension cells, the methodology is the same except that
800 the assay is terminated by fixing settled cells at the bottom of the wells by gently adding 50 µl of 80 %
801 TCA (final concentration, 16 % TCA). Using the seven absorbance measurements [time zero, (Tz), control
802 growth, (C), and test growth in the presence of drug at the five concentration levels (Ti)], the percentage
803 growth is calculated at each of the drug concentrations levels. Percentage growth is calculated as:

804 $[(Ti-Tz)/(C-Tz)] \times 100$ for concentrations for which $Ti \geq Tz$

805 $[(Ti-Tz)/Tz] \times 100$ for concentrations for which $Ti < Tz$.

806

807

808 ***In vitro* experiments**

809 Drug resin affinity chromatography and photocrosslinking experiments, and SDS-PAGE/ silver
810 stain/Western Blot analysis of the results, were conducted by Prosetta Biosciences in San Francisco,
811 California under conditions described in figure legends. Results from disclosed *in vitro* experiments were
812 repeated in triplicate unless otherwise stated. Mass spectrometry analysis of samples were conducted
813 by MS Bioworks in Ann Arbor, Michigan.

814

815 **Method and Analysis Details**

816 **Monkey pox infectious virus assay**

817 BSC-40 cells of 95% confluence in 24-well plates were infected with100 pfu of MPXV Zaire 79 diluted in
818 Eagle's Minimum Essential Medium with 2% fetal bovine serum and incubated in 37 degrees Celsius in
819 5% CO_2 for 1 hour. The viral inocula were removed and replaced with the test compounds in six half log
820 dilutions (0.1 ml per well) and the cells were overlaid with 1% methylcellulose in growth media (1 ml per
821 well). The media and virus control cells received growth medium containing 1% methylcellulose. After
822 three days of infection, when plaques appeared, cells were stained with crystal violet for an hour and
823 then washed with water and dried overnight. The plaques were counted the next day and virus-only
824 wells were compared with the compound-added wells to determine percentage protection. Infected
825 cells were stained with crystal violet and viral plaques were counted. Averages and standard deviation
826 for plaques observed under different treatment conditions were calculated in Microsoft Excel and
827 graphed as the percent inhibition in PAV-617 treated cells compared to untreated cells.

828

829

830 **Human immunodeficiency virus infectious virus assay**

831 MT-2 cells were preseeded in 96-well plates in 100 ul of complete RPMI. Multiple concentrations of
832 PAV-951 were serially diluted in DMSO then into an infection media prepared by diluting NL4-3 Rluc
833 virus stock to 400 IU/100 ul with complete RPMI, which was transferred onto the MT-2 cells with a final
834 MOI of 0.02 and final DMSO concentration of 1% in infected places. One well received DMSO only,
835 instead of PAV-951, and one well received medium only for normalization and background collection.
836 Cells were incubated at 37 degrees Celsius for 96 hours. 100ul of medium was removed and discarded
837 and 10 ul of 15 uM EnduRen luciferase substrate was added to each well, followed by incubation for 1.5
838 hours at 37 degrees Celsius. Plates were read on a luminescence plate reader. Bioluminescence intensity
839 was read on a Synergy H1 BioTek plate reader. Averages and standard deviation for viral titer observed
840 under different treatment conditions were calculated in Microsoft Excel and graphed as the percent
841 inhibition in PAV-951 treated cells compared to untreated cells.

842

843 **Apoptosis Screen**

844 A 96 well plate was seeded with Hennes 20 cells at 500 cells per well, CHO-K1 cells at 500 cells per well,
845 LNCaP C-33 cells at 2000 cells per well, and LNCaP C-81 cells at 2000 cells per well. Cells were grown in
846 100uL minimum essential media for three days then three wells of each cell line received treatment with
847 1% DMSO. 12 hours after drug treatment, a mixture of 25 ul media and 25 ul Apo-ONE reagent
848 (Promega) was added then the plate was covered and placed on a shaker at room temperature for six
849 hours. The plate was read on a microplate reader for fluorescence at 499/521. Values were averaged
850 and standard deviations were calculated for each triplicate condition and graphed on Microsoft Excel.

851 **High density/ low density assay**

852 Two 96 well plates were seeded with Hennes 20 cells in parallel where one was plated at a density of
853 500 cells/well and the other was plated at a density of 15,000 cells/well. 90 ul of minimum essential
854 media was added to each well and plates were placed in a 37 degrees Celsius incubator for 24 hours.
855 The next day, 10ul of media containing dilutions of compound in DMSO were added to each plate in
856 triplicate with final concentrations of 0.025 uM PAV-617, 0.05 uM PAV-617, 0.1 uM PAV-617, 0.5 uM
857 PAV-617, 0.02 uM PAV-951, 0.3 uM PAV-951, 0.4 uM PAV-951, or 0.5 uM PAV-951. Six wells on each
858 plate received 10ul of media containing only DMSO. Each well was gently mixed 5 times with a 100ul
859 pipette. Plates were incubated at 37 degrees Celsius for 72 hours then 10 uL of alamarBlue was added to
860 each well. Wells were mixed 5 times then incubated at 37 degrees Celsius for 72 hours. Plates were then
861 read at 530/590. Values were averaged and standard deviations were calculated for each triplicate
862 condition and graphed on Microsoft Excel.

863

864 **Cell growth recovery assay**

865 A 96 well plate was seeded with either Hennes 20 or LNCaP C-33 cells at 500 cells/well in 90 uL of
866 minimum essential media and incubated at 37 degrees Celsius for 24 hours. 0.5% DMSO was diluted in
867 media and added to 6 control wells for each plate. PAV-617 was diluted in media and added to three
868 wells at a concentration of 0.3 uM. PAV-951 was diluted in media and added to concentration of 0.4 uM.
869 After 24 hours of PAV-617 treatment or 6 hours of PAV-951 treatment, the medium containing
870 compound was removed and replaced with fresh media. After 72 hours (day 5), plates were assayed
871 with alamarBlue and fluorescence was read at 530/590. The medium containing alamarBlue was
872 removed and replaced with fresh media. After another 72 hours (day 8) plates were assayed with
873 alamarBlue and fluorescence again, then medium containing alamarBlue was removed and replaced

874 with fresh media. After a final 72 hour incubation (day 11) plates were assayed with alamarBlue one
875 more time. Average fluorescence for each day and treatment condition were plotted on Microsoft Excel
876 with standard deviation calculated to provide error bars.

877

878 **Human tumor cell proliferation assay**

879 A panel of human tumor cell lines (A172, BFTC-905, COR-L105, DB, FaDu, H9, Hs 294T, MCF7, MDA MB
880 436, MeWo, MHH-PREB-1, SJSA1-OSA, SW1353, and U2OS) were grown in RPMI 1640, 10% FBS, 2 mM L-
881 alanyl-L-glutamine, 1 mM Na pyruvate. Cells were seeded into 384-well plates and incubated in a
882 humidified atmosphere with 5% CO2 at 37C. After 24 hours of incubation DMSO, PAV-617, or PAV-951
883 was added at concentrations of 5 uM, 1 uM, and 0.2 uM and plates were incubated for 3 days. Then
884 cells were lysed with CellTiter-Glo (Promega) which generates a bioluminescence signal relative to ATP
885 levels and is used as a measurement of viable cells. Bioluminescence was read by a PerkinElmer Envision
886 microplate reader. Bioluminescence intensity was measured by a PerkinElmer Envision microplate
887 reader and transformed to a percent of control (POC) using the formula: POC=(Ix/I0)*100, where Ix is
888 the whole well signal intensity at a given treatment, and I0 is the average intensity of the untreated
889 vehicle wells. Values were averaged for each triplicate condition and graphed on Microsoft Excel.

890

891 **Screening for SAR**

892 A549 and PANC1 cells were seeded at a density of 1,250 cell/well in 100ulo of media/well. Plates were
893 incubated at 37 degrees Celsius and 5% CO2 for 24 hours. DMSO, PAV-951, or its analog PAV-442 were
894 added to media at final concentrations of 0.1 uM, 0.5uM, 2.5 uM, or 12.5uM in 0.5% DMSO. The treated
895 plates were incubated at 37 degrees celsius for 72 hours than analyzed by AlamarBlue.

896 **Mouse maximum tolerated dose determination**

897 For the intraperitoneal MTD study, female Balb/c mice aged 8-10 weeks were randomly divided into
898 treatment groups with three animals per group. Animals in each treatment group were weighed and
899 received one IP injection of 0.1-0.15mL containing either vehicle (10% DMSO, 45% propylene glycol, 45%
900 sterile water), 1mg/kg PAV-617, 2 mg/kg PAV-617, 5 mg/kg PAV-617, 10 mg/kg PAV-617, 1mg/kg PAV-
901 951, 2.5 mg/kg PAV-951, 5mg/kg PAV-951 or 10 mg/kg PAV-951. Animals were observed from day 0
902 until day 3 for clinical signs of toxicity. Animals were euthanized after 72 hours and were examined
903 externally and internally by a pathologist for abnormalities in organ weight and tissue damage. Blood
904 samples were sent for a complete blood count bioanalysis. MTD was determined to be the dose at
905 which no signs of toxicity were observed by any parameters.

906 For the oral MTD study, female CD1 mice aged 5-6 weeks were given either an oral dose of vehicle (10%
907 DMSO, 45% propylene glycol, 45% sterile water) or either 10 mg/kg or 20 mg/kg of PAV-617 or PAV-951.
908 The vehicle and 20 mg/kg groups had three animals each, while the 10 mg/kg groups only had one
909 animal. Animals were observed for clinical signs and after a week they were euthanized and examined
910 externally and internally by a pathologist for changes related to toxicity.

911

912 **Pharmacokinetics studies**

913 For the IP and IV PK studies, male Sprague Dawley rats aged 8-10 weeks were randomly divided into
914 treatment groups with four animals per group. Animals in each treatment group were weighed and
915 received one 2.4 mL intravenous dose of either vehicle (10% DMSO, 45% propylene glycol, 45% sterile
916 water), 1mg/kg PAV-617, or 0.5 mg/kg PAV-951, or one intraperitoneal dose of either vehicle (100%
917 labrasol), 5mg/kg PAV-617 or 2.5 mg/kg PAV-951 . Blood was collected from a pre-cannulated line
918 before dosing, and subsequently 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 24

919 hours, and 30 hours post-dosing. Concentration of drug in the plasma over time was measured using a
920 Waters Acquity TQD LCMS/MS. Maximum concentration (Cmax) was determined to be the maximum
921 concentration detected in a dataset. Half life, area under the curve, and mean residence time were
922 calculated with Phoenix WinNolin software.

923 For the oral PK studies, male CD1 mice aged 5-6 weeks were randomly divided into treatment groups
924 with three animals per group. Animals in each treatment group were weighed and received, via oral
925 gavage needle, either one oral dose of vehicle (10% DMSO, 45% propylene glycol, 45% sterile water), 10
926 mg/kg PAV-617, or 10 mg/kg PAV-951. Blood was collected from a pre-cannulated line before dosing,
927 and subsequently 2 minutes, 5 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8
928 hours, and 24 hours post-dosing. Concentration of drug in the plasma over time was measured using a
929 Waters Acquity TQD LCMS/MS. Maximum concentration (Cmax) was determined to be the maximum
930 concentration detected in a dataset. Half life, area under the curve, and mean residence time were
931 calculated with Phoenix WinNolin software.

932

933 **A549 xenograft studies**

934 A549 cells growing in RPMI-1640 medium were suspended with Matrigel in PBS. 0.1 ml of cell
935 suspension containing 1 x 10^6 cells were injected subcutaneously into the left flank region of female, 6-8
936 weeks old nude mice (CrTac: Ncr-Foxn1nu). After 30 days of tumor establishment, mice were divided
937 randomly into treatment groups. In the PAV-617 study, 6 animals were treated with vehicle only (10%
938 DMSO, 10% propylene glycol, 80% sterile water) by IP once daily, 6 animals were treated with 100
939 mg/kg Gemcitabine Hydrochloride by IP twice weekly, and 6 animals were treated with 10 mg/kg PAV-
940 617 by IP once daily for 28 days. In the PAV-951 study, 6 animals were treated with vehicle only (10%
941 DMSO, 10% propylene glycol, 80% sterile water) by IV once daily, 6 animals were treated with 100

942 mg/kg Gemcitabine by IV twice weekly, and 6 animals were treated with 1.5 mg/kg PAV-951 by IV once
943 daily for 14 days. In both studies, mice were weighed and their tumors were measured using a digital
944 Vernier caliper. Tumor volume was calculated using the formula: $(L \times W^2)/2$ where L is the largest
945 diameter and W is the smallest diameter of the tumor. Statistical analysis was performed using Graph
946 Pad Prism (Ver. 5.03). Statistical analysis of tumor growth inhibition between the Control and Treated
947 groups was performed by using One-way ANOVA followed by Dunnett's test.

948

949 **HT-29 xenograft study**

950 HT-29 cells growing in HBSS medium were suspended in Matrigel. 0.1 mL of the cell suspension
951 containing 5×10^6 cells were injected subcutaneously into the left flank region of male, 6-8 week old SCID
952 mice. After tumor establishment, animals were divided randomly into groups of 6 and treated daily by IV
953 with vehicle daily for 17 days, 3 mg/kg PAV-951 three times per week for 17 days, or treated by IP with
954 60 mg/kg Irinotecan every 4 days for 14 days. Tumor volume was calculated using the formula: $(L \times$
955 $W^2)/2$ where L is the largest diameter and W is the smallest diameter of the tumor. Statistical analysis
956 was performed using Graph Pad Prism (Ver. 5.03). Statistical analysis of tumor growth inhibition
957 between the Control and Treated groups was performed by using One-way ANOVA followed by
958 Dunnett's test.

959

960 **Drug Resin affinity chromatography**

961 LNCaP C-33 cells were grown in minimum essential media (UCSF) with 10% FBS and 1% Penstrep for 24
962 hours then treated with 500nM PAV-617, 500nM PAV-951, or DMSO for 22 hours. Cells were scraped
963 into cold phosphate buffered saline (PBS) (10mM sodium phosphate, 150 mM sodium chloride pH 7.4),

964 then spun at 1,000 rpm for 10 minutes until pelleted. The PBS was decanted and the pellet resuspended
965 in a low salt buffer (10mM HEPES pH 7.6, 10mM NaCl, 1mM MgAc with 0.35% Tritonx100) then
966 centrifuged at 10,000 rpm for 10 minutes at 4o C. The post-mitochondrial supernatant was removed and
967 adjusted to a concentration of approximately 10 mg/ml and equilibrated in a physiologic column buffer
968 (50 mM Hepes ph 7.6, 100 mM KAc, 6 mM MgAc, 1 mM EDTA, 4mM TGA). In some conditions, the
969 extract was supplemented with an energy cocktail (to a final concentration of 1mM rATP, 1mM rGTP,
970 1mM rCTP, 1mM rUTP, and 5 ug/mL creatine kinase). 30 ul or 230 ul of extract was then incubated for
971 one hour at either 4°C or 22°C on 30 ul or 230 ul of affigel resin coupled to either PAV-617, PAV-951, or a
972 4% agarose matrix (control). The input material was collected and the resin was then washed with 3 ml
973 column bufffer. The resins were eluted overnight at either 4 ^{o}C or at 22°C in 100ul or 330ul column
974 buffer containing either 100uM PAV-617 or 100uM PAV-951 or DMSO, with or without the energy
975 cocktail. Eluates were run on SDS-PAGE with samples for silver stain and/or western blot or sent for
976 mass spectrometry analysis.

977

978 **Chemical photocrosslinking**

979 A549 extract was prepared as above then adjusted to a protein concentration of approximately 1 mg/ml
980 in column buffer containing 0.01% triton and supplemented with the energy cocktail (to a final
981 concentration of 1mM rATP, 1mM rGTP, 1mM rCTP, 1mM UTP, and 5 ug/mL creatine kinase).
982 Photocrosslinker analogs of PAV-617 and PAV-951 chemically modified to contain biotin and a diazirine
983 group or 1% DMSO were added to 67 ul of extract at 100uM, incubated for one hour at 22°C followed by
984 20 minutes on ice, then exposed to ultraviolet light for 5 minutes at 22°C. After crosslinking, samples
985 were divided in two 30 ul aliquots and one set was denatured by adding 5 ul of 10% SDS, 0.625 ul DTT,
986 and boiling for 5 minutes. Both native and denatured aliquots were then diluted in 800 ul column buffer

987 containing 0.1% triton. 2.5 ul of magnetic streptavidin beads (Pierce) were added to all samples and
988 mixed for one hour at room temperature to capture all biotinylated proteins and co-associated proteins.
989 Samples were placed on a magnetic rack to hold the beads in placed and washed three times with 800 ul
990 of column buffer containing 0.1% triton. After washing, beads were resuspended in 80 ul of gel loading
991 buffer containing SDS and analyzed by western blot or blot for affinity purified streptavidin. Samples
992 were analyzed by western blot.

993

994 **Silver Stain**

995 SDS/PAGE gels were incubated overnight in a fixative (50% methanol, 10% acetic acid, 40% water), then
996 for an hour in 50% methanol (done as two washes), and an hour in water (done as two washes). The gels
997 were sensitized in 0.02% sodium thiosulfate for one minute then washed twice for 30 seconds with
998 water. The gels were incubated for 30 minutes in cold 0.1% silver nitrate with 0.02% formaldehyde then
999 washed twice for 30 seconds. The gels were developed in 3% sodium carbonate with 0.02%
1000 formaldehyde. The developed gels showing the pattern of protein bands was scanned and the image
1001 was analyzed.

1002

1003 **Western blotting**

1004 SDS/PAGE gels were transferred in Towbin buffer (25mM Tris, 192mM glycine, 20% w/v methanol) to
1005 polyvinylidene fluoride membrane, blocked in 1% bovine serum albumin (BSA) in PBS, incubated
1006 overnight at 4 degrees Celsius in a 1:1,000 dilution of 100ug/mL affinity-purified primary IGG to KAP-1,
1007 MTHFD1, hnrnpK, TUBB, or PDI in 1% BSA in PBS containing 0.1% Tween-20 (PBST). Membranes were
1008 then washed twice in PBST and incubated for two hours at room temperature in a 1:5000 dilution of

1009 secondary anti-rabbit or anti-mouse antibody coupled to alkaline phosphatase in PBST. Membranes
1010 were washed two more times in PBST then incubated in a developer solution prepared from 100 uL of
1011 7.5 mg/mL 5-bromo-4-chloro-3-indolyl phosphate dissolved in 60% dimethyl formamide (DMF) in water
1012 and 100ul of 15 mg/ml nitro blue tetrazolium dissolved in 70% DMF in water, adjusted to 50mL with 0.1
1013 Tris (pH 9.5) and 0.1 mM magnesium chloride. Membranes were scanned and the integrated density of
1014 protein band was measured on ImageJ. Averages and the standard deviation between repeated
1015 experiments were calculated and plotted on Microsoft Excel.

1016

1017 **Tandem mass spectrometry**

1018 Samples were processed by SDS PAGE using a 10% Bis-ttris NuPAGE gel with the MES buffer system. The
1019 mobility region was excised and washed with 25 mM ammonium bicarbonate followed by 15mM
1020 acetonitrile. Samples were reduced with 10 mM dithoithreitol and 60 degrees Celsius followed by
1021 alkylation with 5o mM iodacetamide at room temperature. Samples were then digested with trypsin
1022 (Promega) overnight (18 hours) at 37 ^{o}C then quenched with formic acid and desalted using an Empore
1023 SD plate. Half of each digested sample was analyzed by LC-MS/MS with a Waters NanoAcquity HPLC
1024 system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted
1025 over a 75uM analytical column at 350 nL/min packed with Luna C18 resin (Phenomenex). The mass
1026 spectrometer was operated in a data dependent mode, with the Oribtrap operating at 60,000 FWHM
1027 and 15,000 FWHM for MS and MS/MS respectively. The fifteen most abundant ions were selected for
1028 MS/MS.

1029 Data was searched using a local copy of Mascot (Matrix Science) with the following parameters: Enzyme:
1030 Trypzin/P; Database: SwissProt Human (concatenated forward and reverse plus common contaminants);
1031 Fixed modification: Carbamidomethyl (C) Variable modifications: Oxidation (M), Acetyl (N-term), Pyro-

1032 Glu (N-term Q), Deamidation (N/Q) Mass values: Monoisotopic; Peptide Mass Tolerance: 10 ppm;
1033 Fragment Mass Tolerance: 0.02 Da; Max Missed Cleavages: 2. The data was analyzed by label free
1034 quantitation (LFQ) and spectral count methods. LFQ intensity values of each condition were measured in
1035 triplicate and compared against each other to generate $\log_2$ fold change values for each combination of
1036 conditions. Spectral counts were filtered for a 1% protein/peptide false discovery rate requiring 2 unique
1037 peptides per protein and the data set was further adjusted by subtraction of spectral counts for specific
1038 proteins observed in the control resin. Identified proteins were searched in the NURSA database of
1039 protein-protein interactions (https://dknet.org/about/NURSA_Archive) to determine if they interact
1040 with KAP-1, the Bushman labs oncogene database (http://www.bushmanlab.org/links/genelists) to
1041 determine if they were known to be implicated in cancer, and the VirusMentha database
1042 (https://virusmentha.uniroma2.it/) to determine if they interact with HIV. String diagrams of protein-
1043 protein interaction networks were generated using the STRING database (string-db.org).

1044

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1050

1051 **Competing interests:**

1052 Vishwanath R. Lingappa is CEO of Prosetta Biosciences.

1053

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