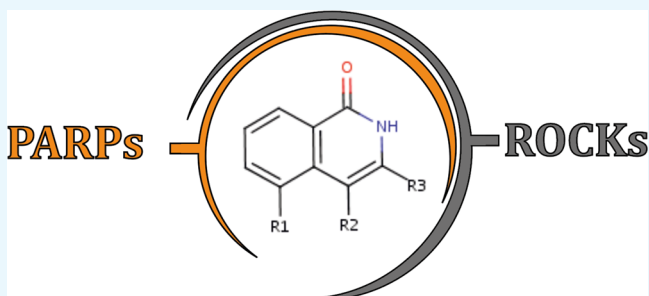


## Dual Inhibitors of PARPs and ROCKs

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**ABSTRACT:** Recent network and system biology analyses suggest that most complex diseases are regulated by robust and highly interconnected pathways that could be better modulated by small molecules binding to multiple biological targets. These pieces of evidence recently led to devote efforts on identifying single chemical entities that bind to two different disease-relevant targets. Here, we first predicted in silico and later confirmed in vitro that UPF 1069, a known bioactive poly(ADP-ribose) polymerase-1/2 (PARP1/2) molecule, and hydroxyfasudil, a known bioactive Rho-associated protein kinase-1/2 (ROCK1/2) molecule, have low-micromolar cross-affinity for ROCK1/2 and PARP1/2, respectively. These molecules can now be regarded as chemical seeds from which pharmacological tools could be generated to study the impact of dual inhibition of PARPs and ROCKs in preclinical models of a variety of complex diseases where both targets are involved.



### INTRODUCTION

The modulation of the activity of a specific protein by a small molecule is one of the cornerstones of modern target-based drug discovery.<sup>1</sup> However, it is increasingly recognized that small-molecule drugs often bind to multiple protein targets, a phenomenon termed as polypharmacology that influences both drug efficacy and safety.<sup>2,3</sup> Albeit far less commonly studied, the polypharmacology of drug metabolites and pharmacological tools is increasingly appreciated and can have profound implications in fundamental studies of biological systems using small molecules as pharmacological tools.<sup>4,5</sup> Moreover, evidence from network and system biology analyses suggests that most diseases are regulated by robust and highly interconnected pathways and thus they could be better modulated using small molecules that bind to multiple disease-relevant proteins.<sup>6,7</sup>

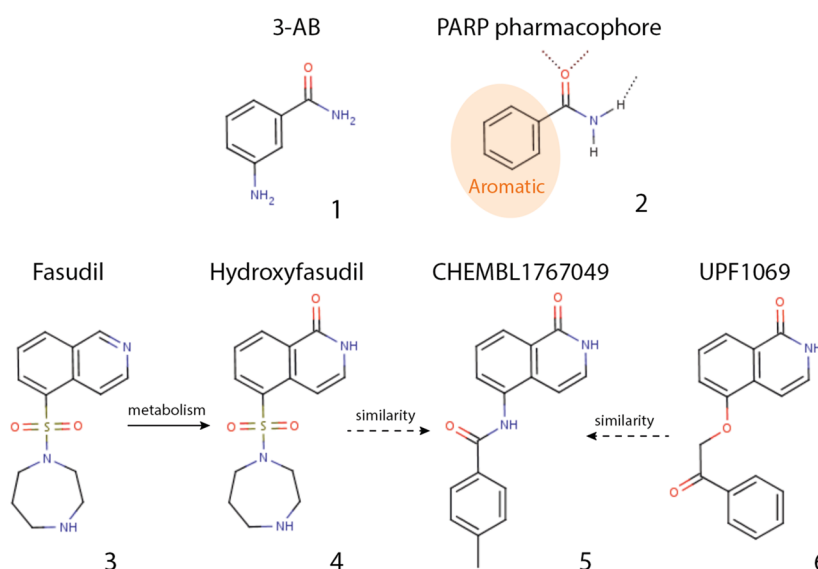
In this respect, there is a growing interest in designing molecules with customized polypharmacological profiles by computational means.<sup>8</sup> In particular, current trends point toward the identification of dual ligands, also referred to as bispecific small molecules, that specifically bind to two different target proteins. Among the several publications describing the design of a bispecific small molecule, the recent attempts to design a cytotoxic agent that also inhibits P-glycoprotein for cancer treatment,<sup>9</sup> a dual inhibitor of H1 and H4 that is a more effective antihistamine,<sup>10</sup> a dual constitutive androstane receptor and pregnane X receptor inhibitor for studying the complex regulation of drug-metabolizing enzymes,<sup>11</sup> and a dual AT1R antagonist and PPAR $\gamma$  partial agonist for the treatment of hypertension and type 2 diabetes illustrate both the diversity of therapeutic areas that could be better addressed with dual inhibitors and the increasing interest in bispecific small molecule design.

Poly(ADP-ribose) polymerase (PARP) inhibitors are a new class of therapeutics that block the DNA-repair activity of several members of the PARP enzyme family.<sup>12,13</sup> PARP inhibitors are designed to compete with the substrate NAD<sup>+</sup> and most are inspired on the early chemical tool 3-aminobenzamide (3-AB; 1), sharing a benzamide pharmacophore (2) that forms a hydrogen-bonding network in the binding site of PARPs (Figure 1).<sup>14</sup> Olaparib was the first PARP inhibitor to receive FDA approval in 2014 for BRCA-mutated ovarian cancer and, more recently, rucaparib and niraparib have been also approved for this indication.<sup>12</sup> After these approvals, the identification of new cancer subtypes and biomarkers that could enable an extended use of PARP inhibitors remains a highly active field of study.<sup>12</sup> With the increase in the understanding of the role of PARPs in the regulation of multiple other physiological and pathophysiological functions, including gene transcription, cell cycle progression, and cell death, the potential therapeutic use of PARP inhibitors beyond cancer has also been investigated.<sup>15,16</sup> For example, it is now widely documented that extensive oxidative or nitrosative stress leads to excessive activation of PARPs, depletion of cellular stores of PARP's substrate NAD, and finally to cell death by necrosis.<sup>15</sup> Accordingly, PARP inhibitors could find applications in therapeutic areas that may benefit from preventing cell death such as cardiovascular or neurodegenerative diseases. However, the full exploitation of PARP inhibitors in the clinic is currently hampered by our yet incomplete understanding of both the biological functions of PARPs and the (poly)pharmacology of PARP inhibitors.<sup>12,13</sup>

Received: September 10, 2018

Accepted: September 24, 2018

Published: October 5, 2018

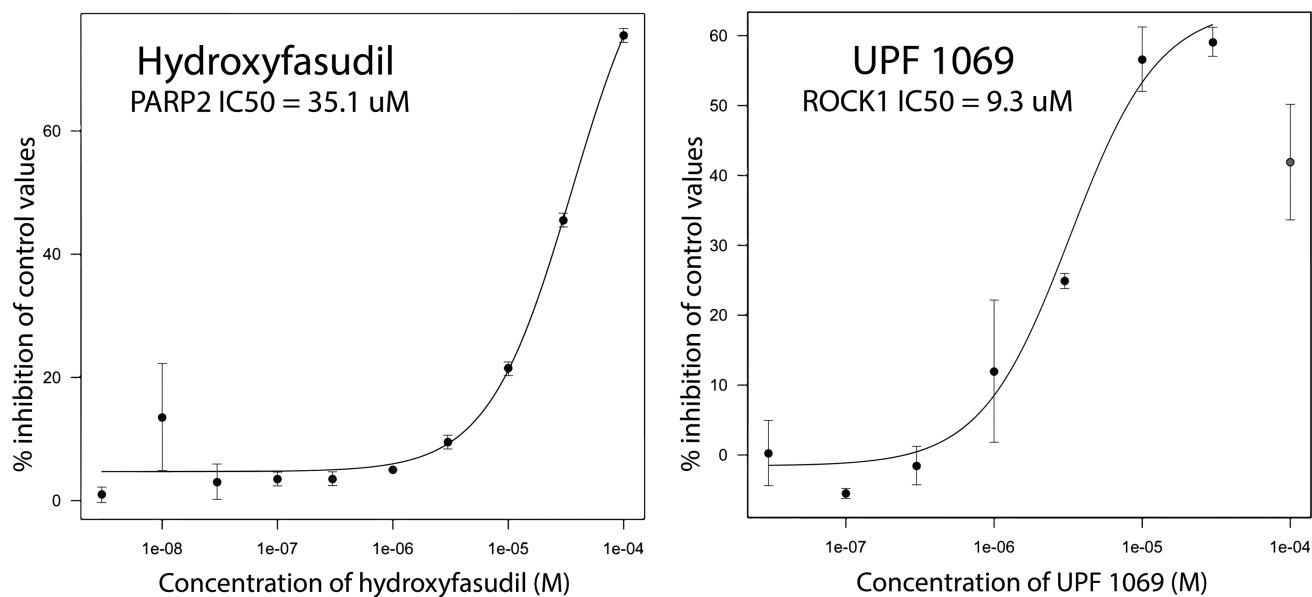


**Figure 1.** Chemical structures of PARP and Rho-associated protein kinase (ROCK) inhibitors. (1) 3-aminobenzamide (3-AB). (2) PARP pharmacophore. (3) Fasudil (ROCK drug). (4) Hydroxyfasudil (metabolite of fasudil). (5) CHEMBL1767049 (PARP inhibitor). (6) UPF 1069 (PARP chemical tool).<sup>27</sup>

**Table 1. Complete Matrix of Affinity Values for Hydroxyfasudil and UPF 1069 against 1, 2, ROCK1, and ROCK2<sup>a</sup>**

compound	PARP1	PARP2	ROCK1	ROCK2
hydroxyfasudil	27 ± 1% @ 10 μM	IC <sub>50</sub> = 35.1 μM	IC <sub>50</sub> = 0.8 μM <sup>32</sup>	IC <sub>50</sub> = 0.6 μM <sup>32</sup>
UPF 1069	IC <sub>50</sub> = 0.1 μM <sup>29</sup>	IC <sub>50</sub> = 1 μM <sup>29</sup>	IC <sub>50</sub> = 9.3 μM	20.5 ± 0.4% @ 10 μM

<sup>a</sup>IC<sub>50</sub> values (μM) were either generated from this work or directly extracted from the literature (in which case the corresponding literature citation is included). Percentage of inhibition values at 10 μM came from this work.



**Figure 2.** Dose–response curves and IC<sub>50</sub> calculation of hydroxyfasudil on PARP2 (left) and UPF 1069 on ROCK1 (right) confirm that both small molecules are dual PARP–ROCK inhibitors.

Recently, the identification of differential kinase off-targets among PARP inhibitors has contributed to explain some of the observed differential cellular effects and facilitate their full exploitation for patient benefit.<sup>13,17–19</sup>

The possibility that PARP inhibitors also bind to kinase off-targets opens an avenue for the generation of dual PARP–kinase inhibitors with potential applications in various therapeutic areas. In this respect, ROCK1 and ROCK2 are

two highly homologous isoforms of Rho-associated protein kinase (ROCK), responsible for translating the signal from Rho GTPases into a wide diversity of functions, including cell motility, cell proliferation, and apoptosis.<sup>20,21</sup> Accordingly, ROCK inhibitors have been extensively investigated in both cardiovascular diseases and cancer, among other therapeutic areas.<sup>21</sup> In 1995, fasudil (3) was the first ROCK inhibitor to be approved in Japan to treat cerebral vasospasm (Figure 1).<sup>20</sup> In

humans, fasudil is rapidly transformed to an active metabolite, hydroxyfasudil (4, Figure 1), that reaches similar concentrations but higher half-life time in plasma and thus plays an important role in fasudil's therapeutic action.<sup>22</sup> A close look at the chemical structure of hydroxyfasudil reveals that it contains the benzamide pharmacophore of PARP inhibitors (Figure 1) and thus it could potentially bind to PARPs as well.

Whereas the off-target kinase polypharmacology of PARP inhibitors has been identified,<sup>15,17</sup> the potential off-target PARP polypharmacology of kinase inhibitors remains unexplored. Most interestingly, it has recently been reported that the Rho-kinase signaling pathway promotes the expression of PARP to accelerate cardiomyocyte apoptosis in ischemia and PARPs and ROCKs also participate in the same pathway to regulate axon growth.<sup>23,24</sup> Therefore, dual PARP–ROCK inhibitors would be invaluable pharmacological tools to study the many pathways in which both PARPs and ROCKs participate and could be used as pathfinder molecules in the development of more efficacious and robust therapeutics to modulate these pathways. These observations prompted us to investigate further the potential cross-pharmacology between PARP and ROCK inhibitors.

## RESULTS

Following on the previous observation, hydroxyfasudil was processed in silico using Chemotargets CLARITY,<sup>25</sup> a widely used and validated predictive pharmacology platform.<sup>5,26</sup> Apart from recovering many of the known interactions with members of the kinase family, the results predicted a potential affinity for the protein PARP2 based on the similarity with the micromolar PARP inhibitor CHEMBL1767049 (5, Figure 1).<sup>27</sup> An in vitro biochemical test was subsequently performed to confirm that indeed hydroxyfasudil inhibits PARP2 in a dose–response manner. Initially, hydroxyfasudil showed 27% of PARP1 and 40% of PARP2 inhibition at 10  $\mu\text{M}$  concentration (Table 1). The dose–response curve of the stronger interaction between hydroxyfasudil and PARP2 was subsequently calculated, yielding a final  $\text{IC}_{50}$  of 35.1  $\mu\text{M}$  (Table 1 and Figure 2). This is an illustrative example of how in silico pharmacology can be used to anticipate potential off-target affinities of an active drug metabolite.

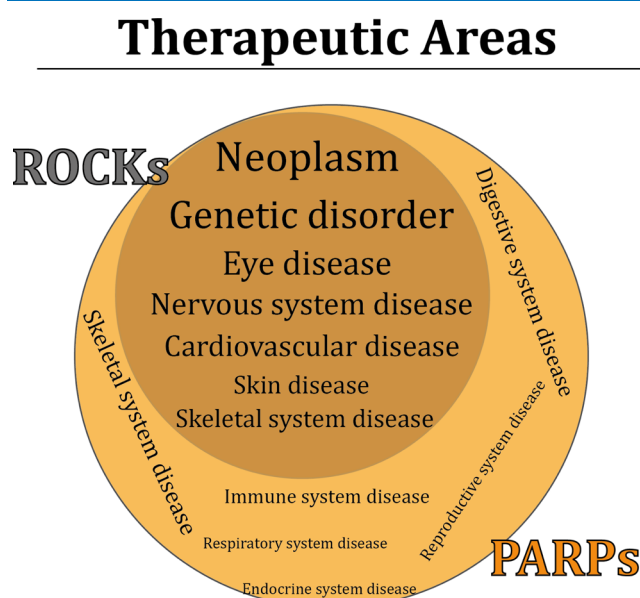
On the basis of the previous results,<sup>13,17</sup> we were also intrigued by the possibility that PARP inhibitors could have some residual ROCK inhibition. Unfortunately, the most similar PARP inhibitor to hydroxyfasudil, CHEMBL1767049 (Figure 1), was not commercially available.<sup>27</sup> However, among the known PARP chemical tools that present a 2*H*-isoquinolin-1-one scaffold substituted at position 7, we found the commercially available chemical probe UPF 1069 (6, Figure 1).<sup>28</sup> UPF 1069 has  $\text{IC}_{50}$  values of 0.1 and 1.0  $\mu\text{M}$  for PARP1 and PARP2, respectively (Table 1).<sup>29</sup> Accordingly, we tested this compound using an in vitro biochemical assay against ROCK1 and ROCK2 and confirmed that UPF 1069 shows also 38% ROCK1 and 20.5% ROCK2 inhibition at 10  $\mu\text{M}$  (Table 1). We subsequently calculated the dose–response curve for the stronger UPF 1069–ROCK1 interaction, which yielded an  $\text{IC}_{50}$  of 9.3  $\mu\text{M}$  (Table 1 and Figure 2).

The identification of two small molecules with low-micromolar affinities for PARPs and ROCKs is an important finding that confirms the existence of a certain level of cross-pharmacology between the members of these two protein families. However, a key question at this stage is whether those affinities could actually have some therapeutic relevance. In

this respect, it is known that, in humans, fasudil is rapidly metabolized to hydroxyfasudil, reaching similar peak plasma concentrations and with a higher half-life time than fasudil.<sup>22,30,31</sup> Specifically, hydroxyfasudil reaches peak plasma concentrations of around 0.84–0.56  $\mu\text{M}$  for continuous infusion of 30–60 mg/min in 30–60 min.<sup>22,30,31</sup> Hydroxyfasudil has  $\text{IC}_{50}$  values of 0.76 and 0.58  $\mu\text{M}$  for ROCK1 and ROCK2, respectively (Table 1).<sup>32</sup> In contrast, the  $\text{IC}_{50}$  values for PARPs are well below their peak plasma concentration (Table 1 and Figure 2). Therefore, PARP2 is unlikely to be involved in the mechanism of action of fasudil at these clinical concentrations. However, even though the dose of 30 mg is the more commonly used in clinical trials, fasudil has also been investigated at 80 mg three times daily in a phase 2 clinical trial of stable angina.<sup>33</sup> Following the result that a dose of 80 mg was found to be safe, this phase 2 trial recommended to explore higher fasudil doses. Accordingly, it may be important to bear in mind in the future that the low-micromolar PARP2 affinity of hydroxyfasudil could become therapeutically relevant at higher clinical doses.<sup>33</sup> In fact, it was recently demonstrated that it is possible to redirect metabolism to increase the concentration of desired drug metabolites in humans.<sup>34</sup>

## DISCUSSION

UPF 1069 and hydroxyfasudil could be invaluable pharmacological tools to explore the biological relevance of simultaneous PARP–ROCK inhibition. To gain a deeper insight into its potential therapeutic relevance, we used the Open Targets resource<sup>35</sup> to prioritize disease areas where both targets are involved. Open Targets is a platform that links targets with diseases using public and newly generated data, including text mining, genomics, and transcriptomics, among others. Using PARP1 and ROCK1 as queries, we identified seven therapeutic areas with more than 15 diseases each that are shared between PARP and ROCK, illustrating the involvement of both targets in similar diseases and processes (Figure 3). Not surprisingly,



**Figure 3.** Venn diagram of shared therapeutic areas between PARP1 and ROCK1 extracted from the Open Targets platform.<sup>44</sup> Font size is proportional to the number of shared diseases inside each therapeutic area (see Materials and Methods).



cancer (under the Open Targets therapeutic areas of “neoplasms” and “genetic diseases”) emerged as the therapeutic area with the highest number of shared diseases, given the approval of PARP inhibitors in ovarian cancer and its investigation in several other types of cancer and the vast literature linking ROCKs to cancer.<sup>20,21</sup> Interestingly, simultaneous inhibition of ROCKs could be beneficial in most of the cancer types where PARP inhibitors are being investigated in last-stage clinical trials, such as prostate and breast cancer, mainly due to the involvement of ROCKs in extracellular matrix remodeling and metastasis.<sup>21</sup>

Dual PARP–ROCK inhibition could also be beneficial in several eye diseases, including diabetic retinopathy, a serious chronic microvascular complication of diabetes mellitus and a leading cause of blindness in industrialized regions.<sup>36,37</sup> ROCK1 kinase upregulation has been implicated in the microvascular endothelial cell dysfunction associated with diabetic retinopathy, whereas a PARP1 knockout in mice has demonstrated the role of PARP1 in the inflammatory response that drives this disease; thus, simultaneous inhibition of PARPs and ROCKs could be beneficial to treat diabetic retinopathy.<sup>36,37</sup>

Nervous system diseases also emerge as complex diseases that could benefit from dual inhibition of PARPs and ROCKs. From this analysis, Alzheimer’s disease (AD) emerges as a potential candidate for a dual inhibitor of ROCKs and PARPs. On the one side, the peptide  $\beta$ -amyloid ( $A\beta$ ), which is itself an AD hallmark and toxic, has been shown to also activate PARP1, leading to neuronal cell death.<sup>38</sup> On the other side, ROCK1 has also been suggested as a potential therapeutic target in AD, given its role in regulating  $A\beta$  production, secretion, and post-translational modification.<sup>39</sup> Given the high unmet need that AD represents and the clinical failures due to the lack of efficacy of all AD therapies investigated so far, dual inhibitors of PARPs and ROCKs could facilitate the simultaneous study of these two therapeutic strategies in an attempt to increase success rates. In addition, it has been recently shown that PARP1 acts in the RhoA/ROCK pathway that inhibits axon growth and thus the dual inhibition of PARPs and ROCKs could also facilitate axon regrowth after acute axonal injury.<sup>24</sup>

Several cardiovascular diseases are also modulated by both ROCKs and PARPs, including ischemia–reperfusion injury. PARP1 hyperactivation has long been implicated in cell death after ischemia–reperfusion injury due to the depletion of  $NAD^+$ .<sup>40</sup> Moreover, ROCKs have been shown to reduce myocardial infarction size and prevent cardiomyocyte apoptosis.<sup>23</sup> Even more interestingly, a very recent study suggests that ROCKs regulate PARP1 expression and participate in the same pathway to accelerate cardiomyocyte apoptosis, suggesting potential synergistic effects of dual PARP–ROCK inhibitors on blocking cell death after ischemia–reperfusion.<sup>23</sup> To complete the list of therapeutic areas that are shared between PARPs and ROCKs, several skin and skeletal system diseases could also benefit from simultaneous modulation of PARPs and ROCKs, thus highlighting the wide diversity of opportunities that dual PARP–ROCK chemical tools could unlock toward designing more effective therapeutics.

## CONCLUSIONS

We have identified hydroxyfasudil and UPF 1069 as interesting complementary chemical seeds from which small-molecule

tools with optimized dual PARP–ROCK inhibition could be generated. When administered at high concentrations, they could already be used to study the biological relevance of dual PARP–ROCK inhibition, which could potentially be exploited in a large number of complex therapeutic areas where both targets are involved. We hope that these findings contribute to increase our understanding of PARP–kinase cross-pharmacology and unlock the design of more efficacious therapeutics that are capable of modulating robust pathways altered in diseases of high unmet medical need.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Hydroxyfasudil hydrochloride (Catalog No. 14413) was obtained from Cayman Chemical (Ann Arbor, MI), and UPF 1069 (Catalog No. S8038) was purchased from SelleckChem (Houston, TX), both with guaranteed purity of >98%.

**In Silico Target Profiling.** Chemical structures were processed with Chemotargets CLARITY v1.0.4.<sup>25</sup> Given the two-dimensional structure of a molecule (smiles or sd/mol file), CLARITY returns the predicted affinities for those targets for which ligand information is available in selected public and patent sources of pharmacological data. Six ligand-based approaches are implemented in CLARITY v1.0.4 that rely on molecular similarity, an implementation of the similarity ensemble approach, fuzzy fragment-based mapping, quantitative structure–activity relationships, machine learning methods (including support vector machine, random forest, and neural networks), and target cross-pharmacology.<sup>41</sup> The method has been successfully and extensively used and validated both retrospectively and prospectively.<sup>5,26,41</sup>

**In Vitro Biochemical Assays of ROCK1 and ROCK2 Activities.** The effects of compounds on the activity of the human ROCK1 and ROCK2 were quantified by measuring the phosphorylation of the substrate Ulight-RRRSLL (PLK) using a human recombinant enzyme and the LANCE detection method. Both assays were performed at the contract research organization company Eurofins Pharma Discovery Services.<sup>42</sup>

The test compound, reference compound, or water (control) were mixed with the enzyme (8.2 ng of ROCK1; 4.52 ng of ROCK2) in a buffer containing 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/Tris (pH 7.4), 0.8 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)- $N,N,N',N'$ -tetraacetic acid/Tris, 8 mM  $MgCl_2$ , 1.6 mM dithiothreitol, and 0.008% Tween 20. Thereafter, the reaction was initiated by adding the substrate Ulight-RRRSLL (50 nM for ROCK1; 100 nM for ROCK2) and ATP (1  $\mu$ M for ROCK1; 10  $\mu$ M for ROCK2), and the mixture was incubated (20 min for ROCK1; 15 min for ROCK2) at room temperature. For control basal measurements, the enzyme was omitted from the reaction mixture. Following incubation, the reaction was stopped by adding 13 mM ethylenediaminetetraacetic acid. After 5 min, the anti-phospho-PLK antibody labeled with europium chelate was added. After 60 more min, the fluorescence transfer was measured at  $\lambda_{ex} = 337$  nm,  $\lambda_{em} = 620$  nm, and  $\lambda_{em} = 665$  nm using a microplate reader (Envision, PerkinElmer). The enzyme activity was determined by dividing the signal measured at 665 nm by that measured at 620 nm (ratio). The results were expressed as a percent inhibition of the control enzyme activity. The standard inhibitory reference compound used was staurosporine, which was tested in each experiment at several concentrations to obtain an inhibition curve from which its  $IC_{50}$  value was calculated.

**In Vitro Biochemical Assays of PARP1 and PARP2 Activities.** Both assays were performed by following the BPS PARP assay kit protocols.<sup>43</sup> The enzymatic reactions were conducted at room temperature for 1 h in a 96-well plate coated with the histone substrate. The reaction buffer (50  $\mu$ L) (Tris–HCl, pH 8.0) contains 2.5  $\mu$ M NAD<sup>+</sup>, 2.5  $\mu$ M biotinylated NAD<sup>+</sup>, 0.026 mg/mL activated DNA, the corresponding human PARP enzyme (10 ng of PARP1, catalog number 80501 (Lot number 160524B); 25 ng of PARP2, catalog number 80502 (Lot number 140729-P4)), and the test compound at the corresponding concentration (hydroxyfasudil hydrochloride hydrate). After enzymatic reactions, 50  $\mu$ L of streptavidin–horseradish peroxidase was added to each well, and the plate was incubated at room temperature for an additional 30 min. Developer reagents (100  $\mu$ L) were added to wells, and luminescence was measured using a BioTek Synergy 2 microplate reader.

The luminescence data were analyzed using computer software Graphpad Prism. In the absence of the compound, the luminescence ( $L_t$ ) in each data set was defined as 100% activity. In the absence of the PARP, the luminescence ( $L_b$ ) in each data set was defined as 0% activity. The percent activity in the presence of each compound was calculated according to the following equation: % activity =  $[(L - L_b)/(L_t - L_b)] \times 100$ , where  $L$  is the luminescence in the presence of the compound,  $L_b$  is the luminescence in the absence of the PARP, and  $L_t$  is the luminescence in the absence of the compound. The percent inhibition was calculated according to the following equation: % inhibition = 100 – % activity.

**Analysis of Therapeutic Areas Using the Open Target Platform.** PARP1 and ROCK1 were used as queries in the Open Targets platform website.<sup>44</sup> Next, the number of diseases for each therapeutic area was obtained from the resource. Only therapeutic areas with more than 15 positively associated diseases with both PARP1 and ROCK1 were considered further. Finally, the evidence provided in the Open Targets platform was further investigated, focusing on the literature references that were carefully read to identify the diseases where simultaneous inhibition of PARP1 and ROCK1 could be beneficial.

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### Notes

The authors declare the following competing financial interest(s): J.M. is the founder of Chemotargets S.L., the company that develops the Chemotargets CLARITY platform.

## ACKNOWLEDGMENTS

This research was funded by the People Program (Marie Curie Actions) of the 7th Framework Program of the European Union (FP7/2007–2013) under REA grant agreement no. 600388 (TECNIOspring program) (A.A.A.); the Agency of Business Competitiveness of the Government of Catalonia,

ACCIO (A.A.A.); the Wellcome Trust Sir Henry Wellcome Postdoctoral Fellowship (204735/Z/16/Z) (A.A.A.); and the Spanish Ministerio de Economía y Competitividad grant BIO2014-54404-R (J.M.).

## ABBREVIATIONS:

PARP, poly(ADP-ribose)polymerase; ROCK, Rho-associated protein kinase

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