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3-[2-(Aminomethyl)-5-[(pyridin-4-yl)carbamoyl]phenyl] benzoates as soft ROCK inhibitors

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ABSTRACT

Clinical development of ROCK inhibitors has so far been limited by systemic or local ROCK-associated side effects. A soft drug approach, which involves predictable metabolic inactivation of an active compound to a nontoxic metabolite, could represent an attractive way to obtain ROCK inhibitors with improved tolerability. We herein report the design and synthesis of a new series of soft ROCK inhibitors structurally related to the ROCK inhibitor Y-27632. These inhibitors contain carboxylic ester moieties which allow inactivation by esterases. While the parent esters display strong activity in enzymatic (ROCK2) and cellular (MLC phosphorylation) assays, their corresponding carboxylic acid metabolites have negligible functional activity. Compound **32** combined strong efficacy (ROCK2 IC_{50} = 2.5 nM) with rapid inactivation in plasma ($t_{1/2}$ <5′). Compound **32** also demonstrated in vivo efficacy when evaluated as an IOP-lowering agent in ocular normotensive New-Zealand White rabbits, without ocular side effects.

Rho-associated, coiled coil containing protein kinase (ROCK)[1] is a serine/threonine kinase belonging to the AGC family.[1] In humans, ROCK consists of two isoforms, namely ROCK1 and ROCK2. These isoforms share an overall homology of 65%, while being 95% homologous in the kinase domain. ROCK plays an important role in numerous cellular processes including smooth muscle cell contraction, cell proliferation, adhesion and migration.[2] Consequently, ROCK inhibitors are of potential interest for the treatment of multiple indications including cardiovascular diseases,[3] inflammatory and autoimmune diseases[4–6] (e.g., asthma, Chronic Obstructive Pulmonary Disease, Inflammatory Bowel Disease), or eye diseases[7] (e.g., glaucoma). The ROCK inhibitor fasudil has been approved in Japan for the treatment of cerebral vasospasm. However, ROCK inhibitors cause a pronounced decrease in blood pressure,[7] thereby limiting their usefulness for the systemic treatment of non-cardiovascular indications.[7] As a consequence, recent development of ROCK inhibitors has typically been restricted to topical applications, which results in lower systemic exposure. Several ROCK inhibitors have been clinically tested as intraocular pressure (IOP)-lowering agents for topical treatment of glaucoma.[8] Those include Y-39983 (Novartis, Basel, Switzerland), K-115 (Kowa Company Ltd, Nagoya, Japan), AR-12286 & AR-13324 (Aerie Pharmaceuticals, Inc., Bedminster, USA) and ATS907 (Altheos, Inc., South San Francisco, USA). Even when used topically, ROCK inhibitors are not devoid of side effects. In the context of ophthalmology, a common adverse event resulting from topical administration of ROCK inhibitors is mild to severe conjunctival hyperemia (redness) due to mechanism-based smooth muscle cell relaxation in conjunctival blood vessels.[8–10] As a consequence, most available ROCK inhibitors have a narrow therapeutic window.

In view of the potential of ROCK inhibitors for generating side effects, a soft drug approach could represent an attractive way towards novel ROCK inhibitors with improved tolerability. Soft drugs, sometimes known as antedrugs, are biologically active compounds that are designed to undergo metabolic inactivation by controlled conversion of the parent molecule into a predictable, nontoxic metabolite.[11,12] Herein, we report the design and initial evaluation of soft ROCK inhibitors.

Compound **1**[13] (Fig. 1) was taken as a starting point for the design of soft ROCK inhibitors. **1** is structurally related to Y-27632, a well-described ROCK inhibitor known for its good selectivity towards ROCK.[14] The crystal structure of a ROCK/Y-27632, revealed the importance of the pyridine nitrogen and primary amine for activity.[15] Consequently, those pharmacophore points were not altered. Modification of the 4-fluorophenyl moiety was instead preferred. Introduction of ester side chains provided an inactivation mechanism for the resulting derivatives, by making them potential substrates for esterases.

A number of ROCK inhibitors structurally derived from **1** were synthesized. They were prepared in ca. 5–10 steps, starting from the commercially available 3-bromo-4-methylbenzoic acid **2**

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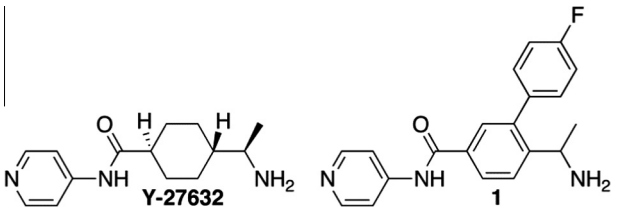


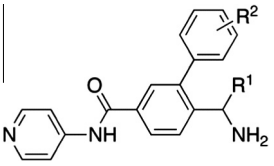
Figure 1. Structure of compounds Y-27632 and **1**.

(Fig. 2). Fisher esterification of **2**, followed by radical bromination of the benzylic group yielded the intermediate benzyl bromide **3**. This compound was transformed into the Boc-protected benzylamine **4** through a sequence of three steps, comprising the substitution of **3** by $(Boc)_2NH$, selective removal of one Boc group by trifluoroacetic acid (TFA), and finally hydrolysis of the methyl ester under basic conditions. The resulting free acid **4** is reacted with aminopyridines, using HATU/DMAP as coupling reagent to provide intermediates **5–6**. The key step to introduce the aromatic ring displaying the carboxylic acid is a Pd-catalyzed Suzuki reaction, using commercially available boronic acids. Further esterification of the resulting carboxylic derivatives **7–9** with a selection of alcohols can be achieved through various methods, including TBTU, Steglich method or the use of Ghosez reagent, to activate the acid. Final deprotection by TFA or HCl gas wraps up the synthesis of this initial series of compounds.

In a first step, we checked what the optimal position was for the introduction of an ester side chain on the phenyl ring of **1**. Replacement of the 4-fluoro by a methyl ester yielded compound **10**, which had significantly reduced potency compared to **1**. Introduction of the same methyl ester on position 3 was however tolerated, resulting in the potent compound **11** (Table 1).

Molecular docking of **1** and **11** in ROCK explains the observed difference of potency between compounds **10** and **11**. The proposed binding mode for compounds **1** and **11** (Fig. 3) is similar to Y-27632, with the additional phenyl moiety positioned under the P-loop (Gly-rich loop) of ROCK. Position 4 (4-F in **1**) closely

Table 1
ROCK2 inhibition data for compounds **1**, **10** and **11**



Compd	R^1	R^2	ROCK2 IC_{50}[a] (nM)
1	Me	4-F	1.2
10	H	4-CO_2Me	102
11	H	3-CO_2Me	2.9

[a] Values are means of two experiments.

contacts residues from the P-loop, discouraging introduction of bulky functional groups. In contrast, position 3 provides access to a solvent-exposed cleft which can accommodate the ester group with minimal perturbation of the binding mode.

In view of these initial results, priority was given to position 3 for further optimization of the series. On-target potency of the candidate soft ROCK inhibitors was evaluated externally, with Y-27632 as comparator. While Y-27632 appeared relatively potent in this assay compared to literature data, its activity remained consistent throughout independent experiments. Activity data for representative compounds is provided in Table 2. Degradation of the compounds in human plasma was used as a first estimate of their soft drug characteristics.

Ester derivatives displaying C_1–C_6 linear alkyls were first synthesized and evaluated. While such chains were initially tolerated, increased length ultimately resulted in decreased activity, as illustrated by **16**. A plausible explanation to this observation is that the solvent-exposed cleft accommodating the ester chain has limited affinity towards lipophilic groups. Flexible alkyl chains could also lose conformational freedom upon binding, resulting in a substantial loss of entropy and thereby in reduced potency. This hypothesis was tested by replacing the *n*-hexyl moiety of **16** by a

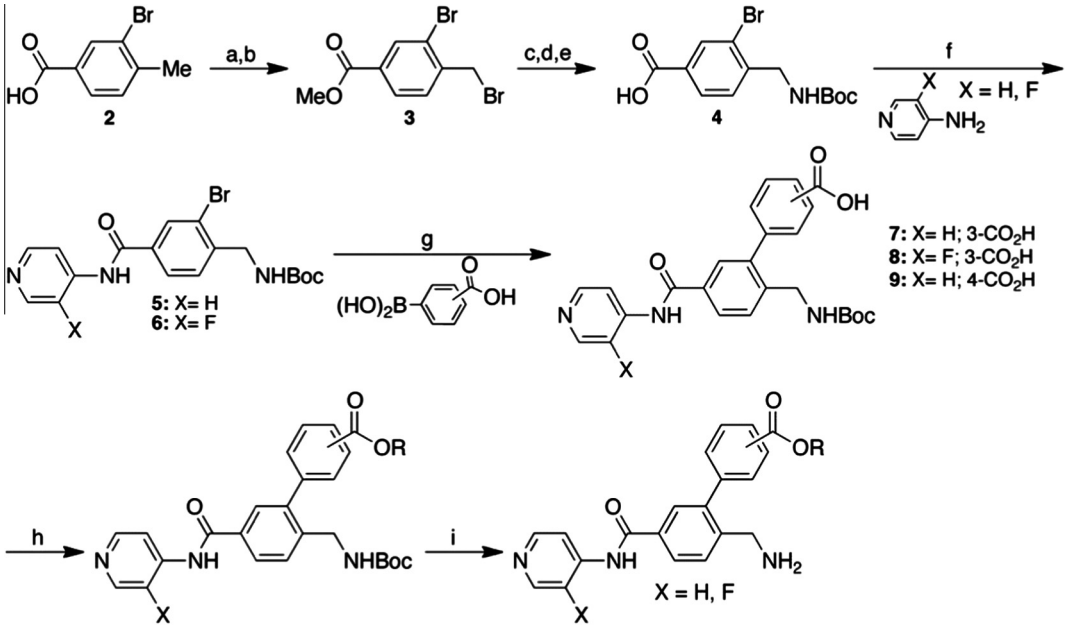


Figure 2. Synthetic scheme for synthesis of compounds **10–35**. (a) H_2SO_4, MeOH, 60 °C, 16 h; (b) NBS, AIBN, CCl_4, reflux, 16 h; (c) Boc_2NH, *t*-BuOK, DMF, rt, 16 h; (d) DCM/TFA (50:1), 0 °C → rt, 4 h; (e) NaOH, MeOH, 50 °C, 2 h; (f) HATU, DMAP, NEt_3, DMA, 30 °C, 16 h; (g) $Pd(dppf)Cl_2$, Na_2CO_3, H_2O, DMF, 100 °C; 16 h; (h) ROH, TBTU, HOBT, DIEA, DMF, rt, 16 h or ROH, DCC, DMAP, DCM, rt, 16 h or $Me_2C{=}C(Cl)NMe_2$, THF or DCM, rt, followed by ROH, 16 h; (i) DCM/TFA (7:1), 30 °C, 16 h or $HCl_{(g)}$ in DCM, 30 °C, 16 h.

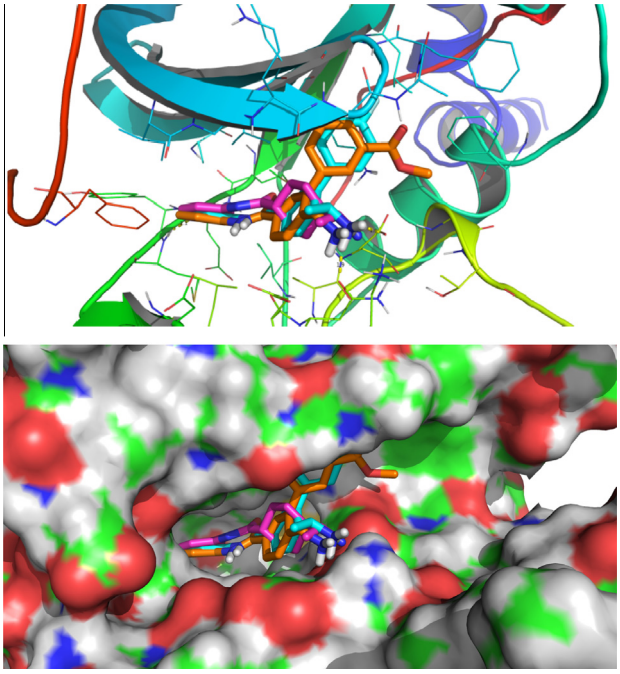


Figure 3. Molecular modeling of compounds **1** (cyan sticks) and **11** (orange sticks) in ROCK1 (PDB 2ETR). Y-27632 is depicted as purple sticks for comparison.

cyclohexyl, but compound **19** did not improve over **16**. However, introduction of a cyclopentylmethyl in **21** led to a 4-fold increase in potency. This finding prompted the synthesis of additional cycloalkylmethyl derivatives. Increasing the ring size yielded **22**, which displayed an activity comparable to Y-27632. On the other hand, reducing ring size delivered **24** and **25**, which had single digit nanomolar potency. Replacement of the cyclohexylmethyl by a benzyl did not restore activity (**26**). Introduction of heteroatoms was generally well tolerated, with the linear ether derivatives **27** and **28** displaying an on-target activity similar or slightly better than their isosteric alkyl analogs **14** and **15**. As with previous derivatives, extension of the ester chain with further lipophilic groups resulted in decreased potency (**27**). However, introduction of a hydroxyl group generated the potent compound **30**. Similarly, cyclic ethers turned out to be viable alternatives to cycloalkyls (**31**–**33**). 3-Fluoro-4-aminopyridine derivatives had slightly reduced potency (2 to 3-fold) when compared to the corresponding 4-aminopyridine analogs (**11** vs **17**; **13** vs **18**; **21** vs **23**).

Myosin light chain (MLC) phosphorylation status is a direct marker of the ROCK enzymatic activity in cells.[16] Low ROCK activity results in decreased MLC phosphorylation. Evaluation of selected compounds in a cellular MLC phosphorylation assay confirmed their functional efficacy. The most potent representatives in the series (**11**, **13** and **32**) displayed EC_{50} values in the low nanomolar range, and were comparable to compound **1** and clearly lower than with Y-27632.

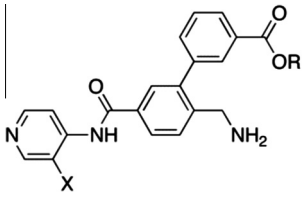
An important aspect of the soft drug approach resides in the decreased activity of the pre-defined metabolite. Compounds **34** and **35**, which represent the metabolites resulting from ester hydrolysis of **11**–**33**, had lower on-target potency than most of their parent compounds. This reduction in activity was further apparent in the MLC phosphorylation assay, where **34** and **35** were almost devoid of functional efficacy. Interestingly, compounds **34** and **35** both displayed very low permeability (P_{app} <0.01 10^{-6} cm/s). This result is consistent with literature data indicating lower membrane permeability for acidic or zwitterionic species compared to neutral or basic species.[17] As ROCK is an intracellular target, it is therefore possible that reduced membrane permeability plays a role in the lower functional efficacy observed for these compounds. Exposure to alcohols resulting from hydrolysis of compounds **11**–**33** should also be taken into account when designing/selecting soft ROCK inhibitors. However, such exposure will highly depend on use, dose and mode of administration of the compound. Such considerations go beyond the scope of this exploratory work.

The soft drug concept involves conversion of the parent compound to the inactive metabolite. This aspect was initially assessed by evaluating the plasma stability of compounds **11**–**33**. We observed a clear influence of linear alkyl chain length on stability (**11**–**16**). Derivatives displaying shorter alkyl chains were hydrolyzed at a relatively slow, although measurable rate. Plasma stability decreased with increasing chain length, to reach a minimum with **13**, which displayed a half life of 18 min. The stability of the cycloalkylmethyl derivatives was, with respect to their linear analogs, similar (**25** vs **14**) or lower (**21** and **23** vs **16**; **24** vs **15**). The cyclohexyl ester **19** displayed significantly increased plasma stability compared to its *n*-hexyl analog **16**. Replacement of cyclohexyl moieties by phenyl had a variable effect. While a fast hydrolysis was observed for the phenyl ester **20**, the difference between the benzyl derivative **26** and its cyclohexylmethyl analog **22** was not striking. Replacement of alkyl chains by ether chains resulted in compounds with relatively short plasma half-life. This was especially clear for compounds **31** (vs **19**) and **32** (vs **23**). In contrast, the hydroxyethyl derivative **30** was more stable than the closely related methoxyethyl, propyl or butyl analogs. Surprisingly, some 3-fluoro-4-amino-pyridine derivatives were hydrolyzed at a slightly increased rate with respect to their 4-aminopyridine analogs. Importantly, the carboxylic derivatives **34** and **35** were not significantly degraded (<5%) over the course of the experiment, indicating that compound degradation resulted, as intended, from ester hydrolysis. This was further confirmed by simultaneously monitoring disappearance of selected compounds and appearance of the corresponding metabolite in plasma samples (data not shown). One can therefore conclude that once in plasma, most compounds will be rapidly converted into the functionally inactive **34** or **35**.

The collected stability data clearly indicate that the degradation of compounds **11**–**33** in plasma is due, as intended, to enzymatic hydrolysis of the carboxylic ester moiety present in those compounds. Firstly, the structurally related compounds **1**, **34** and **35** were stable in plasma (<5% disappearance of parent compound) over the course of the experiment. Secondly, the rapid hydrolysis of compounds **11**–**33** in plasma was not reproduced in phosphate buffered saline (PBS) pH 7.4, although some degree of hydrolysis was noticed for some compounds such as **19** (data not shown). Finally, the observation that increasing linear alkyl chain length initially results in faster hydrolysis rates in plasma differs from common rules governing the chemical stability of benzoic esters wherein the derivatives displaying the shorter, less hindered alkyl chains are more rapidly hydrolyzed;[18] and are instead reminiscent of substrate preferences observed with some hydrolytic enzymes, including esterases.[19]

Among the various hydrolytic enzymes, carboxylic ester hydrolases (EC 3.1.1) probably play a major role in the metabolism of compounds **11**–**33** in plasma. While being essentially devoid of carboxylesterase activity, human plasma contains two main esterases (butyrylcholinesterase and paraoxonase 1) and a pseudoesterase[20] (serum albumin). Simultaneous involvement of these esterases in drug hydrolysis has been demonstrated for compounds as simple as aspirin.[21] At this stage, the esterase(s) responsible for hydrolysis of soft ROCK inhibitors has/have not been formally attributed for each compound. Biochemical intervention with specific inhibitors is a common way to identify the esterase(s) involved in drug or prodrug metabolism,[21] which could provide further elucidation of the metabolism of compounds **11**–**33**.

Table 2
On-target potency, cellular efficacy and stability data for **11–35**



Compd	X	R	ROCK2 IC_{50}[a] (nM)	MLC-PP EC_{50}[b] (nM)	$t_{1/2}$ Plasma (min)
Y-27632	NA	NA	54	200	>120[d]
1	NA	NA	1.2	6.1	>120[d]
11	H	Me	2.9	8.0	91
12	H	Et	<1.0[c]	ND	41
13	H	*n*Pr	1.4	9.1	18
14	H	nBu	5.5	ND	22
15	H	nPen	4.9	ND	58
16	H	nHex	22	ND	50
17	F	Me	7.8	130	65
18	F	nPr	5.1	ND	19
19	H	cHex	20	ND	>120
20	F	Phe	6.2	ND	7
21	H	CH_2-cPen	5.0	70	31
22	H	CH_2-cHex	48	ND	99
23	F	CH_2-cPen	13	52	17
24	F	CH_2-cBu	4.3	ND	13
25	F	CH_2-cPr	2.7	ND	18
26	H	Benzyl	45	ND	64
27	H	$(CH_2)_2$OMe	3.6	170	12
28	H	$(CH_2)_3$OMe	1.5	28	26
29	F	$(CH_2)_2$OiPr	37	ND	6
30	F	$(CH_2)_2$OH	1.4	ND	81
31	F	Oxan-3-yl	8.7	ND	18
32	F	CH_2-oxolan-2-yl	2.5	16	<5
33	F	CH_2-oxan-2-yl	12	ND	7
34	H	H	167	>1000	>120[d]
35	F	H	55	>1000	>120[d]

[a] Values are means of at least two experiments.
[b] Values are means of three experiments.
[c] ROCK2 concentration in the assay was 1 nM.
[d] <5% Degradation over the course of the experiment.

Compound **32** was selected for in vivo evaluation since it combined strong efficacy with rapid hydrolysis in plasma. Topical administration to ocular normotensive New Zealand White (NZW) rabbits and monitoring of the IOP was selected for this first proof of concept. This animal model is easily implemented, corresponds to a potential indication for ROCK inhibitors (IOP-lowering agents for the treatment of glaucoma) and allows for an easy monitoring of the expected side effect, conjunctival hyperemia.

The hydrolytic activity of rabbit eye tissues towards **32** was first assessed by incubating the compound at a concentration of 20 μM, in presence of tissue samples. A specific hydrolytic activity was calculated for each tissue (Table 3) by normalizing the observed degradation rate of **32** with respect to tissue weight. Low hydrolytic activity (0.12 pmol min^{-1} mg^{-1}) was found in aqueous humor, corresponding to a half-life of 116 min in undiluted aqueous humor. This result was in line with the $t_{1/2}$ value obtained when incubating **32** with undiluted, pooled AH samples from NZW rabbits (Sera Laboratories International, Haywards Heath, UK). Higher hydrolytic activities were observed in cornea, conjunctiva and sclera, indicating that **32** could actually be metabolized and behave as a soft ROCK inhibitor in those tissues.

Table 3
Specific hydrolytic activity of rabbit eye tissues towards **32**

Tissue	Specific hydrolytic activity (pmol min^{-1} mg^{-1})
Aqueous humor	0.12
Vitreous humor	0.22
Conjunctiva	1.52
Sclera	1.00
Cornea	2.71

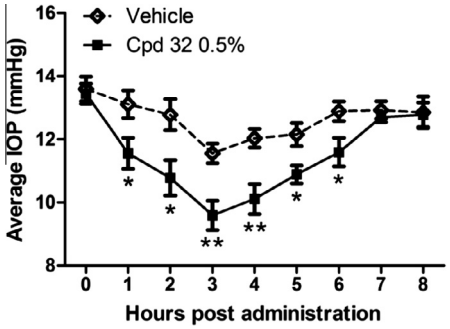


Figure 4. IOP-lowering effect of **32** (0.5% solution) following topical administration to ocular normotensive NZW rabbits. $^{*}P$ <0.05. $^{**}P$ <0.01.

In spite of its metabolically labile nature, **32** had a pronounced IOP-lowering effect when administered topically to ocular normotensive NZW rabbits (40 μl of a 0.5% w/v solution in 1:1 PEG:water vehicle with pH adjusted to 7.0). A significant IOP decrease vs. the vehicle-treated contralateral eye was observed during the six hours following administration (Fig. 4). The highest average IOP reduction was achieved 3 h after instillation, and was 28.7% from baseline (−3.85 mmHg). No obvious hyperemia was noticed over the course of the experiment, suggesting that soft ROCK inhibitors such as **32** could potentially separate the desired IOP-lowering effect of ROCK inhibitors from this commonly observed side effect. Further development and evaluation of this compound series resulted in the discovery of AMA0076, Amakem's clinical candidate for the treatment of glaucoma. A detailed evaluation of AMA0076, including dose ranging studies, rigorous scoring of hyperemia and comparison to other IOP-lowering agents will be presented in an upcoming dedicated paper.

In conclusion, we herein reported the first examples of soft ROCK inhibitors. These compounds are structurally related to Y-27632, but contain carboxylic ester functions allowing their rapid hydrolysis by esterases, resulting in a functionally inactive metabolite. A first in vivo proof of concept illustrating the potential of soft ROCK inhibitors as IOP-lowering agents with reduced hyperemic effects was also presented. Clinical development of ROCK inhibitors for multiple indications, such as asthma, COPD, IBD or even glaucoma, has so far been limited by ROCK-associated side effects. In this context, we believe that soft ROCK inhibitors displaying an improved therapeutic window might represent a novel therapeutic approach towards many of these indications, especially those compatible with topical or local drug delivery.

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Supplementary data

Supplementary data (experimental protocols regarding synthesis of compounds **11–35**, full characterization of compound **32** and of its metabolite **35** and experimental protocols regarding in vitro and in vivo evaluation of compounds **11–35**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.09.040.

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