Immunogenic cell death in mice expressing caspase-resistant ROCK1 is not replicated by ROCK inhibitors

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Running title: ROCK1 cleavage limits liver immunogenic cell death

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Abstract

The characteristic changes in morphology during apoptosis help facilitate "immunologically-silent" cell death by limiting the release of cellular contents and enabling efficient clearance of apoptotic cell bodies. Caspase-mediated cleavage of the ROCK1 protein kinase results in its constitutive activation, which drives the rapid and forceful contraction of apoptotic cells. We previously showed in Julian *et al*. [1] that when ROCK1 was mutated to render it caspase-resistant, there was greater liver damage and neutrophil recruitment after acute treatment with the hepatotoxin diethylnitrosamine (DEN). We now show that acute DEN-induced liver damage resulted in greater induction of pro-inflammatory cytokines/chemokines, indicative of immunogenic cell death (ICD), in mice expressing non-cleavable ROCK1 (ROCK1nc). Hepatocellular carcinoma (HCC) tumours in ROCK1nc mice had higher grade steatosis, and more neutrophils and CD8^{+} T cells relative to mice expressing wild-type ROCK1 (ROCK1wt), indicating that spontaneous tumour cell death also was more immunogenic. Since the induction of ICD has been proposed to be tumour-suppressive, the effects of two structurally distinct pharmacological ROCK inhibitors on HCC tumours was examined. Both fasudil and AT13148 significantly decreased tumour numbers and area, but neither treatment resulted in greater numbers of neutrophils or CD8^{+} T cells to be recruited. In the context of acute DEN-induced liver damage, AT13148 reduced circulating platelet and lymphocyte numbers, and inhibited the recruitment of dendritic, natural killer and CD8^{+} T cells to livers. These observations indicate that there is an important role for ROCK1 cleavage to limit immunogenic cell death which was not replicated by systemic administration of pharmacological ROCK inhibitors. As a result, adjuvant administration

49 of ROCK inhibitors with cancer therapeutics would be unlikely to result in therapeutic

50 benefit by inducing ICD to increase anti-tumour immune responses.

Introduction

Apoptotic cell death is associated with profound morphological changes including cell

contraction, blebbing, nuclear disintegration and apoptotic body release [2]. In addition,

apoptotic cells display a variety of "find me" and "eat me" signals that aid in the efficient

recognition and clearance of cell corpses by phagocytic cells including macrophages [3–

5]. These events reduce the risk of cellular contents being released by dying cells in order

to limit the induction of acute inflammatory and adaptive immune responses over time [6].

In the absence of efficient clearance of apoptotic cells, immunogenic cell death (ICD) may

result in which the release of damage-associated molecular patterns (DAMPs) and

induction of cytokines can promote the recruitment and activation of antigen-presenting

cells and subsequent activation of cytotoxic T-lymphocyte (CTL) mediated adaptive

immunity [7]. Although cancers typically employ several strategies to evade detection

and/or destruction by the immune system, inducing tumour cell ICD can improve the

efficacy of cancer therapeutic agents by promoting anti-tumour immunity [8,9]. Some anti-

cancer agents act both by blocking essential targets in tumour cells and by inducing ICD

[10]. In addition, the physical interventions of irradiation, photodynamic therapy and high

hydrostatic pressure have all been shown to induce cancer cell ICD by promoting the

release of DAMPs and the induction of pro-inflammatory cytokines [11]. Therefore,

therapeutic agents that directly promote ICD could have beneficial effects for cancer

treatment.

The biochemical processes induced by pro-apoptotic stimuli culminate in the

activation of executioner caspases that cleave substrate proteins at specific target

sequences [12]. We and others discovered that caspase cleavage of the ROCK1 protein

kinase at a highly conserved DETD sequence resulted in the generation of a

constitutively-active fragment [13,14]. Caspase-mediated ROCK1 activation led to

increased myosin light chain (MLC) phosphorylation and actin-myosin contraction that

were necessary for rapid cell contraction, membrane blebbing and nuclear fragmentation

during apoptosis [1,13,15]. Pharmacological inhibition of ROCK activity in apoptotic cells

was sufficient to block increased MLC phosphorylation, membrane blebbing and nuclear

disruption without affecting biochemical aspects of apoptotic cell death including caspase

activation, DNA fragmentation, cytochrome C release and phosphatidylserine

externalization [13,15,16]. A genetically modified mouse model in which a single amino

acid change in the DETD caspase cleavage site that rendered ROCK1 insensitive to

caspase cleavage revealed the obligatory role of ROCK1 cleavage and activation for MLC

phosphorylation, the generation of cellular contractile force and the morphological

aspects of apoptotic cell death [1]. Treatment of mice expressing the non-cleavable

ROCK1 (ROCK1nc) with the liver selective hepatotoxin diethylnitrosamine (DEN) [17] that

undergoes CYP2E1-mediated oxidation to become an active genotoxin [18] resulted in

greater acute liver damage and neutrophil recruitment relative to mice expressing wild-

type ROCK1 (ROCK1wt) [1]. Apoptotic ROCK1nc cells released more of the DAMP

protein high mobility group B1 (HMGB1) than ROCK1wt cells, and DEN-induced acute

liver damage and neutrophil recruitment could be significantly reduced by inhibiting

HMGB1 or its cognate pattern recognition receptor (PRR) toll-like receptor 4 (TLR4) [1].

Taken together, these findings indicated that the inability of cells to undergo typical

apoptotic morphological changes due to the absence of caspase-mediated ROCK1

activation induced a form of ICD.

DEN induction of hepatocyte apoptosis and tissue damage can progress to

hepatocellular carcinoma (HCC) over time [17]. While both ROCK genotypes developed

tumours 6 or 9 months after DEN treatment, there were fewer HCC tumours in ROCK1nc

mice at both time points [1]. These observations suggested that the ROCK1nc mutation

which affected apoptotic cell morphologies and resulted in acute hepatocyte ICD following

DEN-treatment could also have a tumour-suppressive effect. Furthermore, these results

raised the possibility that pharmacological ROCK inhibition could also have similar tumour

suppressive effects by inducing ICD.

In this study, we observed that the ROCK1nc mutation altered the typical

morphological changes of apoptotic hepatocytes *in vitro*, and resulted in greater induction

of pro-inflammatory cytokines in ROCK1nc mice relative to ROCK1wt mice following

acute DEN treatment, consistent with the absence of ROCK1 cleavage leading to ICD.

DEN-induced HCC tumours in ROCK1nc mice displayed higher grade steatosis, which

was accompanied by significantly more neutrophils and CD8$^+$ T cells relative to ROCK1wt

mice, while levels of Foxp3$^+$ regulatory T cells (Tregs) were not different. Taken together,

these results indicated that the ROCK1nc mutation induced immune responses that have

been associated with tumour suppression and improved patient outcomes [8]. To

determine if pharmacological ROCK inhibition could phenocopy the effect of the

ROCK1nc mutation, wild-type ROCK1 mice were treated with the ROCK inhibitors fasudil

[19] or AT13148 [20–22] following DEN-initiation of HCC. Although both ROCK inhibitors

significantly reduced HCC tumour numbers and areas, neither increased neutrophil or

CD8^{+} T cell recruitment relative to vehicle controls. By examining the effect of AT13148

on acute DEN-induced liver damage, it was observed that the numbers of circulating

platelets and lymphocytes were decreased, as were the proportion of dendritic, natural

killer and CD8^{+} T cells recruited to damaged livers. Taken together, these observations

indicate that the caspase-cleavage of ROCK1 is required to limit innate and adaptive

immune responses to acute DEN-induced liver damage and spontaneous apoptosis in

HCC tumours. However, potential therapeutic benefits from the tumour-suppressive

actions of the ROCK1nc mutation could not be recapitulated by systemic pharmacological

ROCK inhibition. As a result, it is unlikely that the addition of ROCK inhibitors as a cancer

therapy adjuvant would improve therapeutic efficacy by inducing ICD and promoting anti-

cancer immune responses. Nevertheless, ROCK inhibitor monotherapy did have a

significant effect on reducing HCC tumour growth, suggesting that there may be clinical

utility for ROCK inhibitors in the treatment of liver cancer, similar to the positive effects

observed in pre-clinical studies for other cancer types including pancreatic ductal

adenocarcinoma [22–24] and squamous cell carcinoma [25].

Results

Caspase cleavage of ROCK1 affects hepatocyte morphology, liver gene

expression and cytokine release following genotoxin treatment

The apoptotic death of mouse embryo fibroblasts (MEFs) homozygous for the ROCK1nc

genetic modification resulted in less contractile force generation relative to wild-type

MEFs, leading to fewer apoptotic blebs [1]. To determine if there were similar effects on

the apoptotic death of hepatocytes, primary hepatocytes from ROCK1wt and ROCK1nc

mice were isolated, treated with the apoptotic stimulus of the Bcl2 selective BH3 mimetic

ABT-199 (1 μM), then imaged using bright field microscopy at 3-minute time intervals.

While ROCK1wt hepatocytes underwent rapid and forceful contraction that produced

many blebs (Figure 1A, left panels; Video 1), apoptotic ROCK1nc hepatocytes were less

compact, had more residual contraction fibres and fewer apoptotic blebs (Figure 1A, right

panels; Video 2). Scanning electron microscopy revealed that the cell bodies of

ROCK1WT hepatocytes were studded with large blebs, while ROCK1nc were essentially

devoid of blebs and had large patches of membrane adjacent to the cell (Figure 1B).

Therefore, the ROCK1nc mutation that renders ROCK1 resistant to caspase mediated

proteolysis and activation similarly affected the apoptotic morphological features of

contraction and blebbing for hepatocytes and fibroblasts [1].

The liver-selective pro-hepatotoxin diethylnitrosamine (DEN) [17] can be

metabolized by CYP2E1 [18] to become genotoxic [26], resulting in hepatocyte apoptosis

and tissue damage that can progress to cirrhosis and hepatocellular carcinoma (HCC)

[17]. The toxic effects of DEN occur with high penetrance in male mice, with female mice

being relatively insensitive [17]. After intraperitoneal (IP) injection of 10 week old male

mice with 100 mg/kg DEN, haematoxylin and eosin (H&E) staining of livers isolated 72

hours later revealed markedly greater levels of steatosis in ROCK1nc mice compared to

livers from ROCK1wt mice (Figure 2A), consistent with previous observations [1]. RNA

was isolated from 4 untreated ROCK1wt and ROCK1nc livers, and from 4 ROCK1wt and

ROCK1nc livers 72 hours after DEN treatment, and polyA$^+$-enriched RNA was

sequenced. There were 2515 genes that were significantly altered in expression by at

least 1.5 fold (adjusted p < 0.05) between untreated and DEN treated ROCK1wt livers

(Figure 2–figure supplement 1A), and 1255 genes that were similarly altered in

expression between untreated and DEN treated ROCK1nc livers (Figure 2–figure

supplement 1B), of which 740 were in common (Figure 2B). The direction of change was

consistent for 96.5% of the 740 common genes (Figure 2–figure supplement 1C),

although the magnitude of positive and negative gene expression changes was

significantly greater for ROCK1wt than for ROCK1nc livers (Figure 2–figure supplement

1D). Gene set enrichment analysis revealed that there were four Reactome gene sets

(out of 1604 total gene sets) [27,28] that were significantly (nominal p < 0.05) altered in

both ROCK1wt and ROCK1nc livers (Figure 2C). In addition to the top hit "Transcriptional

Regulation by tp53" gene set that likely reflects the DNA damaging actions of DEN [29],

the next three gene sets were all related to immune signalling pathways. To determine if

there were differences in cytokine induction between ROCK1wt and ROCK1nc mice

following DEN treatment, the levels of 14 cytokines/chemokines were measured by

cytokine arrays. By comparing the fold-change induction of each cytokine/chemokine for

vehicle versus DEN treatment for each genotype, there was significantly greater induction

in ROCK1nc mice of interleukins (IL) IL-6, IL-1β, IL-10, tumour necrosis factor α (TNFα),

IL-12-p40, IL-4, IL-2, IL-1α, and C-C motif chemokine ligand 2 (CCL2) (Figure 2D). The

induction of IL-5, CCL3, IL12-p70, C-X-C motif chemokine ligand 9 (CXCL9) and

interferon γ (IFNγ) was not different between ROCK1wt and ROCK1nc mice, and no

cytokines/chemokines were induced to a significantly greater extent in ROCKwt mice

(Figure 2D). Taken together, these observations indicate preventing caspase-mediated

activation of ROCK1 leads to aberrant morphological changes during apoptotic

hepatocyte death and greater DEN-induced steatosis and inflammation.

Mutation of ROCK1 caspase cleavage site increases neutrophil, and CD3^{+} and CD8^{+}

T cell recruitment to hepatocellular carcinoma tumours.

Treatment of 14 day old male mice with 400 μg DEN by IP injection results in the

development of HCC with high penetrance [1,17], with evidence of spontaneous

apoptosis in tumours [30]. ROCK1nc mice were previously shown to develop fewer HCC

tumours than ROCK1wt mice, suggesting that tumour development was affected by the

alterations in apoptotic cell morphologies due to absence of caspase-mediated ROCK1

activation [1]. Similar to there being more steatosis in ROCK1nc livers following acute

DEN-treatment [1], there was a significantly ($p < 0.001$, Chi-square test) higher proportion

of high grade (≥ 3) steatotic HCC tumours in ROCK1nc livers relative to ROCK1wt livers

(Figure 3A). In addition, steatosis was detectable in liver tumours of 12/12 ROCKnc mice

and only 10/12 ROCKwt mice. Despite the greater steatosis in ROCK1nc tumours, there

were no significant difference in biochemical markers of liver function and health between

ROCK1wt and ROCK1nc mice (Figure 3A–figure supplement 1A-H).

Immunohistochemical (IHC) analysis revealed that there were significantly more S100A9[+]

neutrophils in ROCK1nc tumours relative to ROCK1wt tumours (Figure 3B), and no

difference in F4/80[+] macrophages between the genotypes (Figure 3C), comparable to the

observations for acute DEN treatment [1]. Since previous studies have reported that low

ratios of Foxp3^{+} regulatory T cells (Tregs) to either CD3^{+} or CD8^{+} T cells were correlated

with better prognosis for patients with HCC [31–34], the levels of each cell types was

assessed in ROCK1wt and ROCK1nc HCC tumours. Both CD3^{+} (Figure 4A) and CD8^{+} T

cells (Figure 4B) were detected at significantly higher levels in ROCK1nc liver tumours

than in ROCK1wt tumours, while there was no difference in Foxp3$^+$ Treg numbers (Figure

4C). These results indicate that the enhanced pro-inflammatory cytokine responses

observed in ROCK1nc mouse livers after acute DEN-treatment (Figure 2D) are paralleled

by greater CD8$^+$ T cell responses in HCC tumours (Figures 3-4).

Pharmacological ROCK inhibition reduces DEN-induced liver tumour numbers

without increased immune cell recruitment.

A proposed cancer treatment strategy is to enable the immune system to more effectively

target cancers by increasing the immunogenicity of tumour cells by transforming

spontaneous or therapy-induced cell death from immunologically-silent apoptosis to a

more inflammatory necrotic-like form [6,35–37]. Results from the ROCK1nc mouse model

indicate that blocking caspase-induced ROCK1 activation leads to increased acute

inflammation (Figure 2D) that results in greater innate and adaptive immune responses

(Figures 3-4), which were correlated with reduced tumour numbers [1]. These

observations suggest that pharmacological ROCK inhibition could have a similar effect

as the ROCK1nc mutation in promoting tumour suppressive immune responses, which

could result in a novel clinical use for ROCK inhibitors as pro-immunogenic agents for

cancer therapy. To test this possibility, 14 day old male ROCK1wt mice were IP injected

with 400 μg DEN, and then at 10 weeks mice were orally administered either with water

vehicle or 2 mg of the ROCK inhibitor fasudil [19] twice daily until mice reached 41 weeks

(Figure 5A). Fasudil treatment had no significant effects on whole body, liver, lung or

spleen weights (Figure 5–figure supplement 1A-G). In addition, fasudil treatment had no

significant effects on blood haemoglobin levels or the numbers of circulating platelets,

white cells, lymphocytes or monocytes, but did result in a reduction in neutrophil numbers

(Figure 5–figure supplement 2A-F), and no effect on biochemical markers of liver health

and function except for decreased serum levels of aspartate aminotransferase (Figure 5–

figure supplement 2G-I). While liver tumours were observed in all mice for both treatment

groups (Figure 5B), tumour numbers (Figure 5C) and total tumour areas (Figure 5D) were

significantly lower in the fasudil treated mice. IHC analysis revealed no significant

differences in S100A9$^+$ neutrophils (Figure 5E) in liver tumours. A trend towards fewer

CD8$^+$ T cells in the livers of fasudil treated mice (Figure 5F) did not reach statistical

significance. To corroborate these observations, a comparable experiment substituted

the structurally unrelated ROCK inhibitor AT13148 [20–22] for fasudil. ROCK1wt mice

that had been injected IP at 14 days with 400 µg DEN were then orally administered three

times per week with either vehicle (10% DMSO, 1% Tween 20 in water) or 40 mg/kg

AT13148 until mice reached 41 weeks (Figure 6A). AT13148 treatment had no significant

effects on whole body, liver, lung or spleen weights (Figure 6–figure supplement 1A-G).

In addition, AT13148 had no significant effect on blood haemoglobin levels or the

numbers of circulating platelets, lymphocytes, monocytes or neutrophils, although white

cell numbers were significantly decreased (Figure 6–figure supplement 2A-F). AT13148

had no significant effects on biochemical markers of liver health and function (Figure 6–

figure supplement 2G-L). All livers from both treatment groups had tumours (Figure 6B),

although there were fewer tumours (Figure 6C) and smaller tumour areas (Figure 6D) for

the AT13148 treated mice. IHC analysis showed that there were no significant differences

in neutrophil numbers between the treatment groups (Figure 6E), but AT13148 treatment

resulted in fewer CD8$^+$ T cells in liver tumours (Figure 6F). Although both ROCK inhibitors

fasudil and AT13148 had similar anti-tumour effects (Figures 5-6), neither treatment

increased neutrophil or CD8$^+$ T cell recruitment to tumours in contrast to the observations

in ROCK1nc mice (Figures 3-4).

To characterize the effect of ROCK inhibition on immune cell recruitment to the

liver in response to acute DEN treatment, 10 week old male ROCK1wt mice were orally

administered three times per week with either vehicle or 40 mg/kg AT13148 for 10 days,

with all mice receiving IP injections of 100 µg DEN on the 7th day. The addition of AT13148

with DEN did not result in significant differences in whole body or liver weight, or in blood

haemoglobin levels or numbers of circulating white cells, monocytes or neutrophils

relative to DEN treatment alone (Figure 7–figure supplement 1A-G). However, AT13148

did significantly reduce the number of circulating platelets (Figure 7A) and lymphocytes

(Figure 7B). Analysis of immune cell numbers in dissociated livers by flow cytometry

revealed that AT13148 significantly reduced the number of dendritic cells (Figure 7C), NK

cells (Figure 7D), CD8$^+$ T cells (Figure 7E) and Kupffer cells (Figure 7G) relative to vehicle

treated mice. The numbers of recruited macrophages, neutrophils, plasmacytoid dendritic

cells, CD4$^+$ T cells, Tregs, γδ T cells and NKT cells in DEN treated livers were not different

between vehicle and AT13148 treated mice (Figure 7-figure supplement 2A-H). These

findings indicate that the ROCK inhibitor AT13148 had an overall negative effect on

immune cell recruitment following acute DEN treatment.

Discussion

One of the hallmarks of cancer is the ability to evade destruction by the immune system [38]. Immunoevasion is accomplished by a variety of means, but the strategy of reactivating the immune system as an cancer therapeutic strategy has largely focussed on the blocking the checkpoint inhibitor PD-1/PD-L1 and CTLA-4 pathways to enable tumour-reactive T cells to muster effective antitumor responses [39]. An additional strategy that is still in the process of becoming widely adopted is to intentionally induce ICD to stimulate the immune system in concert with conventional targeted therapies [9]. The effect of ICD is to promote the action of dying cells as immunological adjuvants through the release of DAMPs that promote the recruitment and activation of antigen-presenting cells, ultimately resulting in activation of cytotoxic T lymphocyte-mediated adaptive immunity and the establishment of immunological memory of tumour antigens and neoantigens [7]. Cancer therapies are typically developed through an iterative process that includes testing on established tumour cell lines *in vitro* and xenograft tumours grown in immunocompromised mice to examine their anti-cancer effects, which would not necessarily include assessments of their potential immunostimulatory or immunosuppressive effects [40]. Nevertheless, some commonly used cancer chemotherapies do in fact induce ICD including bleomycin, bortezomib, doxorubicin, and oxaliplatin, although whether immunostimulation is a meaningful component of their therapeutic mechanisms of action is not clear [40].

ROCK1nc mice express a mutated form of ROCK1 in which the caspase cleavage site DETD (amino acids 1110-1113) was changed to DETA, making the protein resistant to caspase cleavage [1,13]. The apoptotic death of ROCK1nc cells is characterized by

lower MLC phosphorylation, reduced cellular contractile force, altered morphologies and

greater HMGB1 release relative to cells expressing wild-type ROCK1 [1]. In ROCK1nc

mice, treatment with the hepatoxin DEN resulted in greater liver damage and neutrophil

recruitment than in ROCK1wt mice, both of which were reduced through inhibition of

HMGB1 or its receptor TLR4 [1]. In this study, acute DEN-induced liver damage was

associated with greater induction of pro-inflammatory cytokines in ROCK1nc mice

compared to ROCK1wt mice (Figure 2D). Taken together, these results are consistent

with cell death in ROCK1nc cells being immunogenic. In the long term, DEN-induced

fewer HCC tumours in ROCK1nc mice [1], with more high grade steatosis (Figure 3A)

and more neutrophil (Figure 3B) and CD8$^+$ T cells (Figure 4B) in ROCK1nc tumours

relative to ROCK1wt tumours. Levels of F4/80$^+$ macrophages (Figure 3C) and Foxp3$^+$

Treg cells (Figure 4C) were not significantly different between the genotypes. Clinical

studies in human HCC patients have previously determined that high CD8$^+$ T cells and/or

high CD8$^+$/Foxp3$^+$ ratios are associated with increased overall and disease-free survival

of HCC patients [32–34], suggesting that ICD in ROCK1nc tumours which resulted in high

CD8$^+$ T cell numbers and CD8$^+$/Foxp3$^+$ ratios induced tumour-suppressive immunity.

From a clinical perspective, the obvious question is whether pharmacological inhibition of

ROCK activity would provoke similar phenotypes.

Two structurally unrelated ROCK inhibitors, fasudil and AT13148, were selected

due to their previous efficacy in mouse tumour studies [22,24] and because both have

been shown to be safe for administration to human patients. Fasudil has been approved

and safely used in Japan and China for many years for the treatment of cerebral

vasospasm associated with subarachnoid hemorrhage [41], and there have been

additional proposed applications of fasudil as a therapy for neurodegenerative diseases

such as Amyotrophic Lateral Sclerosis [42,43] and cardiovascular diseases [44].

Development of AT13148 reached Phase I testing in patients with solid tumours [21].

Both fasudil and AT13148 reduced HCC tumour numbers and areas (Figures 5C-D, 6C-

D) indicating beneficial therapeutic actions, but neither led to increased numbers of

neutrophils or CD8^{+} T cells in tumours relative to vehicle controls (Figures 5E-F, 6E-F).

In fact, AT13148 significantly reduced CD8^{+} T cells in HCC tumours (Figure 6F), and

fasudil treatment resulted in a similar trend (Figure 5F). While the ROCK1nc mutation

only inhibits activation by caspase cleavage in apoptotic cells, pharmacological inhibitors

block activation resulting from caspase cleavage and RhoA binding, as well as reducing

basal kinase activity, of both ROCK1 and ROCK2 in all cells in the body [1,20,45]. The

actions of ROCK inhibitors that exceed the effect of the ROCK1nc mutation on kinase

activity, combined with the widespread targeting of apoptotic and non-apoptotic cells,

likely accounts for the differences observed for neutrophil and CD8^{+} T cells in HCC

tumours between the genetic ROCK1nc modification and pharmacological ROCK

inhibition. Given the significant inhibitory effect of AT13148 on CD8^{+} T cell numbers in

HCC tumours, the effects on immune cell recruitment in response to acute DEN damage

was examined. There were significantly fewer circulating platelets and lymphocytes in

AT13148 treated mice relative to vehicle controls (Figure 7A-B), while the numbers of

dendritic, NK and CD8^{+} T cells recruited to ROCK1wt livers in response to DEN treatment

were reduced (Figures 7C-E). These observations are consistent with the reduction in

CD8^{+} T cells found in HCC tumours in AT13148 treated mice (Figure 6F).

Apoptotic ROCK1nc cells released more of the DAMP protein HMGB1 and lactate

dehydrogenase (LDH), a cytosolic protein often used as a surrogate marker of cell death

[1], indicating that their cell death was more necrotic-like than the typical apoptotic death

of ROCK1wt cells. AT13148 development advanced to phase 1 clinical trials in patients

with solid tumours [21]. We previously showed that pharmacological ROCK inhibition

during early apoptosis reduced the release of LDH and the DAMP proteins Histones H1.2,

H2A, H2B, H3 and H4, S100-A4 and S100-A11, and heat shock proteins HSPA4, HSPA5,

HSP8, HSP90AA1, HSP90B1 and HSP90B2 relative to control apoptotic cells [16]. The

reduced release of cellular contents in ROCK inhibitor treated cells during early apoptosis

was attributed to decreased production of apoptotic bodies, which do not consistently re-

form impermeable membranes after detaching from apoptotic cell bodies, thus allowing

for leakage of their contents. As a result, a potential explanation for the differences in

neutrophil and CD8$^+$ T cell numbers in HCC tumours could be due to there being more

DAMP release in ROCK1nc mice than in ROCK inhibitor treated mice. An additional

possibility is that due to the central role of ROCK signalling in cell motility [46],

pharmacological ROCK inhibition impaired the ability of neutrophils and CD8$^+$ T cells to

migrate towards attractant stimuli.

Although the physical disruption of cells by irradiation, photodynamic therapy and

high hydrostatic pressure have been shown to induce ICD and could be used in

combination therapies to stimulate anti-tumour immune responses, altering the

morphological changes associated with apoptotic cell death by pharmacologically

blocking ROCK activity is not likely to be therapeutically beneficial. The ability of ROCK

inhibitors to reduce HCC tumour numbers and areas (Figures 5C-D, 6C-D) may be related

to their effects on blocking invasive tumour growth [22,24]. When the ability of ROCK

inhibitors to improve the efficacy of therapeutic agents by increasing tumour penetration

[23,47] is also considered along with their anti-metastatic properties [48], then there may

still be clinical benefits from the inclusion of ROCK inhibitors in HCC combination

therapies.

Materials and Methods

Mouse Model

ROCK1nc mice were generated at the Cancer Research UK Beatson Institute, and were

bred and maintained as described in [1]. All mouse experiments were approved by the

University of Glasgow College of Medicine, Veterinary and Life Sciences Research Ethics

Committee, and performed under a project license granted by the United Kingdom

government Home Office, in line with the Animals (Scientific Procedures) Act 1986 and

European Union Directive 2010/63/EU in a dedicated barriered facility. ROCK1wt and

ROCK1nc were backcrossed for more than 10 generations into the C57Bl/6J strain. Mice

were assigned to treatment groups by researchers who were not blinded with respect to

genotypes. For routine genotyping, all animals were ear notched at weaning and samples

sent to Transnetyx (Cordova, TN, US) genotyping service for analysis.

Isolation of Hepatocytes

Hepatocytes were isolated from male and female ROCK1nc and ROCK1wt mice by a

collagenase digestion and Percoll gradient method. Isolated cells were seeded on

collagen-coated plates. For coating plates with collagen (Corning, 354249), 50 mg/ml

collagen was prepared in water containing glacial acetic acid (6 µl in 5 ml water). Plates

were coated for 1 hour after which excess liquid was aspirated and plates left to dry in a

fume hood.

For hepatocyte isolation, mice were anesthetized before opening the thoracic and

peritoneal cavities. Livers were perfused with Liver Perfusion Media (Gibco,17701038) at

37°C via the right atrium and out through the portal vein. Once the liver became pale,

Perfusion Media was switched to Liver Digest Media (Gibco, 17703034). Digested livers

were excised, gall bladders removed, and then livers were transferred to dishes

containing Liver Perfusion Media. Livers were gently teased with forceps to release cells

into solution. Cell suspensions were then filtered through 70 µm cell strainers to remove

undigested tissue and connective tissue. Cells were centrifuged at 135 x g for 1 minute

and resuspended in 6 ml of hepatocyte media (William's E media (Gibco, 22551-022) with

3% fetal bovine serum (Gibco, 10270), 100 U/ml penicillin with 100 µg/ml streptomycin

(Gibco, 15140-122) and 2 mM L-glutamine (Gibco, 25030-032).

Hepatocytes were separated using a three-layer discontinuous Percoll (Sigma,

P1644) gradient. The Percoll density solutions used were; 1.12 g/ml, 1.08 g/ml and 1.06

g/ml. To make the 1.12 g/ml solution, 2.63 ml Percoll, 0.3 ml phosphate buffered saline

(PBS; 170 mM NaCl, 3.3 mM KCl, 1.8 mM Na_2HPO_4, 10.6 mM H_2PO_4) and 64.6 µl water

were mixed; for the 1.08 g/ml solution, 2.86 ml Percoll, 0.5 ml PBS and 1.63 ml water

were mixed; and for the 1.06 g/ml solution, 2.085 ml Percoll, 0.5 ml PBS and 2.42 ml

water were mixed. Percoll gradients were generated by layering 3 ml of the 1.12 g/ml

solution at the bottom over which 5 ml of the 1.08 g/ml solution was carefully layered

followed by 5 ml of the 1.06 g/ml solution. Over this, 6 ml of cell suspension was carefully

layered while avoiding gradient disturbance. The cell suspension was fractionated by

centrifugation without brakes at 750 x g for 20 minutes. After centrifugation, the layer of

cells between 1.06 g/ml and 1.08 g/ml that contained live hepatocytes was collected and

added to 10 ml of hepatocyte media. Cell suspensions were centrifuged at 135 x g for 1

minute. Cell pellets were then resuspended in hepatocyte media and seeded on collagen

coated plates for analysis.

Time-lapse Microscopy of Hepatocytes

ROCK1wt and ROCK1nc primary hepatocytes were plated on 6-well glass bottom dishes

pre-coated with collagen. Cells were serum-starved overnight prior to induction of

apoptosis with 1 µM ABT-199 (Selleckchem, S8048) diluted in serum-free media. Bright

field time-lapse microscopy images were acquired with a 10x objective using a Nikon TE

2000 microscope with a heated stage and 5% CO_2 gas line. Immediately after induction

of apoptosis, dishes were transferred to the microscope, 3-4 random positions were

chosen from each well and time lapse images were taken every 3 minutes for 18 hours.

Scanning Electron Microscopy (SEM)

Hepatocytes were cultured on collagen coated 10 mm glass cover slips in 24 well plates.

Apoptosis was induced using UVC radiation (254 nm wavelength at 0.3 J/cm^2) and cells

were processed for SEM at 24 hours post apoptosis induction. Cover slips were washed

twice in filtered PBS then fixed in 1.5% glutaraldehyde (Sigma, G5882) in 0.1 M sodium

cacodylate for 1 hour at room temperature. Samples were then washed twice in 0.1 M

sodium cacodylate and stored in 0.1 M sodium cacodylate. Cells were washed with

phosphate buffer (3 washes for 10 minutes), post-fixed in 1% Osmium Tetroxide/0.1M

Sodium Cacodylate buffer for 1 hour then washed for 3 x 10 minutes in distilled water.

Dehydration steps were through a graded ethanol series (30%, 50%, 70%, 90%) for 10

mins each, then 100% ethanol 4 times for 5 mins each. Samples were dried with

hexamethyldisilazane (Sigma, H4875) before coating with gold/palladium, and were

imaged on a JEOL 6400 Scanning Electron Microscope running at 10Kv. TIFF images

were captured using Olympus Scandium software at the Electron Microscopy Facility at

the University of Glasgow.

Preparation and administration of substances

To prepare diethylnitrosamine (DEN; Sigma, N0258), 100 ml of sterile PBS was injected

into the bottle containing 1 g of DEN to give a final concentration of 10 mg/ml. The

procedure was performed in the fume hood. For short-term studies assessing DEN-

induced hepatic injury, mice were treated with a single dose of DEN at 100 mg/kg body

weight by intraperitoneal injection.

To prepare fasudil, 1 g of fasudil (LC Laboratories, F-4660) was dissolved in 50 ml

distilled water giving a final concentration of 20 mg/ml. Fasudil was then aliquoted and

stored at -20°C until required for dosing. Mice were dosed with 100 μl (2 mg) fasudil twice

daily for the duration of the study.

To prepare AT13148 (Selleckchem, S7563), compound was dissolved in 89%

H_2O/10% (v/v) DMSO (Sigma, D2650)/1% (v/v) Tween 20 (Sigma, P1379) at a

concentration of 8 mg/ml. The dissolved drug was kept at 4°C for a maximum of 2 weeks.

Mice were dosed three times per week (Monday, Wednesday, Friday) for the duration of

studies performed. Mice were dosed initially with 30 mg/kg for the first two weeks, then

40 mg/kg thereafter.

Tissue collection and fixation

Animals were euthanized by carbon dioxide inhalation, weights recorded, and blood

collected for haematology or biochemistry analysis. Necropsy was performed

immediately to prevent tissue autolysis. Tissues collected were fixed for at least 24 hours

by immersing in 10% neutral buffered formalin. For acute liver studies, the left lobe of the

liver was routinely used for histological analysis and fixed in 10% neutral buffered

formalin. Small pieces from the median lobe were frozen using dry ice or collected in

RNALater for RNA analysis. The caudate lobe was frozen using dry ice for making

cryosections.

For DEN-induced hepatocellular carcinoma studies, mice were examined for

visible lesions in all organs, liver weights were recorded and the number of tumours

counted. Tumour foci were measured using digitised vernier callipers. Steatosis was

graded on H&E-stained sections based on semi-quantitative estimation of percentage of

steatotic hepatocytes, according to the following criteria: grade 0, less than 5%; grade 1,

5 to 25%; grade 2, 26 to 50%; grade 3, 51 to 75%; grade 4, more than 75%. Lungs were

also collected to analyse metastatic lesions. Tissue were either fixed in 10% neutral

buffered formalin for histological analysis or maintained in PBS until analysis. Fixed

tissues were then processed, paraffin embedded and sectioned by the Cancer Research

UK Beatson Institute Histology Service.

Histology

Paraffin-embedded tissue sections of 4 µm thickness were cut and routinely stained with

haematoxylin and eosin (H&E) by the Beatson Histology Service. Blank paraffin sections

were stored at 4° C until ready for use. Frozen liver tissue was embedded in OCT and cut

at 12 µm thickness and mounted on super-frost slides. Cryosections were stored at -80°C

until ready to be used.

RNA isolation and sequencing

RNA was isolated using the Qiagen RNasy Mini Kit according to the

manufacturer's instruction. RNA concentration and purity was determined using

NanoDrop 2000 spectrophotometer. RNA quality was confirmed using the Agilent RNA

Screen Tape assay and the Aligent 2200 TapeStation. Total RNA was used to generate

an oligo dT-enriched library with the Illumina TruSeq RNA Library Preparation kit v2.0.

Quality and quantity of the DNA library was assessed using an Agilent 2100 Bioanalyzer

and a Qubit (Thermo Fisher Scientific), respectively. The library was run on the Illumina

NextSeq 500 platform using the High Output 75 cycles kit (2 x 36 cycles, paired-end

reads, single index). RNAseq data were analysed by the Computational Biology

department at the CRUK Beatson Institute. Gene set enrichment analysis (GSEA) was

run using GSEA v4.1.0 for Windows [49].

Quality control checks of raw RNA-Seq data files were done with fastqc

(http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and fastq_screen

(http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/). RNA-Seq reads

were aligned to the mouse genome (GRCm38.75) using TopHat2 [50] and BAM files were

further processed with htseq_count

(http://www.huber.embl.de/users/anders/HTSeq/doc/count.html). Differential analysis of

count data was performed by the DESeq2 package [51].

Multiplex Cytokine Analysis

MILLIPLEX Multiplex Assays Using Luminex (Millipore, MCYTOMAG-70K) were used to

measure the levels of 15 cytokines (INF-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10,

IL12p40, IL12p70, IL-17, CCL2, CCL3, CXCL9, TNFα). Serum levels of these cytokines

were analysed using the Mouse Cytokine/Chemokine Magnetic Bead Panel Kit following

manufacturer's instructions. Briefly, 200 µl of wash buffer was added to each well of 96-

well plates, which were sealed and mixed on a plate shaker for 10 minutes at room

temperature after which the wash buffer was decanted. Inverting the plates and tapping

them onto absorbent towels removed residual wash buffer. 25 µl of standards or controls

were added to the appropriate wells and 25 µl of assay buffer was added to the sample

wells. 25 µl of serum matrix was added to the background, control and standard wells.

25 µl of serum samples diluted 1:2 in assay buffer was added to the sample wells. All

serum samples were measured in duplicate. 25 µl of premixed magnetic beads

conjugated to antibodies for all 15 analytes were added to each well. Plates were sealed,

protected from light and incubated overnight at 4° C on a plate shaker. This was followed

by a wash step (two times with 200 µl of wash buffer) on a magnetic plate washer. 25 µl

of detection antibodies was added to each well, and plates were sealed and incubated

with agitation for 1 hour at room temperature. Streptavidin-phycoerythrin (25 µl) was

added to each well, and plates were sealed and incubated with agitation for another 30

minutes at room temperature, followed by a final wash step. Magnetic beads were then

re-suspended in sheath fluid and assayed on a Luminex 200 analyser using xPOTENT

software.

Immunohistochemistry

Immunohistochemistry was routinely performed on 4 µm thick formalin-fixed paraffin-

embedded (FFPE) sections. Sections were deparaffinised in xylene (3 washes of 5

minutes each) and rehydrated through decreasing concentrations of ethanol (2 washes

of 3 minutes each in 100% ethanol, 1 wash for 3 minutes in 70% ethanol). Slides were

then washed in distilled water (dH2O). To unmask antigens, slides were boiled at 98° C

in a water bath (WB) for the specified time in antigen retrieval buffer. The buffers used

varied with the antibody (Table 1). If Proteinase K (Dako, S2032) was used as the retrieval

agent, then the slides were incubated at room temperature for the specified time (Table

1). Slides were left to cool down to room temperature for at least 30 minutes and then

rinsed in distilled water. Endogenous peroxidase activity was quenched by incubating

sections in 3 % H_2O_2 (Fisher, 10687022) in PBS for 10 minutes. After slides were rinsed

once in distilled water and once in Tris-buffered saline + Tween 20 (TBST; 20 mM Tris-

HCL pH 7.5, 136 mM NaCl, 0.1% (v/v) Tween 20), sections were blocked using blocking

solution containing 5% serum (depending on the species in which the secondary antibody

was raised) in TBST for 30 minutes at room temperature. Primary antibody diluted in

blocking solution was added to the slides and kept in a humidified chamber for the

specified time. Primary antibody dilutions and incubation times are indicated in Table 1.

Slides were rinsed three times in TBST to remove excess/unbound primary antibody.

HRP-labelled secondary antibodies were added to the slides and incubated at room

temperature for 30 minutes at room temperature. For secondary antibody staining, either

ImmPRESS kits (anti-goat, MP-7405; anti-rat, MP-7444) or Envision+ Reagents (Dako:

rabbit, K4002) were used. Slides were rinsed again three times in TBST before staining

with 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Dako, K3467), as chromogen.

Sections were incubated with DAB solution until brown colour develops and the slides

were immediately transferred to TBST to stop the reaction.

Table 1. Antigen retrieval methods, primary antibodies with dilutions, and

incubation conditions for IHC. Citrate buffer (10 mM Sodium citrate, 0.05% (v/v) Tween

20). WB = water bath. RT = room temperature.

Antigen (clone)	Species	Supplier (Catalogue Number)	Antigen Retrieval Solution	Retrieval Method	Antibody Dilution; Time & Temperature
CD3 (SP7)	Rabbit	Vector (VP-RM01)	Citrate buffer pH 6	WB; 98°C; 25 min	1:75; 35 min, RT
F4/80 (Cl:A3-1)	Rat	Abcam (ab6640)	Proteinase K	10 min at RT	1:400; 35 min; RT
Foxp3 (FJK-16s)	Rat	eBioscience (14-5773-80)	Tris-EDTA buffer pH 9	WB; 98°C; 20 min	1:50; O/N; 4°C
S100A9 (M-19)	Goat	Santa Cruz (sc-8115)	Citrate buffer pH 6	WB; 98°C; 25 min	1:1000; 35 min; RT
CD8 (4SM15)	Rat	eBioscience (14-0808-82)	Tris-EDTA buffer pH 9	WB; 98°C; 20 min	1:500; 35 min; RT

For counterstaining, slides were rinsed in distilled water and then stained with

haematoxylin for 1 minute. After rinsing in distilled water, the slides were then transferred

to Scots tap water for 1 minute or until blue colour developed to the desired intensity.

Slides were rinsed in distilled water and the sections were dehydrated using increasing

concentrations of ethanol (1 wash for 3 minutes in 70% ethanol, 2 washes of 3 minutes

each in 100% ethanol). Slides were then cleared by three washes in xylene for 3-minutes

each, before mounting with DPX mountant (Sigma, 44581). In all staining experiments, omitting the primary antibody on one slide served as the negative control. Sections were analysed using bright field microscope (Olympus BX51) and representative images were obtained at different magnifications.

Immunofluorescence staining of frozen sections

Immunofluorescence staining of cryosections was performed on 12 μm thick cryosections. Tissue sections were fixed by immersing the slides in pre-cooled acetone (-20°C) for 10 minutes. Slides were removed from fixative and acetone was allowed to evaporate from the tissue sections for 20 minutes. After slides were rinsed in PBS, cryosections were blocked in blocking buffer (5% serum in PBS) for 1 hour at room temperature. Anti-CD8 primary antibody diluted in blocking buffer (1:100) was added to the slides and kept in a humidified chamber for 2 hours at room temperature. Slides were rinsed twice in PBS before adding Alexa Fluor 594 conjugated goat anti-rat secondary antibody (ThermoFisher Scientific, A48264) diluted 1:500 in blocking buffer and incubated for 1 hour at room temperature. Slides were then rinsed three times in PBS before mounting with Vectashield DAPI hard set (Vector, H-1500). Cryosections were analysed using fluorescence microscope (Olympus BX51) and images were obtained at 20x magnification.

Hepatocellular carcinoma induction

Male mice were injected intraperitoneally (IP) with DEN as a single 40 μl dose (400 μg, equates to ~10 μl/g) at 14 days old. Mice were checked at regular intervals for general health and signs related to hepatocellular carcinoma such as hepatomegaly (swollen

abdomen), pain (hunched, high gait), weight loss (pinched shoulders) or reduced mobility.

The experimental end point was defined as 41 weeks, or in the event of development of

clinical signs, at which point mice were humanely culled and samples collected. Mouse

data such as body weight and organ weight were recorded, liver tumours were counted

and measured using calipers, and lungs and spleen were assessed for metastases. Blood

samples were additionally taken for analysis of haematological parameters and liver

function tests. For FFPE samples, tissues were collected and placed directly into 10%

neutral buffered formalin solution. Samples were placed on a rocker for 24 hours then

moved to a 70% ethanol solution until paraffin embedding. Tumour areas were calculated

using the following formula; Area = π x (Length/2) x (Width/2)

Haematology

Mice were euthanized by carbon dioxide inhalation and blood samples were collected via

cardiac puncture in potassium-EDTA tubes. Haematology analysis was performed by the

Clinical Pathology Lab at the Veterinary School (University of Glasgow).

Liver Function

Mice were euthanized by carbon dioxide inhalation and blood samples were collected via

cardiac puncture in 1.5 ml microcentrifuge tubes. Blood was allowed to clot at room

temperature for at least 30 minutes and then centrifuged at 1500 x g for 15 min at 4° C.

The resulting supernatant (serum) was transferred to fresh tubes for analysis. If the serum

samples were not analysed immediately, they were aliquoted and stored at -20° C until

ready for analysis. Liver function tests were performed by the Clinical Pathology Lab at

the Veterinary School (University of Glasgow), where serum concentrations of alanine

transaminase, total bilirubin, alkaline phosphatase, aspartate aminotransferase, total

protein, albumin and globulin were determined using an automated analyser.

Flow Cytometry

Cell suspensions were generated from mouse livers by first perfusing livers with PBS,

then dissecting them and placing into ice cold PBS. The liver tissues were dissociated

using scissors and incubated in a solution of 5 ml PBS containing 1 mg/ml collagenase D

(Millipore, 11088858001), 0.5 mg/ml dispase (Millipore, D4818) and 0.1 mg/ml

collagenase P (Millipore, 11213857001) at 40°C for 30 minutes shaking at 750 rpm. The

cell suspensions were then diluted in 45 ml RPMI 1640 (Gibco, 31870-025) and passed

through a 70 μM filter twice. The suspensions were then spun at 300 x g for 5 minutes

and resuspended in red blood cell (RBC) lysis buffer (Biolegend, 420302) for 5 minutes.

Following termination or RBC lysis in PBS, the suspensions were then spun down and

resuspended in PBS.

2.5×10^6 cells were stained in a volume of 100 μl unless otherwise stated. Cell

suspensions were incubated in Zombie NIR live dead dye (Biolegend, 423106) at 1:250

in PBS at room temperature for 20 minutes. Suspensions were spun at 300 x g for 3

minutes and washed twice in PBS. Suspensions were then incubated in Fc block at 1:250

in FACS buffer (PBS with 2% (v/v) FBS, 0.5% (w/v) sodium azide, 0.2 mM EDTA) for 20

minutes at room temperature then washed twice in FACS buffer. Cells were stained with

antibodies for 30 minutes at room temperature (Table 2). Cells were then spun down and

washed twice in FACS buffer. Secondary staining containing secondary antibodies and/or

streptavidin fluorophore conjugate was then performed in FACS buffer at room

temperature for 30 minutes. Cells were again spun down and washed twice in FACS

buffer. Cells were then resuspended in FACS buffer and an equal volume 4%

paraformaldehyde/PBS (final concentration 2%) was added for fixation.

Samples were analysed and data collected using an Attune NxT acoustic focusing

flow cytometer. Post-acquisition analysis was performed using FlowJo v10 software.

Table 2. Liver flow cytometry staining panel

Lymphoid Panel				Myeloid Panel			
Antigen (clone)	**Supplier**	**Dilu-tion**	**Fluoro-phore**	**Antigen (clone)**	**Supplier**	**Dilu-tion**	**Fluoro-phore**
B220 (RA3-6B2)	Biolegend	1:200	PerCPCy5.5	CD11b (M1/70)	Biolegend	1:200	BV570
CD3 (17A2)	Biolegend	1:200	FITC	CD11c (N418)	Biolegend	1:200	BV711
CD4 (RN4-5)	eBio-science	1:200	SB600	CD45 (30-F11)	Biolegend	1:200	BV510
CD8 (53-6.7)	Biolegend	1:200	PECy7	CD115 (AFS98)	Biolegend	1:100	BV421
CD19 (6D5)	Biolegend	1:200	PE	Clec4f	Biolegend	1:100	Goat/AF594
CD25 (PC61)	Biolegend	1:100	AF700	F4/80 (BM8)	Biolegend	1:100	Biotin/PE
CD45 (30-F11)	Biolegend	1:200	BV510	Ly6C (HK1.4)	Biolegend	1:400	PECy7
FoxP3 (MF14)	Biolegend	1:100	AF647	Ly6G (1A8)	Biolegend	1:200	AF700
NK1.1 (PK136)	Biolegend	1:200	BV711	MHCII (M5/114.15.2)	Biolegend	1:500	FITC
TCRβ (A57-597)	Biolegend	1:100	BV421	PDCA1 (927)	Biolegend	1:100	PerCPCy5.5
TCRγδ (GL3)	Biolegend	1:100	Biotin/AF594	Siglec H (551)	Biolegend	1:100	APC

633 *Statistical Analysis*

634 Graphpad Prism 9 software was used for statistical analysis of data and generation of

635 graphs. The specific test used for analysis is stated in each figure legend.

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Competing interests

The authors declare that they have no conflict of interest.

Dataset availability

RNA sequencing data is available upon request.

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Figure legends

Figure 1. Altered apoptotic morphologies of ROCK1nc hepatocytes.

A. Primary hepatocytes isolated from ROCK1wt (left panels) or ROCK1nc (right panels)

mice were treated with 1 μM ABT-199 to induce apoptosis, then images were capture

every 3 minutes by bright field microscopy. Representative images shown at indicated

time points. **B.** Scanning electron micrographs of representative ROCK1wt (left) and

ROCK1nc (right) hepatocytes. Scale bars = 10 μm.

Figure 2. Steatosis and cytokine/chemokine induction by acute DEN treatment of

primary hepatocytes.

A. Haematoxylin and eosin (H&E) staining of representative liver sections from ROCK1wt

or ROCK1nc mice 72 hours after DEN injection. Scale bar = 100 μm. **B.** Venn diagram of

the number of genes significantly (adjusted $p < 0.05$) changed in expression by at least

1.5 fold in ROCK1wt and ROCK1nc livers 72 hours after DEN injection. **C.** Reactome

gene sets significantly associated (nominal $p < 0.05$) with genes that were significantly

(adjusted $p < 0.05$) altered in expression by at least 1.5 fold in ROCK1wt or ROCK1nc

livers 72 hours after DEN injection. **D.** Relative induction of cytokines/chemokines 72

hours after DEN injection relative to untreated ROCK1wt and ROCK1nc mice. Means ±

standard deviation, n = 5 independent determinations. Unpaired Student's t-test, p values

less than 0.05 indicated in bold typeface.

Figure 2-figure supplement 1. RNA sequencing of untreated and DEN-treated ROCK1wt and ROCK1nc livers.

A. Log10-log10 scatter plots of mean RNAseq reads for ROCK1wt livers that had been treated for 72 hours with vehicle vs DEN, where $p < 0.05$ and fold-change (FC) differences $> \pm 1.5X$ indicated with green dots, $p \geq 0.05$ or FC $\leq \pm 1.5X$ indicated with grey dots. **B.** Log10-log10 scatter plots of mean RNAseq reads for ROCK1nc livers that had been treated for 72 hours with vehicle vs DEN, where $p < 0.05$ and fold-change (FC) differences $> \pm 1.5X$ indicated with red dots, $p \geq 0.05$ or FC $\leq \pm 1.5X$ indicated with grey dots. **C.** Scatter plot of RNAseq $\log_2$ FC for 72 hour treatment with vehicle versus DEN from ROCK1wt and ROCK1nc mouse livers. Dotted line shows Deming regression line with calculated slope = 0.8313. **D.** RNAseq Log2 FC for sequence reads increased in DEN versus vehicle treated ROCK1wt and ROCK1nc mice (left) and for sequence reads decreased in DEN versus vehicle treated ROCK1wt and ROCK1nc mice (right). Horizontal line = median. Unpaired Student's t-test, p values less than 0.05 indicated in bold typeface.

Figure 3. Greater steatosis and neutrophil numbers in ROCK1nc liver tumours.

A. H&E staining of representative liver tumour sections from ROCK1wt or ROCK1nc mice 9 months after DEN treatment. Steatosis was determined on H&E-stained sections as a percentage of the total area from 10 random fields per section per mouse, and graded according to the following criteria: grade 0, < 5%; grade 1, 5-25%; grade 2, 26-50%; grade 3, 51-75%; grade 4, > 75%. Scale bars = 100 µm. **B.** Representative

Immunohistochemical (IHC) images of anti-S100A9 antibody-stained formalin-fixed

paraffin embedded (FFPE) liver sections showing neutrophils (brown) in liver tumours (left

panels), and corresponding bar graph (right panel). S100A9 positive neutrophils were

scored as number per mm^2 in 5 random fields per section per mouse. **C.** Representative

IHC images of anti-F4/80 antibody-stained liver sections showing macrophages numbers

in liver tumours (left panels), and corresponding bar graph (right panel). F4/80 positive

macrophages were scored as number per mm^2 in 5 random fields per section per mouse.

Scale bars = 100 μm. Means ± standard deviation, ROCK1wt n = 10 mice, ROCK1nc n =

7 mice. Unpaired Student's t-test, p values less than 0.05 indicated in bold typeface.

Figure 3-figure supplement 1. Biochemical markers of liver function and health are

not different between ROCK1wt and ROCK1nc mice with DEN-induced tumours.

Serum levels of: **A.** total bilirubin; **B.** alkaline phosphatase; **C.** aspartate

aminotransferase; **D.** alanine transaminase; **E.** total protein; **F.** albumin; **G.** globulins and

H. ratio of albumin/globulin were not significantly different between ROCK1wt and

ROCK1nc mice with DEN-induced tumours. Means ± standard deviation, ROCK1wt n =

12 mice, ROCK1nc n = 11 mice. Unpaired Student's t-test.

Figure 4. Greater CD3$^+$ and CD8$^+$ T cell numbers in ROCK1nc liver tumours.

A. Representative IHC images of anti-CD3 antibody-stained liver sections showing CD3$^+$

T cells (brown) in liver tumours (left panels), and corresponding bar graph (right panel).

CD3$^+$ positive T cells were scored as number per mm^2 in 5 random fields per section per

mouse. **B.** Representative immunofluorescence images of anti-CD8 antibody-stained

liver sections showing CD8$^+$ T cells (red) and DAPI stained cells (blue) in ROCK1nc

tumours (left panels), and corresponding bar graph (right panel). CD8$^+$ T cells (red) were

scored as a percentage of the total 4′,6-diamidino-2-phenylindole (DAPI; upper panel,

blue) cell numbers from 5 random fields per section per mouse **C.** Representative IHC

images of anti-Foxp3 antibody-stained liver sections showing Foxp3$^+$ Treg cells (brown)

in liver tumours (left panels), and corresponding bar graph (right panel). Foxp3$^+$ Treg cells

were scored as number per mm^2 in 5 random fields per section per mouse. Scale bars =

100 μm. Means ± standard deviation, ROCK1wt n = 10 mice, ROCK1nc n = 7 mice.

Unpaired Student's t-test, p values less than 0.05 indicated in bold typeface.

Figure 5. Fewer liver tumours in fasudil treated mice without increased immune cell

recruitment.

A. Timeline of DEN + fasudil treatment regimen. **B.** Macroscopic view of representative

livers (scale bars = 10 mm) and representative H&E stained tumour sections (scale bars

= 500 μm) from vehicle or fasudil treated mice. **C.** Total tumour numbers in ROCK1wt

mice treated with vehicle or fasudil. Means ± standard deviation, n = 13 mice per

condition. **D.** Total tumour areas in ROCK1wt mice treated with vehicle or fasudil. Means

± standard deviation, n = 13 mice per condition. **E.** Representative IHC images of anti-

S100A9 antibody-stained liver section from fasudil treated mouse showing neutrophils

(brown) in liver tumours (left panels), and corresponding bar graph (right panel). S100A9

positive neutrophils were scored as number per mm^2 in 5 random fields per section per

mouse.   Scale bar = 100 μm. Means ± standard deviation, vehicle n = 13 mice, fasudil n

= 9 mice. **F.** Representative IHC images of anti-CD8 antibody-stained liver section from

fasudil treated mouse showing CD8$^+$ T cells (brown) in liver tumours (left panels), and

corresponding bar graph (right panel). CD8$^+$ T cells were scored as number per mm^2 in 5

random fields per section per mouse. Means ± standard deviation, vehicle n = 12 mice,

fasudil n = 11 mice. Unpaired Student's t-test, p values less than 0.05 indicated in bold

typeface.

Figure 5-figure supplement 1. No differences in body, liver, lung or spleen weights

between mice treated with vehicle or fasudil.

Weights at experimental endpoint of: **A.** whole mice; **B.** livers; **C.** livers as a percentage

of body weight; **D.** lungs; **E.** lungs as a percentage of body weight; **F.** spleens; **G.** spleens

as a percentage of body weight. There were no significant differences between mice

treated with vehicle or fasudil. Means ± standard deviation, vehicle n = 13 mice, fasudil n

= 13 mice. Unpaired Student's t-test.

Figure 5-figure supplement 2. Fewer circulating neutrophils and reduced aspartate

aminotransferase levels in fasudil treated mice relative to vehicle control.

Blood from vehicle or fasudil treated mice was analyzed for: **A.** Haemoglobin levels;

numbers of **B.** platelets; **C.** white cells; **D.** lymphocytes; **E.** monocytes; **F.** neutrophils.

Serum from vehicle or fasudil treated mice was analyzed for markers of liver function and

health: **G.** bilirubin; **H.** alanine transaminase; **I.** albumin; **J.** alkaline phosphatase; **K.**

aspartate aminotransferase; **L.** globulins. Means ± standard deviation, vehicle n = 9 mice,

fasudil n = 9 mice. Unpaired Student's t-test, p values less than 0.05 indicated in bold

typeface.

Figure 6. Fewer liver tumours in AT13148 treated mice without increased immune

cell recruitment.

A. Timeline of DEN + AT13148 treatment regimen. **B.** Macroscopic view of representative livers (scale bars = 10 mm) and representative H&E stained tumour sections (scale bars = 500 μm) from vehicle or AT13148 treated mice. **C.** Total tumour numbers in ROCK1wt mice treated with vehicle or AT13148. Means ± standard deviation, vehicle n = 8 mice, At13148 n = 9 mice. **D.** Total tumour areas in ROCK1wt mice treated with vehicle or AT13148. Means ± standard deviation, vehicle n = 8 mice, At13148 n = 9 mice. **E.** Representative IHC images of anti-S100A9 antibody-stained liver section from AT13148 treated mouse showing neutrophils (brown) in liver tumours (left panels), and corresponding bar graph (right panel). S100A9 positive neutrophils were scored as number per mm^2 in 5 random fields per section per mouse. Scale bar = 100 μm. Means ± standard deviation, vehicle n = 5 mice, AT13148 n = 6 mice. **F.** Representative IHC images of anti-CD8 antibody-stained liver section from AT13148 treated mouse showing $CD8^+$ T cells (brown) in liver tumours (left panels), and corresponding bar graph (right panel). $CD8^+$ T cells were scored as number per mm^2 in 5 random fields per section per mouse. Means ± standard deviation, vehicle n = 5 mice, AT13148 n = 6 mice. Unpaired Student's t-test, p values less than 0.05 indicated in bold typeface.

Figure 6-figure supplement 1. No differences in body, liver or lung weights between

mice treated with vehicle or AT13148.

Weights at experimental endpoint of: **A.** whole mice; **B.** livers; **C.** livers as a percentage

of body weight; **D.** lungs; **E.** lungs as a percentage of body weight; **F.** spleens; **G.** spleens

as a percentage of body weight. There were no significant differences between mice

treated with vehicle or AT13148, with the exception of spleen weights that were

significantly smaller when comparing weights, but not when compared as percentages of

body weights. Means ± standard deviation, vehicle n = 9 mice, AT13148 n = 9 mice.

Unpaired Student's t-test.

Figure 6-figure supplement 2. No significant differences in circulating blood cells

or biochemical markers of liver health and function between vehicle or AT13148

treated mice.

Blood from vehicle or AT13148 treated mice was analyzed for: **A.** haemoglobin levels;

numbers of **B.** platelets; **C.** white cells; **D.** lymphocytes; **E.** monocytes; **F.** neutrophils.

Serum from vehicle or At13148 treated mice was analyzed for markers of liver function

and health: **G.** bilirubin; **H.** alanine transaminase; **I.** albumin; **J.** alkaline phosphatase; **K.**

aspartate aminotransferase; **L.** globulins. Means ± standard deviation, vehicle n = 5 mice,

AT13148 n = 8 mice. Unpaired Student's t-test, p values less than 0.05 indicated in bold

typeface.

Figure 7. ROCK inhibitor AT13148 lowered circulating platelet and lymphocyte numbers, and reduced immune cell recruitment to livers following acute DEN treatment.

ROCK1wt mice were treated with 40 mg/kg AT13148 or vehicle 3 x per week via oral gavage for 10 days, then injected with 100 mg/kg DEN intraperitoneally 3 days prior to sacrifice to induce acute liver damage. Flow cytometry analysis of circulating **A.** platelets and **B.** lymphocytes in vehicle (n = 5) and AT13148 (n = 4) treated mice. Liver cell suspensions were analyzed by flow cytometry to determine the relative proportions of **C.** dendritic cells, **D.** natural killer (NK) cells, **E.** CD8$^+$ T cells and **F.** Kupffer cells from vehicle and AT13148 treated mice (n = 5). Means ± standard deviation. Unpaired Student's t-test, p values less than 0.05 indicated in bold typeface.

Figure 7-figure supplement 1. No significant differences in body or liver weights, blood haemoglobin levels, or circulating white cells, monocytes or neutrophils between vehicle or AT13148 treated mice following acute DEN treatment.

Weights at experimental endpoint of: **A.** whole mice; **B.** livers; **C.** livers as a percentage of body weight. Blood from vehicle or AT13148 treated mice was analyzed for: **D.** haemoglobin levels; numbers of **E.** white cells; **F.** monocytes; **G.** neutrophils. Means ± standard deviation, vehicle n = 5 mice, AT13148 n = 4 mice. Unpaired Student's t-test.

Figure 7-figure supplement 2. Immune cell recruitment to livers following acute DEN treatment in vehicle or AT13148 treated mice.

Liver cell suspensions were analyzed by flow cytometry to determine the proportions of immune cells relative to total cell numbers counted of: **A.** recruited macrophages; **B.** neutrophils; **C.** plasmacytoid dendritic cells; **D.** B cells; **E.** CD4$^+$ T cells; **F.** Tregs; **G.** γδ T cells; **H.** NKT cells. Means ± standard deviation, vehicle n = 5 mice, AT13148 n = 5 mice. Unpaired Student's t-test.

Video 1. Time lapse of apoptotic ROCKwt hepatocytes.

ROCK1wt hepatocytes were plated on 6-well glass bottom dishes that had been coated with collagen. Cells were serum-starved overnight prior to induction of apoptosis with 1 µM ABT-199 diluted in serum-free starvation medium. Immediately after induction of apoptosis, the dishes were transferred to the microscope and brightfield time-lapse microscopy images were acquired at 3 minute intervals for up to 120 minutes with a 10X DIC objective using a Nikon TE 2000 microscope with a heated stage and 5% CO_2 gas line.

Video 2. Time lapse of apoptotic ROCKnc hepatocytes.

ROCK1nc hepatocytes were plated on 6-well glass bottom dishes that had been coated with collagen. Cells were serum-starved overnight prior to induction of apoptosis with 1 µM ABT-199 diluted in serum-free starvation medium. Immediately after induction of apoptosis, the dishes were transferred to the microscope and brightfield time-lapse microscopy images were acquired at 3 minute intervals for up to 120 minutes with a 10X DIC objective using a Nikon TE 2000 microscope with a heated stage and 5% CO_2 gas line.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 2-figure supplement 1

Figure 3-figure supplement 1

Figure 5-figure supplement 1

Figure 5-figure supplement 2

Figure 6-figure supplement 1

Figure 6-figure supplement 2

Figure 7-figure supplement 1

Figure S7-figure supplement 2