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Early View

Research letter

(R)-Crizotinib Predisposes To and Exacerbates Pulmonary Arterial Hypertension in Animal Models

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Title: (R)-Crizotinib Predisposes To and Exacerbates Pulmonary Arterial Hypertension in Animal Models.

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Take-Home Message:
Our study demonstrates that (R)-Crizotinib, a frontline therapy for lung cancer, predisposes to and exacerbates PH in animal models. Our findings suggest that caution and regular follow-up should be exercised in lung cancer patients treated with the compound.

To the Editor:

Pulmonary hypertension (PH) is a life-threatening disease of multiple etiologies. Regardless of the underlying cause, PH is characterized by vasoconstriction and progressive thickening of the pulmonary vessel wall all of which is initiated by the loss of pulmonary artery endothelial cell (PAEC) [1]. Indeed, a large body of works has shown that damaged or apoptotic PAECs initiate the remodeling process through the release of growth, fibrogenic and pro-inflammatory factors that directly induce contraction and enhance survival and proliferation of adjacent PA smooth muscle cells (PASMCs) and fibroblasts [1, 2]. Over the past decade, intense research efforts have been directed at deciphering how PH cells acquire their "cancer-like" properties. As a consequence, the therapeutic potential of numerous anti-neoplastic drugs have been tested in preclinical models with some of them reaching clinical assays [3]. Considering the biphasic pattern of apoptosis that characterizes the disease (i.e. PAEC apoptosis that triggers the disease is followed by an apoptosis-resistant state allowing vascular remodeling [4]), it is not surprising that some anticancer agents can both predispose to and treat pulmonary arterial hypertension (PAH). This is exemplified by studies showing that Dasatinib, a second-generation tyrosine kinase inhibitor (TKI) approved for Philadelphia chromosome positive chronic myeloid leukemia, improves established PAH in multiple animal models [5], while its administration before exposure to PH inducers exacerbates pulmonary vascular remodeling and PA pressures; histological and hemodynamic changes not observed in rats exposed to Dasatinib alone [6].

Recently, several observational studies have highlighted the development of PAH in patients with metastatic non-small cell lung cancer with anaplastic lymphocyte kinase (ALK) rearrangement who received ALK/cMET TKI (including Xalkori, Ceritinib, Brigatinb and Lorlatinib) [7, 8]. Since PH can be associated with multiple diseases including lung cancer [9], the question remains whether development of PAH in lung cancer patients receiving c-MET/ALK TKI represents an adverse drug event or disease spread. To clarify this point and potentially improve our understanding of the pathogenesis underlying PH development, we investigated in different animal models whether (R)-Crizotinib (also known as Xalkori), a standard frontline therapy for c-MET-positive and ALK-rearranged lung cancer [10], exacerbates existing PH and/or predisposes to PH in well-established animal models (ethic approval #VRR-19-018).

We first explored the influence of (R)-Crizotinib therapy on existing PH in the Sugen/Hypoxia (Su/Hx) rat model (Figure 1A, protocol A). We found that one third of (R)-Crizotinib-treated Su/Hx rats died between weeks 4 and 5, whereas none of the Su/Hx rats receiving vehicle died. In agreement with this, treatment with (R)-Crizotinib resulted in a significant increase in right ventricular (RV) systolic pressure (RVSP) and mean PA pressure (mPAP) compared to the

injured vehicle-treated group, as assessed by RV catheterization in closed-chest animals (Figure 1A). Although RV hypertrophy, as measured by Fulton index, and natriuretic peptide A (*Nppa*) and -B (*Nppb*) transcript levels were not significantly different between groups (data not shown), stroke volume (SV) and cardiac output (CO) were more significantly declined in Xalkori-treated rats. Total pulmonary resistance (TPR, calculated by dividing the mPAP by the CO) was augmented and this was reflected by an increase in medial wall thickness of distal PAs (Figure 1B). We next investigated whether (R)-Crizotinib-induced adverse cardiopulmonary effects could be reproduced in a second PAH model; namely the monocrotaline (MCT) rat. In view of the high mortality rate seen in (R)-Crizotinib-treated Su/Hx rats, the duration of (R)-Crizotinib treatment was reduced to one week, initiated two weeks after MCT injection (Figure 1A, protocol B). In comparison with vehicle-treated MCT rats, (R)-Crizotinib treatment significantly increased RVSP and mPAP. No significant differences were observed with regard to SV, CO, TPR, and vascular remodeling (Figure 1A and 1B) nor with RV hypertrophy (data not shown), possibly due to the shorter duration of treatment .

Having demonstrated that treatment with (R)-Crizotinib aggravates existing PH in rodent models, we next adopted a reverse reasoning and investigated whether the drug given to rats prior to exposure to a PH inducer potentiates the development of the disease by exacerbating hemodynamical and structural changes (Figure 1C). Because a single dose of 60mg/kg of MCT produces severe PH that could mask the potential worsening effects of (R)-Crizotinib pretreatment, mild PH was induced by a low dose of MCT (40mg/kg). In this protocol, (R)-Crizotinib-treated rats exhibited an exaggerated pulmonary hypertensive response compared to vehicle-pretreated animals, as demonstrated by a significant increase in RVSP and mPAP, lower SV and reduced CO (Figure 1C) without any impact on the degree of RV hypertrophy (data not shown). Accordingly, TPR was significantly augmented and medial wall thickness of distal PAs was increased by approximately 2-fold in rats who received (R)-Crizotinib (Figure 1C and 1D). It must be noticed that for each protocol, a left heart catheterization was not performed. However, measurement of *Nppa* and *Nppb* expression in left ventricles revealed no significant changes between groups (data not shown). Finally, effects of chronic administration of (R)-Crizotinib alone (100mg/kg/d) for 21 consecutive days were investigated. (R)-Crizotinib-treated rats did not show any hemodynamic difference (i.e. RVSP, mPAP, SV, CO and TPR) when compared with vehicle-treated animals (Figure 1C), indicating that the drug by itself is not sufficient to elicit PH.

Since injury-induced death of PAECs is recognized as a critical initiating event in PAH, we next investigated whether *in vitro* exposure of control human PAECs to a clinically relevant dose of (R)-Crizotinib influences their survival and proliferative capacities. We first verified its capacity

to inhibit basal phosphorylation of its primary target, c-Met, in cultured cells. As expected, (R)-Crizotinib drastically diminished phosphorylation of c-Met and its downstream pro-survival signal AKT (Figure 1E). As revealed by Annexin V staining, terminal transferase-mediated DNA end labelling (TUNEL) assay and immunoblot for cleaved Caspase-3, apoptosis of PAECs was markedly increased upon exposure to (R)-Crizotinib (Figure 1E and 1F). In agreement with this, (R)-Crizotinib elicited anti-proliferative effects, as illustrated by a significant diminution in the proportion of Ki67-positive cells and reduced expression levels of proliferating cell nuclear antigen (PCNA) (Figure 1E and 1F). Surprisingly, exposure to (R)-Crizotinib was associated with an increase in the number of large, flat PAECs containing multiple nuclei or aberrant nuclei clusters (Figure 1F), a feature usually seen in cells that undergo a form of cell death called mitotic catastrophe induced by ionizing radiation and certain anticancer drugs. Accordingly, expression levels of the mitotic regulator Polo-like kinase 1 (PLK1) was nearly abolished in PAECs treated with (R)-Crizotinib (Figure 1E).

Interestingly, HGF/c-MET signaling was documented to elicit pro-survival effects on endothelial cells [11] and activation of cMET signaling, via supplementation in HGF two weeks after MCT injection, was shown to improve vascular remodeling [12]. These data may explain in part why (R)-Crizotinib both predisposes to and aggravates established PH. Whether the deleterious impact of (R)-Crizotinib on PAECs is mediated by on- or off-target effects remains to be explored. Several studies have evidenced that (R)-Crizotinib elicits anti-tumor activity via off-target effects [13, 14], including the inhibition of multiple kinases, such as Src (Figure 1E), Lck, Abl and Yes, all critically involved in PAH development [15]. This suggests that the adverse outcome induced by (R)-Crizotinib on the pulmonary vasculature is likely the consequence of its cumulative effects on multiple targets.

In conclusion, our study shows for the first time that the anticancer agent (R)-Crizotinib may cause endothelial cell injury, and by doing so, amplify the response to well-established PH inducers. Although it remains unclear whether a similar relationship between (R)-Crizotinib-induced endothelial cell injury and PH development in lung cancer patients exists, further study of the affected signaling pathways may provide important information into the pathophysiology of PAH, and potentially new targets to combat this serious condition. In addition, our findings suggest that clinicians should consider further evaluation for PH in lung cancer patients treated with (R)-Crizotinib who develop worsening dyspnea or heart failure symptoms.

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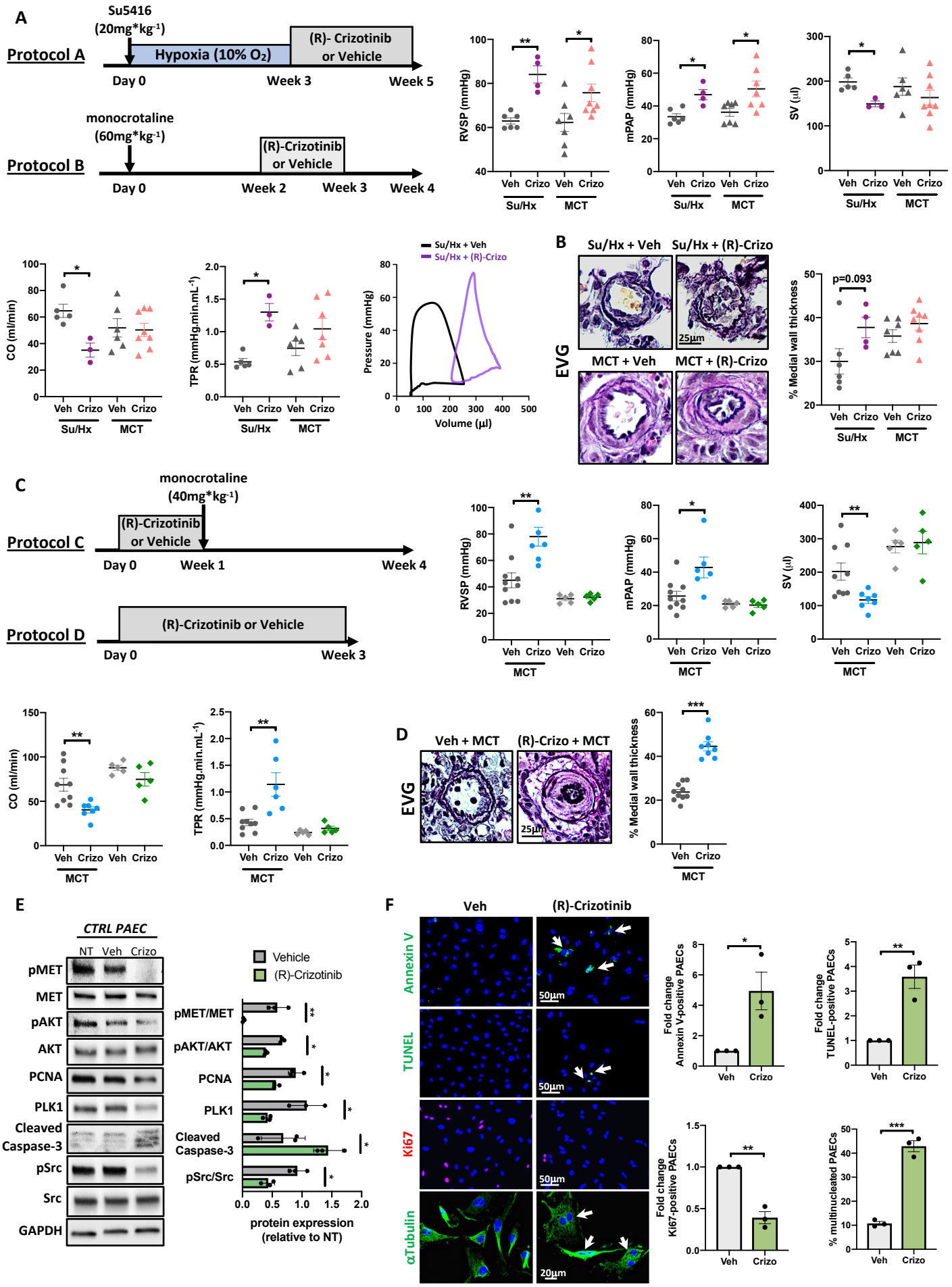
Figure Legend:

(R)-Crizotinib predisposes to and aggravates existing pulmonary hypertension in animal models. (A) Male Sprague Dawley rats (8-9 weeks, 250-300g body weight) were exposed to Sugen (20mg/kg) followed by 3 weeks of hypoxia (10% O_2, protocol A) or were subcutaneously injected with monocrotaline (60mg/kg, protocol B) to induce PH. After PH establishment, MCT-injected or Su/Hx rats were randomly assigned to receive either (R)-Crizotinib (100mg/kg/day) or vehicle by oral gavage, for 2 or 1 week respectively. Two weeks after treatment was started, rats were anesthetized and underwent right heart catheterization with pressure-volume analyses to measure the right ventricular (RV) systolic pressure (RVSP), mean PA pressure (mPAP), strove volume (SV), cardiac output (CO) and total pulmonary resistance (TPR, calculated as mPAP/CO), prior to euthanasia and tissue collection. A representative RV pressure-volume (PV) loop in both vehicle-treated Su/Hx and (R)-Crizotinib-treated Su-Hx rats is shown. **(B)** Paraffin-embedded lung tissue sections were stained with Elastica Van Gieson (EVG) and medial wall thickness of small PAs (<75μm) was quantified. At least 15 PAs per rats were measured. **(C)** Male Sprague Dawley rats were treated with (R)-Crizotinib (100mg/kg/day) or vehicle for 1 week followed by a single subcutaneous injection of low dose monocrotaline (40mg/kg) (protocol C) or received (R)-Crizotinib (100mg/kg/day) or vehicle by oral gavage for 21 consecutive days (protocol D). Before sacrifice, cardiac hemodynamic and RV function were assessed by right heart catheterization. **(D)** Lung tissue sections were stained with EVG to analyze and quantify PA wall thickness. **(E)** Representative Western blots and corresponding densitometric quantifications of phospho-MET (Tyr1234/1235), MET, phospho-AKT (Ser473), AKT, PCNA, Cleaved caspase-3, PLK1, phospho-Src (Tyr416) and Src in control PAECs (n=3) exposed to (R)-Crizotinib (1μM) or its vehicle (DMSO) for 48h. The levels of protein expression were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **(F)** Apoptosis (Annexin V staining and TUNEL labeling) and proliferation (Ki67 staining) were measured in serum-stimulated (10% FBS) control PAECs (n=3) treated with (R)-Crizotinib (1μM) or its corresponding vehicle for 48 hours. Representative images and quantification of alpha-tubulin labeled control PAECs exhibiting multinucleation upon (R)-Crizotinib (1μM) exposure for 48h. For each different PAEC cell line, at least 350 cells per condition from 10 randomly selected visual fields were counted. All the data were analyzed in a blinded fashion without knowledge of the treatment groups. Data are expressed as mean±standard error of the mean. NT: non-treated cells. Statistical significance was calculated using Mann-Whitney U test. P value of less than 0.05 was considered to indicate statistical significance. *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$.


A
Su5416
(20mg*kg-1)
Protocol A
Hypoxia (10% O2)
(R)- Crizotinib
or Vehicle
Day 0
Week 3
Week 5
monocrotaline
(60mg*kg-1)
Protocol B
(R)-Crizotinib
or Vehicle
Day 0
Week 2
Week 3
Week 4
RVSP (mmHg)
mPAP (mmHg)
SV (µl)
Veh Crizo Veh Crizo
Su/Hx
MCT
CO (ml/min)
TPR (mmHg.min.mL-1)
Pressure (mmHg)
Volume (µl)
Su/Hx + Veh
Su/Hx + (R)-Crizo
B
EVG
Su/Hx + Veh
Su/Hx + (R)-Crizo
MCT + Veh
MCT + (R)-Crizo
25µm
% Medial wall thickness
p=0.093
C
monocrotaline
(40mg*kg-1)
Protocol C
(R)-Crizotinib
or Vehicle
Day 0
Week 1
Week 4
Protocol D
(R)-Crizotinib or Vehicle
Day 0
Week 3
D
Veh + MCT
(R)-Crizo + MCT
EVG
25µm
% Medial wall thickness
E
CTRL PAEC
NT Veh Crizo
pMET
MET
pAKT
AKT
PCNA
PLK1
Cleaved Caspase-3
pSrc
Src
GAPDH
Vehicle
(R)-Crizotinib
pMET/MET
pAKT/AKT
PCNA
PLK1
Cleaved Caspase-3
pSrc/Src
protein expression
(relative to NT)
F
Veh
(R)-Crizotinib
Annexin V
TUNEL
Ki67
αTubulin
50µm
20µm
Fold change Annexin V-positive PAECs
Fold change TUNEL-positive PAECs
Fold change Ki67-positive PAECs
% multinucleated PAECs
Veh Crizo