INTRODUCTION

Influenza viruses are a common cause of human respiratory infections, resulting in yearly epidemics of high morbidity and mortality. In the United States, seasonal influenza results in approximately 200,000 hospitalizations and 36,000 deaths in a typical year [1]. Influenza viruses can cause global pandemics, for example the 1918 Spanish flu, the 1957 Asian flu and the 1968 Hong Kong flu, during which rates of illness and death from influenza-related complications among all age groups significantly increased worldwide. Current treatment options for influenza viruses are limited and a high proportion of currently circulating influenza viruses have developed resistance to some of the clinically available drugs [2]: most currently circulating strains are resistant to the adamantanes M2 inhibitors and oseltamivir has demonstrated potential for resistance development as observed in pre-2009 pandemic H1N1 strains [3]. While it is true that currently circulating human strains are sensitive to neuraminidase inhibitors, the overall frequency of A(H1N1)pdm09 viruses containing NA mutations associated with drug resistance was approximately 1.8% in the 2015–2016 season, which is slightly higher than 0.5% in the previous 2014–2015 season [4]. The development of novel and effective therapeutic strategies is therefore urgently required to improve the medical treatment options for influenza.

Targeting host cell proteins as opposed to viral proteins may be advantageous because it could allow the identification of
novel targets with a broad spectrum of activity and limit the generation of resistance mutations introduced by the error prone RNA-dependent RNA polymerase. However, targeting host cell proteins could increase the potential for toxicity in an infectious setting. One such host protein that could be targeted is p38 mitogen-activated protein kinase (MAPK). In vitro, influenza virus infection has been shown to activate MAPK in human airway cells [5, 6]. The onset of influenza is associated with symptoms such as lethargy and fever with the cytokine response to infection believed to be responsible for these clinical signs [7], and it is known that inhibition of p38 MAPK can attenuate the production of pro-inflammatory cytokines such as TNFα [8]. A prior publication suggested that inhibition of p38 MAPK can reduce influenza viral replication [9]. Therefore, p38 MAP kinase inhibition could affect both viral replication and the inflammatory response to infection which have advantages over current influenza therapies. BCT197 is a novel low molecular weight p38 MAPK inhibitor with an IC_{50} value of <1 µM [10], which has progressed to the clinic. In a healthy human volunteer study, BCT197 potently inhibited influenza infection in a mouse model where it was compared to vehicle treatment.

METHODS

Cell culture and viruses
Influenza A virus strain H3N2 A/Udorn/307/72 (Udorn) and H1N1 A/PR/8/34 (PR8) and influenza B virus strain B/Lee/40 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Madin–Darby canine kidney (MDCK) epithelial cells (CCL-34) (ATCC) were cultured in Dubecco’s modified Eagle’s medium (DMEM) (Mediatech, Manassas, VA, USA) supplemented with 10 % v/v fetal bovine serum (Omega Scientific, Tarzand, CA, USA), 1 % v/v penicillin-streptomycin (Cellgro, Manassas, VA, USA), supplemented with 1 % v/v sodium pyruvate (Cellgro).

Influenza virus titration by plaque assay
MDCK cells were seeded at 3 × 10^5 cells well^{-1} in 12-well tissue culture plates. At 100 % confluence (2 days post-seeding), the cells were washed twice with PBS and six serial dilutions of the virus (1/10 each time) were added to the cells. Following infection with 0.3 ml well^{-1} of each dilution, plates were incubated at 37 °C for 1 h, and the cells were washed with PBS before the addition of 2 % w/v agar (Sea-Plaque Agarose, Lonza, Walkersville, MD, USA) containing 1 µg ml^{-1} tosyl phenylalanyl chloromethyl ketone-trypsin (Sigma-Aldrich, St. Louis, MO, USA) to the cell surface. Plates were left at room temperature for 20–30 min to allow for the overlay to set, and were then incubated at 37 °C for 72 h. Cells were fixed with 4 % v/v paraformaldehyde (Affymetrix USB, Santa Clara, CA, USA) before both the fixative and agar were removed and the cells stained with 0.1 % w/v Crystal Violet (Fisher Chemical, Watham, MA, USA) in 20 % v/v ethanol. Plaque titres were determined as plaque forming units (p.f.u.) per ml.

Infection of polarized primary human bronchial epithelial cells (HBECs)
HBECs (Lonza) were grown in BEBM media (Lonza) supplemented with BCGM singlequots (Lonza) until 80–90 % confluent. Cells were washed, then seeded apically into 12-well transwell plates (2 × 10^5 cells well^{-1}) (Millicell-CM with 1 µM pore semi permeable membrane, Millipore) in differentiation media [1:1 mix of 2XBCGM singlequots minus triiodothyronine (T3) in BEBM:DMEM high glucose hyclone with all-trans-retinoic acid at 5 × 10^{-8}M prepared in 100 % ethanol (Sigma-Aldrich)]. Then, 7 days after seeding an airway–liquid interface (ALI) was initiated by removing the apical culture medium. When cultures were >3 weeks old trans-epithelial resistance was measured as an indication of having reached a well-differentiated state prior to use.

Prior to infection, p38 MAPK inhibitor BCT194 or nucleozin, an influenza A inhibitor, were added to the basolateral compartment and incubated for 2 h at 37 °C. Cells were infected apically at m.o.i. of 0.01 for 1 h at 37 °C before the virus was removed, cells were washed three times with the medium and returned to 37 °C. Virus was collected every 24 h at the apical surface and titred by a plaque assay.

Animal infection and treatment
All studies were approved by the Institutional Animal Care and Use Committee of Novartis Institutes for BioMedical Research, Emeryville, CA, USA. Female BALB/c mice (18–20 g; Harlan Laboratories, Livermore, CA, USA) were infected by 50 µl intranasal administration of 150 pfu Influenza H1N1 A/PR/8/34 (PR8), under isofluorane (Pirana Healthcare, Bethlehem, PA, USA) anesthesia. Body weight was measured daily with animals removed from the study if the animal lost ≥25 % of its body weight as compared to its pre-infection values.

Oral administration of the compound began 24 h post infection (p.i.) for 5 days with either a p38 MAP kinase inhibitor [BCT197, 18 and 6 mg kg^{-1} quaque die (QD) formulated with 0.5 % hydroxypropyl methylcellulose (HPMC) 60 : 0.5 % Tween 80], dexamethasone (3 mg kg^{-1} QD, Medisca, Las Vegas, NV, USA, formulated with PBS) or the approved antiviral oseltamivir (Tamiflu, 5 mg kg^{-1} BID Roche Laboratories, Indianapolis, IN, USA, formulated with PBS). Vehicle-treated animals received 0.5 % HPMC 60 : 0.5 % Tween 80. The dose of oseltamivir was based on prior mouse studies [13], and the doses of BCT197 approximated human exposure which was either equal to (6 mg kg^{-1}) or higher (18 mg kg^{-1}) than the COPD clinical trials which had demonstrated a significant anti-inflammatory effect from P38MAPK inhibition [11].
Sample collection and assessment

Animals were euthanized 2, 4, 7 and 9 days p.i. to examine viral load, cytokines, total and differential cell counts, protein and lactate dehydrogenase (LDH); a subset (five animals on day 7) were processed for microscopic evaluation. For viral titre measurement, animals were euthanized using CO₂, the lungs removed, and homogenized for 30 s before centrifugation at 1942 g for 10 min at 4 °C; the supernatant was collected and the viral titre determined using a plaque assay. For inflammatory parameters, animals were anesthetized by an intraperitoneal administration of a mixture of ketamine (Mylan, Canonsburg, PA, USA), xylazine (Lloyd, Shenandoah, IA, USA) and acepromazine (Phoenix Pharmaceuticals, Burlingame, CA, USA) (100, 20 and 10 mg kg⁻¹ respectively). A blood sample was collected from the abdominal vena cava, the trachea cannulated and a bronchoalveolar lavage (BAL) performed using sterile saline (0.9 % w/v). The lungs were removed, inflated and fixed in 10 % neutral buffered formalin (VWR, Visalia, CA, USA) at a constant pressure of 20 cm water for histopathological analysis. Lung sections were stained with alcian blue and haematoxylin and eosin and scored from grade 1 to grade 5 (described in Table S1, available in the online version of this article). Blood samples were centrifuged at 9503 g for 10 min at 4 °C and the serum collected. An aliquot of BAL fluid (BALF) was removed for differential cell counting and using cytospin slides and cytospin funnels (Azer Scientific, Morgantown, PA, USA) samples were prepared and centrifuged at 700 r.p.m. with slow acceleration for 5 min (Shandon Cytospin 4, Thermoscientific, Hudson, NH, USA). Slides were stained with Wright’s stain using an automated slide stainer (Hematek, Bayer, Berkeley, CA, USA) and overlaid with a coverslip. The number of macrophages, neutrophils, lymphocytes and eosinophils in a total of 200 cells were determined at ×40 magnification. The remaining BAL was centrifuged at 380 g for 5 min at 4 °C, and the supernatant removed for cytokine measurement. The cell pellet was re-suspended in 0.5 ml of methyl violet fixative [0.01 % w/v methyl violet (Fluka Chemical, Gillingham, UK), 1.5 % v/v acetic acid (Sigma-Aldrich)] and a total cell count performed using a multi-chamber haemocytometer. The total cell count was subsequently used to extrapolate back the number of macrophages, neutrophils, lymphocytes and eosinophils present in the sample. BALF LDH (Roche Laboratories), total protein (VWR) and the cytokines TNFα, IL-1β, IFNγ, IL-6, IL-12p70, IL-10, keratinocyte-derived cytokine (KC) (Mesoscale Discovery, Rockville, MD, USA) and RANTES (Mesoscale Discovery) were measured using specialized kits according to the manufacturer’s instructions. Corresponding serum cytokines were also measured.

Data analysis

All data are presented as mean±SEM and were analysed in GraphPad Prism 5.0 (GraphPad software, San Diego, CA, USA). Comparisons were made using a Kruskall–Wallis one-way ANOVA followed by a Dunn multiple comparison test with P<0.05 taken to be significant. The Kaplan–Meier method was used to assess survival. Animals not surviving to day 9 were excluded from analysis unless otherwise noted. For the viral titre analysis, the last-observation-carried-forward analysis method was used.

RESULTS

The effect of p38 MAPK inhibition on viral replication in polarized HBECs

To evaluate if p38 MAPK inhibition could have an impact on viral entry, replication or propagation, differentiated HBECs at an ALI were infected, in the presence and absence of BCT197 analogue BCT194 (0.5, 5 and 50 µM, basolaterally) and A/Udorn and B/Lee viral titres followed over 4 days. None of the concentrations tested affected viral replication as measured by a plaque assay (Fig. 1). As an additional control, nucleozin, a compound targeting the NP protein of influenza A virus [14, 15], was used. Nucleozin has an EC₅₀ of 0.7 µM (data not shown) in a cytopathic effect assay with A/Udorn.

![Fig. 1. Human bronchial epithelial polarized cells were treated with the compound (basolateral chamber) for 2 h prior to infection with influenza virus A/Udorn/H3N2 and B/Lee at m.o.i. 0.01. New virions were collected every 24 h at the apical side of the infected cell surface and titred by a plaque assay in MDCK cells. Data presented as the mean of n=2 replicates.](image-url)
A basolateral concentration of 5 µM of nucleozin effectively reduced the viral titre of A/Udorn below the detection limit of the plaque assay. To assess the impact of p38 MAPK inhibition in the presence of both innate and adaptive immunity BCT197, a close analogue of BCT194, was subsequently examined in a murine influenza model. BCT197 was chosen because it was more suitable for in vivo testing based on improved mouse PK (data not shown), and has been shown to be efficacious in vivo [16].

**The effect of p38 MAPK inhibition in a murine influenza model**

All vehicle-treated animals were removed from study and euthanized by day 9 due to ≥25% body weight loss (Fig. 2). Compared to vehicle, a protective effect was observed with 31% (5/16) of the 6 mg kg⁻¹ BCT197, 67% (10/15) dexamethasone and 94% (15/16) oseltamivir-treated animals surviving to the end of study. However, only 12.5% (2/16) survival was observed in the 18 mg kg⁻¹ BCT197 group.
(Fig. 2b). All of the curves were statistically significantly different from vehicle ($P<0.0125$, using Bonferroni correction for multiple comparisons as part of the Kaplan–Meier analysis).

Viral titre was measured in lung samples collected 2, 7 and 9 days p.i. In the vehicle-treated group, viral titre remained stable between days 2 and 7. Seven days p.i., treatment with dexamethasone or 18 mg kg $^{-1}$ BCT197 appeared to increase viral load while 6 mg kg $^{-1}$ BCT197 (the more clinically relevant dose) had a similar effect to that of oseltamivir (Fig. 3). None of these changes were statistically significant when compared to vehicle, analysed using a non-parametric Kruskall–Wallis ANOVA with Dunn’s multiple comparison test.

Significant changes in BALF cell numbers were observed at 4 and 7 days p.i. (Fig. S1). The only change in BALF cells induced by BCT197 (6 mg kg $^{-1}$ dose) occurred 4 days p.i. where treatment reduced the number of macrophages present in BALF compared to vehicle treatment ($P<0.05$, $n=8$ per group, Figs 4a and S1). Oseltamivir and dexamethasone significantly reduced both BALF macrophages and lymphocytes when compared to vehicle at 4 days p.i. (Figs 4a and S1), and oseltamivir significantly reduced BALF total cells ($5.13\pm0.50\times10^5$ cells and $2.51\pm0.45\times10^5$ cells, $P<0.01$, $n=8$ per group). Seven days p.i., dexamethasone treatment significantly increased total BALF cells ($3.17\pm0.68\times10^5$ cells and $6.51\pm0.40\times10^5$ cells, $P<0.01$, $n=8$ per group, Fig. S1) and this was reflected by a significant increase in neutrophils (Fig. 4c).

At day 7 p.i., microscopic changes associated with influenza virus infection included degeneration and necrosis of the bronchi and bronchioles with epithelial ulceration, neutrophilic inflammation of the bronchi and bronchioles, alveolar-interstitial inflammation with type II pneumocyte hypertrophy, peribronchial and perivascular inflammatory infiltrates and periarterial edema. There was no meaningful difference in main microscopic changes associated with influenza infection between the two doses (6 or 18 mg kg $^{-1}$ day $^{-1}$) of BCT197 (Table S2). There was no meaningful improvement of influenza-related microscopic changes in animals administered BCT197 when compared to controls (Fig. 5). The only difference was a slight increase in severity of periarterial edema in BCT197-treated animals. When compared to vehicle controls, the administration of oseltamivir did not induce meaningful differences other than a slight reduction in the severity of perivascular edema. Administration of dexamethasone decreased the severity of alveolar-interstitial inflammation and peribronchial/perivascular inflammatory infiltration while increasing the severity of bronchial/bronchiolar degeneration and necrosis. In addition, dexamethasone induced a slightly increased severity of bronchial/bronchiolar inflammation correlating with a significant increase in neutrophil counts noted in the BALF cell count (Fig. 4c).

All cytokine data are presented in Figs 6 and 7. The only measureable cytokines in the serum were KC and IFN$\gamma$. IL-12p70 was not measurable in either BAL or the serum. Two days p.i., compared to vehicle, BCT197 (6 mg kg $^{-1}$), oseltamivir and dexamethasone significantly reduced IFN$\gamma$ levels present in BALF (2.2-, 2.5- and 8-fold lower respectively) (Fig. 6a). This reduction was maintained with oseltamivir and dexamethasone at day 4, while BCT197 groups were similar to vehicle-treated animals. By day 7, IFN$\gamma$ levels in the oseltamivir-treated group had increased and were approximately twofold higher than vehicle-treated animals. Oseltamivir also significantly reduced KC levels at day 2.
and both KC and RANTES (regulated on activation, normal T cell expressed and secreted) by day 4 p.i. (Fig. 6 b, c). The biggest impact for dexamethasone treatment was observed 7 days p.i. with significant increases in TNFα (1.6-fold), KC (2.1-fold), IL-6 and RANTES (both 2.7-fold) when compared to vehicle-treated animals (Fig. 6c–e). RANTES levels in mice treated with BCT197 at 18 mg kg⁻¹ were also approximately twofold higher than vehicle treated animals at day 7 (Fig. 6c).

Considering the serum cytokines, the only significant difference observed across the groups was at day 7 when the IFNγ levels for both BCT197 doses were approximately twofold higher than vehicle-treated animals (Fig. 7).

Measurement of total protein and LDH in the BALF was used as a marker of edema and cytotoxicity respectively. Both protein and LDH levels did increase over the course of the study; the only significant change was observed in protein levels on day 2 where 6 mg kg⁻¹ BCT197 treatment significantly increased total protein levels in BALF compared to vehicle-treated animals (210.29±29.0 versus 131.5±7.8 µg ml⁻¹, P<0.05, n=8 animals per group).

**DISCUSSION**

This study determined that the p38 MAPK inhibitor BCT194 did not prevent viral replication in HBECs, and also determined the effect of the related P38MAPK inhibitor BCT197 in a murine model of influenza. BCT197 improved survival and weight loss compared to vehicle, but to a lesser degree than either oseltamivir or dexamethasone. It was also demonstrated that both dexamethasone and the higher dose of BCT197 increased viral load 7 days p.i. The lower dose of BCT197, which is more clinically relevant, remained comparable to vehicle treatment. However, unlike dexamethasone, BCT197 treatment did not increase neutrophil or cytokine levels present in BALF compared to vehicle treatment. Finally, BCT197 did not improve the histological features of influenza infection 7 days p.i.

The p38 MAPK pathway is quickly activated in immortalized human airway epithelial cells during influenza virus infection, and its inhibition was shown to slow down viral trafficking to the nucleus during viral entry [9]. Here we tested BCT194 in influenza virus infection of human primary epithelial cells differentiated at an ALI. Despite the low m.o.i. used in these experiments, we could not detect inhibition of virus production over a 4 day period. This result might be explained by several factors including differences in the cell types, viral strains used, or that in a robust replication system such as infection of polarized HBECs, a moderate slow-down of viral entry does not translate into significant reduction of viral titres.

The effect of p38 MAPK inhibition has been investigated in mouse models of avian and swine influenza [17, 18]. Animals infected with H5N1 A/Thailand/KAN-1/2004 and treated with a p38 MAPK inhibitor had reduced body weight loss and improved survival outcomes [17] which, although more
Fig. 5. Haematoxylin and eosin-stained sections representative of the extent of inflammation on day 7 p.i. (asterisks). (a) Influenza H1N1 at 150 p.f.u./mouse+vehicle. (b) Influenza H1N1 at 150 p.f.u./mouse+BCT197 at 18 mg kg$^{-1}$ QD. (c) Influenza H1N1 at 150 p.f.u./mouse+oseltamivir at 5 mg kg$^{-1}$ BID. (d) Influenza H1N1 at 150 p.f.u./mouse+dexamethasone at 3 mg kg$^{-1}$ QD. There was a similar extent of inflammation between control (a), BCT197 (b) and oseltamivir (c) treated animals and decreased severity of alveolar-interstitial and peribronchial/perivascular inflammation with administration of dexamethasone (d). Inset of (a) is higher magnification depicting an area of bronchiolar epithelial degeneration and necrosis (br) with mixed cell inflammation (mainly macrophages and neutrophils). (f) Higher magnification depicting an area of bronchiolar epithelial degeneration and necrosis with mixed cell inflammation (mainly macrophages with fewer neutrophils) and perivascular edema. Scale bar 1 mm (a–d); scale bar 60 microns (f).
Fig. 6. Levels of IL-1β (a), TNFα (b), IL-6 (c), KC (d), RANTES (e), IFNγ (f) and IL-10 (g) present in BALF 2, 4, 7 and 9 days p.i. Data presented as mean±SEM. *, P<0.05; **, P<0.01; ***, P<0.001 versus vehicle n=2–8 animals/group.
marked, is a finding similar to that following BCT197 treatment. Unlike BCT197 in our influenza model, significant reduction of multiple cytokines was observed 2 days p.i. after SB 202190 treatment. Wei et al. [18] reported that treatment with SB203580 significantly reduced lung tissue cytokine levels compared to H9N2 A/swine/HeBei/012/2008-infected controls, although <2-fold reduction in cytokine levels were observed. Slightly less histopathological lesions occurred following treatment, as well as a non-significant reduction in lung edema as measured by wet/dry ratio. Although the study was conducted for 14 days, the authors make no report on the effect of infection and treatment on the body weight of the animals. Despite using a different influenza strain, Börgeling et al. [17] demonstrated that SB203580 did indeed improve body weight loss and survival. One thing of note is that both of these studies began treatment with p38 MAPK inhibitor either before or directly after inoculation with influenza. This is in contrast with BCT197 which was administered therapeutically, 24h p.i. This may, in part, explain the differences between the treatments on cytokine reduction.

Dose may also play a role in the therapeutic efficacy of p38 MAPK inhibition, as indicated by differences in inflammatory responses observed between the two different dosing groups of BCT197 in our influenza model. A randomized, adaptive design, double-blind, placebo-controlled, parallel-group multicenter trial evaluating the effect of p38 MAPK inhibitor PH-797804 in adults with mild to moderate COPD, found a significant difference in FEV1 (compared to the placebo) was achieved treating with 3 and 6mg PH-797804 but not 10mg [19]. A more in-depth analysis of drug exposure and response in our model may help elucidate the differences between dose and effect and elucidate any differential effects of dose on p38 MAPK signalling pathways. Dose may also play a role in the careful balance required between the reduction of aberrant inflammation and the prevention of infection. Although not significant, treatment with 18mg kg\(^{-1}\) BCT197 resulted in a trend towards an increased lung viral load 7 and 9 days p.i. when compared to vehicle. As with other treatments tested (and vehicle) viral load continued to decrease between days 7 and 9 suggesting viral clearance was not impaired. Clinically, PH-797804 treatment with 10mg had a higher percentage of COPD exacerbation (13%) compared to the 6mg (1%) and 3mg (6%) doses over the duration of the trial [19]. The cause(s) of the exacerbations are not recorded but may indeed highlight that a careful selection of dose is required.

Cases of severe influenza can be associated with a hyperinduction of pro-inflammatory cytokines (hypercytokinemia or ‘cytokine storm’) and the development of acute lung injury or acute respiratory distress syndrome [20]. There have been a number of studies investigating the effect of immunomodulatory agents in models of influenza or in humans to determine if modulating the immune response could change the impact on lung injury. For example, therapeutic treatment with a sphingosine analogue [21], a prostanoid [22], the host defense cationic peptide LL-37 [23] and a SIP1 agonist [24] have been shown to have beneficial effect on survival in mouse models of influenza. However, unlike BCT197 in our influenza model, these agents significantly reduced multiple cytokines and at more than one time point. All these compounds bar the prostanoid, were administered in a prophylactic manner, suggesting time of treatment in conjunction with mechanism of action may be important for exerting effects on cytokine levels in mouse models of influenza. Interestingly, dexamethasone has been previously shown to have minimal effects on inflammation in models of influenza at the low doses used [25, 26], but here we show treatment with this corticosteroid increased viral titres, neutrophils and cytokines present in BALF 7 days p.i. While this finding is not completely understood, increase in neutrophils after treatment with a corticosteroid has been previously observed in other infection models [27].

p38 MAPK inhibitors have not been tested clinically for influenza treatment. However, the use of corticosteroids, as immune modulators, in the clinical treatment of influenza has had mixed effects (dependent on time of administration during the disease progression and dose given). Quispe-Laime et al. in a small open label study [28] found that corticosteroids were well tolerated in severe influenza, while...
other much larger studies [29–31] have demonstrated that corticosteroid treatment in severe influenza increased mortality or secondary infection. A recent meta-analysis [30] of 16 observational trials (3039 individuals), concluded that corticosteroid treatment of presumed influenza-associated complications is associated with a significantly increased risk of mortality (OR, 2.12; 95% CI, 1.36–3.29). However, to date, no large randomized clinical trial has been run with corticosteroids [32]. In humans, treatment of influenza with the approved antiviral zanamavir as compared with a placebo was associated with significant reduction of cytokines induced by influenza infection [33].

In conclusion, inhibition of p38 MAP kinase with BCT197, under the conditions tested, did not offer an improved outcome over dexamethasone or oseltamivir in terms of impacting viral replication or disease course in the mouse influenza model. However, treatment with BCT197 did improve the disease course when compared with vehicle treatment. While the use of inhibitors of innate inflammatory pathways may raise concerns of negative effects on infection regulation, the improved weight loss, survival and lack of impaired viral control (when compared to untreated animals) at BCT197 doses relevant to those being used in clinical trials in acute exacerbations of COPD is encouraging.

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Conflicts of interest
All authors were employed by Novartis at the time the work was performed.

Ethical statement
All studies were approved by the Institutional Animal Care and Use Committee of Novartis Institutes for BioMedical Research, Emeryville, CA, USA.

References


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