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Modulating Innate and Adaptive Immunity by (R)-Roscovitine: Potential Therapeutic Opportunity in Cystic Fibrosis

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Key Words

CFTR · Corrector · Cystic fibrosis · Infection · Inflammation · Innate immunity · *Pseudomonas aeruginosa* · Roscovitine · Seliciclib · TRPC6

Abstract

(R)-Roscovitine, a pharmacological inhibitor of kinases, is currently in phase II clinical trial as a drug candidate for the treatment of cancers, Cushing's disease and rheumatoid ar-

thritis. We here review the data that support the investigation of (R)-roscovitine as a potential therapeutic agent for the treatment of cystic fibrosis (CF). (R)-Roscovitine displays four independent properties that may favorably combine against CF: (1) it partially protects F508del-CFTR from proteolytic degradation and favors its trafficking to the plasma membrane; (2) by increasing membrane targeting of the TRPC6 ion channel, it rescues acidification in phagolysosomes of CF alveolar macrophages (which show abnormally high pH) and consequently restores their bactericidal activ-

ity; (3) its effects on neutrophils (induction of apoptosis), eosinophils (inhibition of degranulation/induction of apoptosis) and lymphocytes (modification of the Th17/Treg balance in favor of the differentiation of anti-inflammatory lymphocytes and reduced production of various interleukins, notably IL-17A) contribute to the resolution of inflammation and restoration of innate immunity, and (4) roscovitine displays analgesic properties in animal pain models. The fact that (R)-roscovitine has undergone extensive preclinical safety/pharmacology studies, and phase I and II clinical trials in cancer patients, encourages its repurposing as a CF drug candidate.

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Introduction

Cystic fibrosis (CF) is a genetic disease affecting the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) ion channel (7q31.2, 1,480 amino acids, 168 kDa), allowing the passage of chloride and bicarbonate ions across the apical membrane of epithelial cells. The CFTR channel displays five domains: two hydrophobic membrane-spanning domains (each constituted of six transmembrane helical segments), two hydrophilic nucleotide-binding domains (NBD) and a cytoplasmic regulatory domain which is encoded by exon 13 and contains numerous charged residues and most of the potential phosphorylation sites. The most frequent mutation site (F508del) is localized in NBD1. Loss of function of CFTR translates into pulmonary problems, including dehydration and overproduction of mucus, respiratory difficulties, chronic infection (*Pseudomonas aeruginosa* in particular) and inflammation. Good overviews on various aspects of CF can be found in several recent reviews [1–6].

Lung damage secondary to chronic infection is the main cause of death in CF patients. Treatment of lung disease to reduce the impact of dysregulated innate immunity, infections, inflammation and subsequent lung injury is, therefore, of major importance [7–13]. Improved survival and increased mean age of CF patients worldwide is encouraging [14], but pulmonary infections remain the main problem for CF patients, as mortality in CF directly relates to compromised respiratory function. Despite some progress in the treatment of CF in recent years, transplantation remains the only therapeutic option for subjects reaching the terminal phase of pulmonary disease. Currently, conventional medical treatment has little to offer to these late-stage CF patients and effective new agents need to be identified. The current devel-

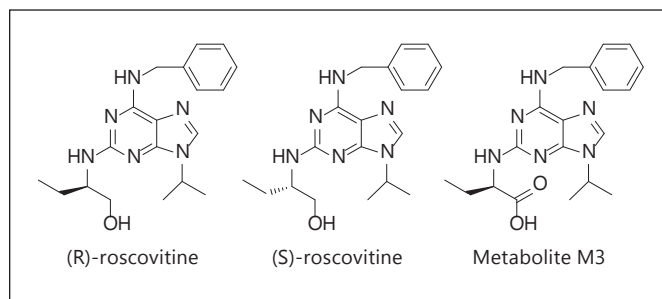


Fig. 1. Structure of the two isomers of roscovitine and M3, the major metabolite of (R)-roscovitine. Isomer R of roscovitine has been developed as a cancer drug candidate under the name seliciclib (CYC202).

opment of new drugs with antimicrobial or anti-inflammatory properties, and the recent discovery and use of CFTR correctors and potentiators provide increasing hope for the treatment of CF [15–23].

We here review recent evidence showing that roscovitine, a protein kinase inhibitor developed as a clinical phase II anticancer drug, rescues the trafficking defect of the F508del-CFTR protein, positively affects various aspects of the biology of innate immune cells, leading to potentiation of the antimicrobial defense and down-regulation of the inflammatory process, and displays analgesic properties. This body of results advocates in favor of the evaluation of roscovitine for the treatment of CF.

Roscovitine: A Wide-Potential Kinase Inhibitor

The 2,6,9-trisubstituted purine (R)-roscovitine (referred to as roscovitine above and in the rest of the article; fig. 1) was discovered in 1997 as a pharmacological inhibitor of cyclin-dependent kinases (CDKs) [24, 25; reviews: 26–29], a class of regulators essential for cell division and other major cellular functions [reviews: 29–31]. Its selectivity has been extensively studied: it interacts with various CDKs, casein kinases (CK1), dual specificity tyrosine phosphorylation-regulated kinases (DYRKs) as well as with pyridoxal kinase [32–34]. Roscovitine was cocrystallized with CDK2, CDK5, CDK9 and pyridoxal kinase [24, 33, 35, 36].

Roscovitine has been patented (its synthesis and some derivatives) in the USA, Europe and Japan for several applications [37]. The ‘Centre National de la Recherche Scientifique’ (CNRS) holds exclusive rights to the patent that applies to cancers, infections and inflammatory dis-

eases as granted to Cyclacel Pharmaceuticals. A second patent covers the use of roscovitine for the treatment of cerebrovascular conditions (e.g. stroke) and was licensed by the CNRS to Neurokin [38]. Finally, a third patent proposing the use of roscovitine for the treatment of CF was purchased from the CNRS and the University of Poitiers by ManRos Therapeutics [39]. The synthesis of roscovitine and related analogues has been largely described and optimized [40].

The therapeutic potential of roscovitine has been evaluated for numerous medical and veterinary indications. In addition to cancer, we can cite stroke [41], Parkinson's disease [42], Alzheimer's disease [L.H. Tsai, pers. commun.], cranial trauma [43], pain signaling (see 'Roscovitine Has Analgesic Properties', below), various viral infections [44], polycystic kidney disease [45, 46], glomerulonephritis [47–50], glaucoma [51, 52], Lambert-Eaton syndrome [53–55], deafness [56], Timothy syndrome [57–59], fibrosis [60], Cushing's disease [61, 62] and diabetes [63]. These studies have made it to preclinical trials, with the exception of glaucoma, glomerulonephritis and Cushing's disease, where roscovitine entered clinical trials. In cancer research, Cyclacel Pharmaceuticals has conducted preclinical, clinical phase I [64–67] and clinical phase II [68] trials with roscovitine under the name seliciclib or CYC202. Non-small cell lung cancer, breast cancer and nasopharyngeal cancer have been the main indications [68]. Recently, roscovitine has entered clinical trials for the treatment of Cushing's disease [61, 62, 69] and rheumatoid arthritis [70]. In the animal breeding field, roscovitine has been used as a tool to synchronize nucleus donor cells for the cloning of numerous mammals [71, 72].

Kinetic biodistribution analysis in rats revealed that the highest area under the curve for roscovitine was observed in the lungs [73]. Several mouse models of lung inflammation or injury were efficiently treated with roscovitine by intraperitoneal administration: bleomycin-induced lung injury [74], lipoteichoic acid- and *Streptococcus pneumoniae*-induced lung inflammation [75], and lung injury induced by mechanical ventilation [76]. Furthermore, roscovitine has been evaluated in phase IIa clinical trials against non-small cell lung cancer, where a substantial increase in overall survival was observed (388 vs. 218 days in the placebo arm) despite no difference in progression-free survival [68]. Altogether, these data demonstrate that the lung is a viable target for roscovitine.

Roscovitine is orally bioavailable in man [66–68] and rodents [73, 77]. Once in the organism, roscovitine is rap-

idly metabolized by the liver, essentially by oxidation [27]. The main metabolite is the carboxylate product (M3; fig. 1) [66, 67, 73, 77], which does not inhibit the kinases targeted by roscovitine but may account for other effects of the drug [77–79]. Although the half-life of M3 in humans is similar to that of roscovitine [66, 67], its CF-favorable biological activity could extend that of roscovitine in CF treatment. Alternatively, the M3 compound could feasibly be developed as a drug candidate per se. Indeed, since it is essentially 'kinase dead', the toxic effects of roscovitine associated with its antiproliferative effects should thus be considerably reduced, permitting chronic administration of M3 over long periods and/or an increase in the treatment dose. As inhibition of CDKs appears to be important regarding the effects exerted by roscovitine on neutrophils, this could limit the anti-inflammatory action of M3 on CF, thus normalizing the inflammatory response in CF rather than completely abrogating it. It seems, therefore, possible to envisage the development of M3, or one of its analogues, as an alternative CF drug candidate derived from roscovitine.

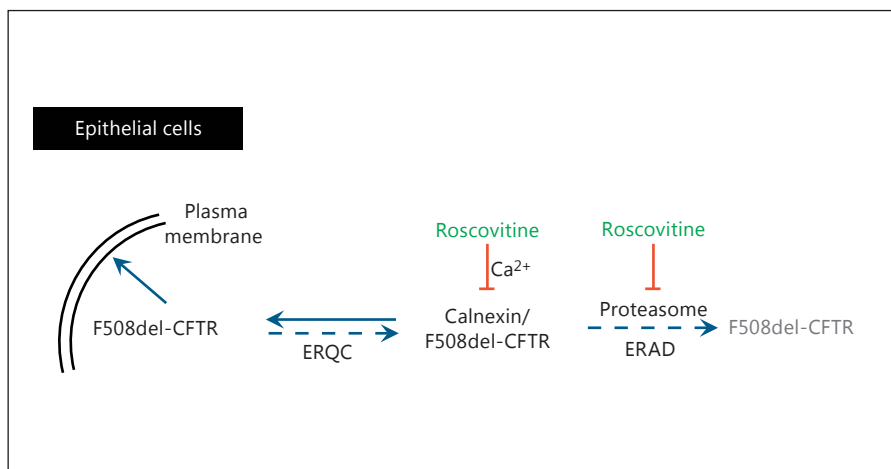
Roscovitine and CF

Roscovitine Protects the Chloride Channel F508del-CFTR from Proteolytic Degradation and Acts as a Corrector for Its Membrane Localization

The described CFTR mutations are grouped into class I (mutations leading to lack of CFTR protein synthesis), class II (mutations leading to anomalies in CFTR processing, such as disruption of folding and trafficking to the surface), class III (mutations leading to defective regulation or gating of CFTR), class IV (mutations leading to defective chloride conductance) and class V (mutations leading to alternative splicing and production of insufficient quantity of CFTR polypeptide) [17, 18]. Alterations in CFTR activity in CF, thus, originate from different causes, depending on the type of mutation. Although 2002 mutations have been described in CFTR (<http://www.genet.sickkids.on.ca/StatisticsPage.html>), deletion of the codon corresponding to phenylalanine 508 (F508del-CFTR) is by far the most frequent, representing almost 70% of all CF cases. Only five other mutations (G542X, G551D, W1282X, N1303K and R553X) represent more than 1% of all CF cases. All other mutations are rare and even exceptional, often uniquely detected in a single family.

The F508del-CFTR protein is expressed normally but, due to misfolding, it is not transported to the apical mem-

Fig. 2. Roscovitine corrects the trafficking defect of F508del-CFTR by regulating its proteolytic degradation. Abnormally folded F508del-CFTR is taken up by the ER quality control (ERQC) system. It binds to the calnexin chaperone in a calcium-dependent manner. The complex is then taken up by the ER-associated degradation (ERAD) pathway for proteolytic degradation by the proteasome. Roscovitine depletes ER Ca^{2+} stores, reducing the interaction of F508del-CFTR with calnexin. In addition, roscovitine lowers proteasomal activity in a Ca^{2+} -independent manner. Altogether, this favors the stabilization of F508del-CFTR and its trafficking to the plasma membrane.



brane of epithelial cells. The mutation is temperature sensitive, meaning that physiological activity of F508del-CFTR is partially restored when cells are cultured at a low temperature (27°C). This is probably linked to proper folding, partial restoration of trafficking and correct translocation to the plasma membrane. The F508del-CFTR protein is, thus, potentially functional but, at physiological temperatures, deletion of F508 prevents the correct folding and subsequent correct localization of CFTR to the plasma membrane. Correctors are usually low-molecular-weight molecules that allow the localization of F508del-CFTR to the plasma membrane. Correctors are diverse in terms of chemical structure, mechanism of action and potency to rescue the abnormal trafficking and function of F508del-CFTR. However, all of these compounds are only partial correctors [15–19]. We have recently shown that roscovitine also acts as a partial corrector of F508del-CFTR [78]. This corrector effect seems to originate both from a negative effect on the recognition of F508del-CFTR by the endoplasmic reticulum (ER) conformation-based quality control pathway and from a partial inhibition of F508del-CFTR proteolysis by the ER-associated degradation pathway (fig. 2). Depletion of ER Ca^{2+} stores by roscovitine reduces the Ca^{2+} -dependent interaction of F508del-CFTR with calnexin, preventing F508del-CFTR to be taken up by the ER-associated degradation pathway to proteolysis. In parallel, roscovitine reduces proteolytic degradation of F508del-CFTR by the proteasome in a Ca^{2+} -independent manner. This increases the availability of F508del-CFTR for translocation to the plasma membrane (fig. 2). The resulting corrector effect does not require the kinase-inhibitory activities of roscovitine as M3, the main hepatic metabolite of roscovitine

(fig. 1) [73, 77], also displays corrector properties. Furthermore, other roscovitine derivatives which are active on kinases (CR8, olomoucine) do not show a corrector activity. Recently, a screen to detect potential correctors among a chemical library of 231 kinase inhibitors revealed several corrector products (active at $10\ \mu\text{M}$), notably kenpaullone and alsterpaullone, two inhibitors of CDK/GSK-3 [80]. These compounds, which we also identified as correctors [unpubl. results], were developed during a long-term collaboration between our laboratory in Roscoff and Prof. Conrad Kunick's team in Braunschweig [81].

Roscovitine Reduces the Intraphagolysosomal pH in CFTR-Deficient Macrophages and Restores Their Bactericidal Properties

For several years, treatment of CF has aimed at correcting the epithelial defect due to CFTR absence or dysfunction. Several lines of evidence are converging to a novel paradigm of a dysregulated innate immunity resulting in the defects in bacterial clearance observed in CF [7–12]. Pivotal to these processes are neutrophils and macrophages [12].

Intraphagolysosomal pH and Bactericidal Abilities of Macrophages

The intraphagolysosomal pH of CFTR-deficient or *cfr-/-* macrophages has been shown to be abnormally high (pH 6.5–7.2) when compared to the intraphagolysosomal pH of non-CF macrophages (pH 4.5–5.2; fig. 3a, b) [82]. Neither the phagocytic capacity of macrophages nor the fusion of phagosomes with lysosomes are affected by the mutant CFTR [82]. However, bacteria, once phagocy-

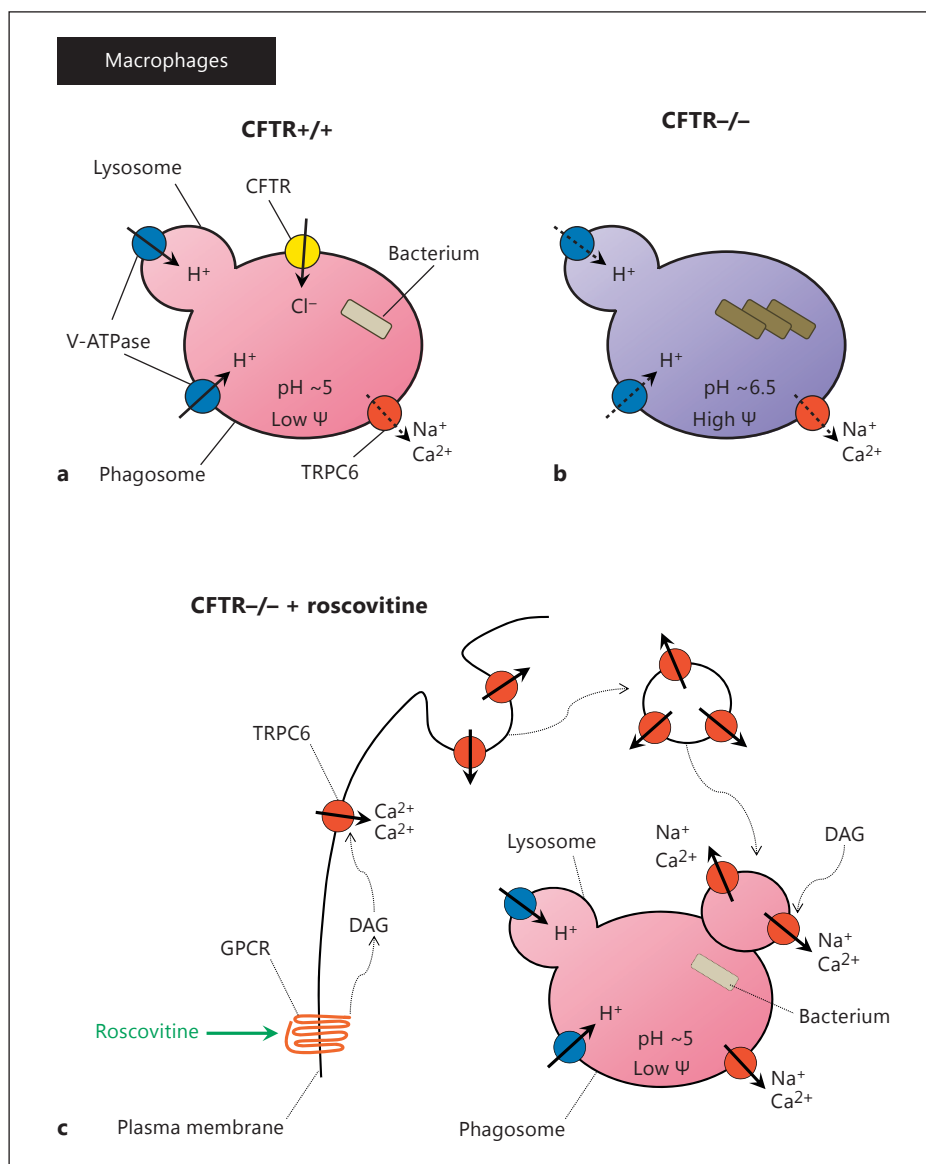


Fig. 3. Schematic overview of TRPC6 rescue of microbicidal activity in CFTR-deficient AMs through GPCR (G protein-coupled receptor) activation with (R)-roscovitine. Ionic fluxes in alveolar phagosomal membranes are permissive for intraluminal acidification and the development of a microbicidal environment. GPCR stimulation with (R)-roscovitine sets sequential intracellular signaling events in motion, leading to vesicle-mediated TRPC6 translocation and insertion. Calcium-dependent TRPC6 insertion into the plasma membrane and subsequent uptake into phagosomes determines the production of an intraluminal microbicidal environment. **a** CFTR^{+/+} AMs. Phagosomal acidification is driven by the flow of protons into the phagosomal lumen through V-ATPase activity, which, if uncompensated, produces charge buildup in the confined intraluminal compartment. Charge compensation is provided by Cl⁻ influx through CFTR allowing for a decrease in phagosomal membrane potential and enhanced acidification. The phagosomal lumen pH is approximately 5, and the phagosomal membrane potential (Ψ) is low (approx. +28 mV). The acidified

phagosomal lumen supports the proteolytic activity of lysosomal enzymes, leading to bacterial killing. **b** CFTR^{-/-} AMs. The absence of a Cl⁻ influx pathway reduces the level of acidification, and the phagosomal lumen pH reaches near-neutral levels, leaving a high phagosomal membrane potential. The lack of an acidified phagosomal lumen prevents bacterial lysis and supports bacterial growth. The elevated phagosomal membrane potential reduces proton movement into the phagosomal lumen. **c** CFTR^{-/-} AMs exposed to roscovitine. Recruitment of the cation channel TRPC6 to the plasma membrane and subsequently to the phagosomal membrane upon particle engulfment provides an alternative charge shunt pathway in the absence of CFTR expression. Activation of TRPC6 in the phagosomal membrane by (R)-roscovitine-generated diacylglycerol (DAG) opens a cation exit pathway from the phagosomal lumen acting as an alternate charge shunt, thereby allowing for pH regulation and acidification. The phagosomal pH is maintained at a level of approximately 5 and the membrane potential is low. These conditions support microbicidal activity [adapted from 79].

tosed, are not destroyed in the phagolysosomes [82, 83]. Bacteria are even able to multiply within the phagolysosomes [83], which sit at neutral pH far from the normal acidic pH optimum for lysosomal lipases and proteases. As a consequence, the inability of F508del-CFTR macrophages to destroy bacteria can aggravate infections that affect CF patients. Elevation of intraphagolysosomal pH by 2 units is also observed in *cftr*^{-/-} macrophages which completely lack expression of this ion channel.

The pH abnormalities in CF are now being documented in different cellular compartments, cells and tissues [82–89] despite earlier resistance in certain camps [90–94]. Recent findings from Zhang et al. [88] identified a population of secretory lysosomes that exhibit a higher pH in alveolar macrophages (AMs) deficient in *cftr*^{-/-} than in wild-type lung macrophages. The role of CFTR in bacterial clearance in the lung is underscored in recent studies on CF pigs which develop human-like CF lung disease [95–97]. Newborn pigs do not exhibit signs of airway inflammation but already display a defect in their ability to eliminate bacteria, which leads to the accumulation of bacteria in the lungs [reviewed in 98]. These studies provide further validation for our data which established that AMs express functional CFTR and cells from CFTR null as well as mutant mice exhibit defective bactericidal activity [82, 86]. The cause of this deficiency is apparently a failure of lysosomes and phagosomes to acidify properly in the knockout model. The severity of the acidification phenotype scales with the mutant genotype with F508del-CFTR being the most severe [86]. Phagocytosis per se is not affected and it does not appear that CFTR affects phagolysosomal fusion or reactive oxygen species production. Interestingly, only AMs showed a dependence of lysosomal acidification upon CFTR expression. Recently published data demonstrate that the macrophage tissue source determines dependence of intracellular acidification on CFTR expression [86]. We surmise that other Cl⁻ channels may play a similar role in phagosomal function in other innate immune cells. Mice null for CLC-3 are susceptible to sepsis, and Moreland et al. [99] suggested that CLC-3 is crucial for normal host defense by mechanisms that may involve phagocytic and secretory behavior in neutrophils, observations which are in conflict with those of Painter et al. [100–102], who maintained that CFTR mediates halide transport in human neutrophils.

In collaboration with ManRos Therapeutics, the University of Chicago group (V.R., A.G.G. and D.N.) demonstrated that roscovitine reduces the intraphagolysosomal pH of F508del-CFTR macrophages by more than 1 unit

(fig. 3c). This effect is also observed with the M3 metabolite of roscovitine. We initially thought that roscovitine was acting as a corrector in F508del-CFTR AMs with the F508del-CFTR being addressed to the phagolysosome membranes following uptake of the bacterial cargo, and thus correcting the intraphagolysosomal pH. Unexpectedly, roscovitine also reduced the intraphagolysosomal pH of *cftr*^{-/-} macrophages. This demonstrates that the CFTR channel is not involved in the acidifying effect of roscovitine in phagolysosomes. This effect could thus, in principle, take place in any macrophage that shows a neutral intraphagolysosomal pH linked to a functional inactivation of CFTR, in other words independently of the mutation involved. In terms of therapeutic applications, this signifies that roscovitine could, therefore, have a macrophage phagolysosomal pH-correcting effect in many forms of CF, regardless of the mutation affecting the CFTR gene and channel functionality.

The consequence of the intraphagolysosomal pH rescue, even if partial, is a marked improvement in the ability of *cftr*^{-/-} and F508del-CFTR macrophages to eliminate *P. aeruginosa*, the major pathogen in CF (fig. 4). Improvement in the bactericidal properties of these macrophages lacking CFTR or functional CFTR, by treatment with roscovitine, is therefore independent of CFTR and – perhaps more importantly – of the antibiotic resistance profile of bacterial isolates. Roscovitine could, therefore, have a general bactericidal effect on most CF forms. Improvement in the bactericidal properties is observed with the M3 metabolite, S-CR8, N⁶-methyl-roscovitine and O⁶-benzyl-roscovitine, but is not observed with S-roscovitine, miglustat, olomoucine, finisterine, perharidine or purvalanol A [79].

Molecular Mechanisms of Action: Indirect Targeting of the TRPC6 Ion Channel

Recent results suggest that the effects of roscovitine on the intraphagolysosomal pH of macrophages could be explained by an action mediated by the Ca²⁺-permeable channel TRPC6 [79]. TRPC6 belongs to the TRP (transient receptor potential) family of ion channels, particularly important in respiratory system diseases. The TRP family comprises 28 members, which are grouped into several different classes: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPA (ankyrin) [reviewed in 103–108]. The TRPC channels comprise 6 members, TRPC1, TRPC3, TRPC4, TRPC5, TRPC6 and TRPC7. TRPC6 is a channel activated by diacylglycerol derived from the hydrolysis of phospholipids (phosphoinositides) by phospholipase C.

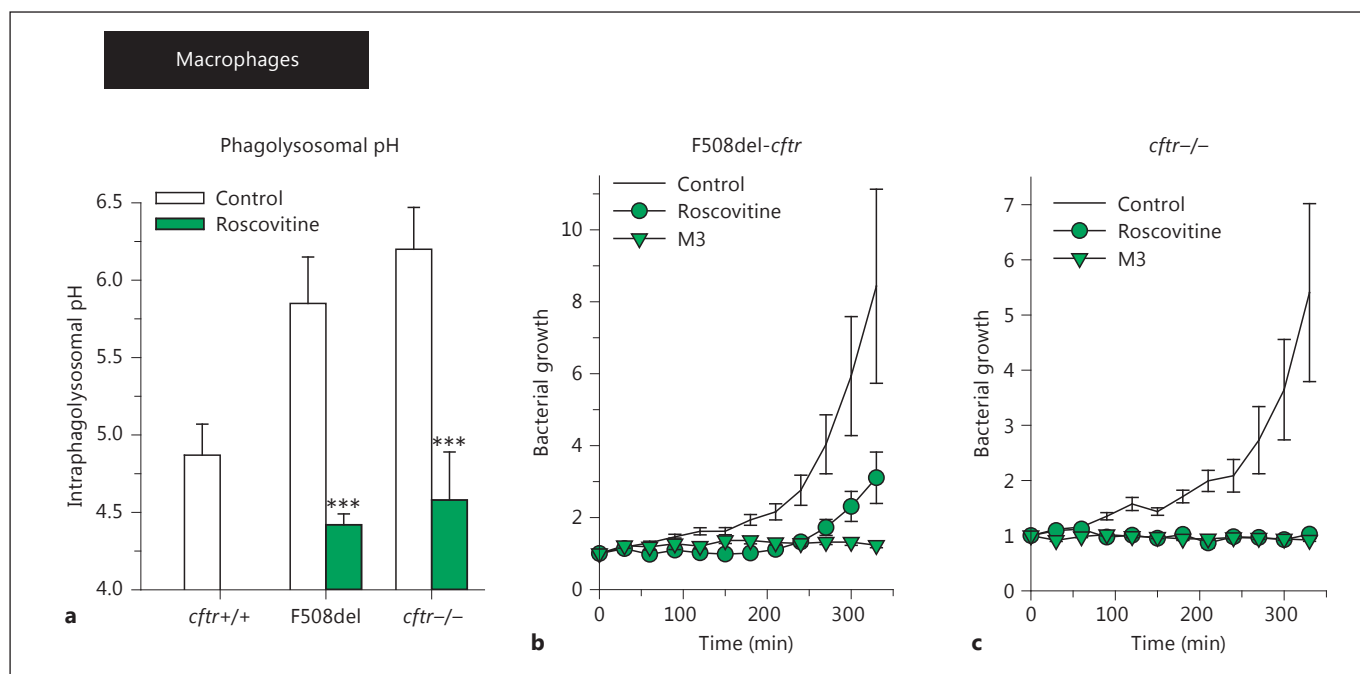


Fig. 4. Roscovitine acidifies the phagolysosomes of F508del-CFTR and *cftr*^{-/-} macrophages and prevents bacterial growth (**b, c**). **a** Intraphagolysosomal pH in *cftr*^{+/+}, F508del-CFTR or *cftr*^{-/-} mouse AMs. Mutation or absence of CFTR leads to increased pH. Exposure to roscovitine results in phagolysosomal acidification. Means \pm SEM. *** $p < 0.001$ vs. control (two-way ANOVA). **b, c** F508del-CFTR (**b**) or *cftr*^{-/-} (**c**) AMs were exposed to DsRed-labeled bacteria, and fluorescence intensity at 607 ± 20 nm was

monitored over time following exposure to 20 μ M roscovitine, M3 or corresponding amounts of vehicle (control). Bacterial growth is shown as fold increase in DsRed fluorescence. Summary data from at least 3 separate experiments [adapted from 79]. Mean fluorescence intensities \pm SEM. Bacterial growth is prevented by *cftr*^{+/+} AM (data not shown), but not by F508del-CFTR or *cftr*^{-/-} AM. Bacterial growth is prevented by both roscovitine and its metabolite.

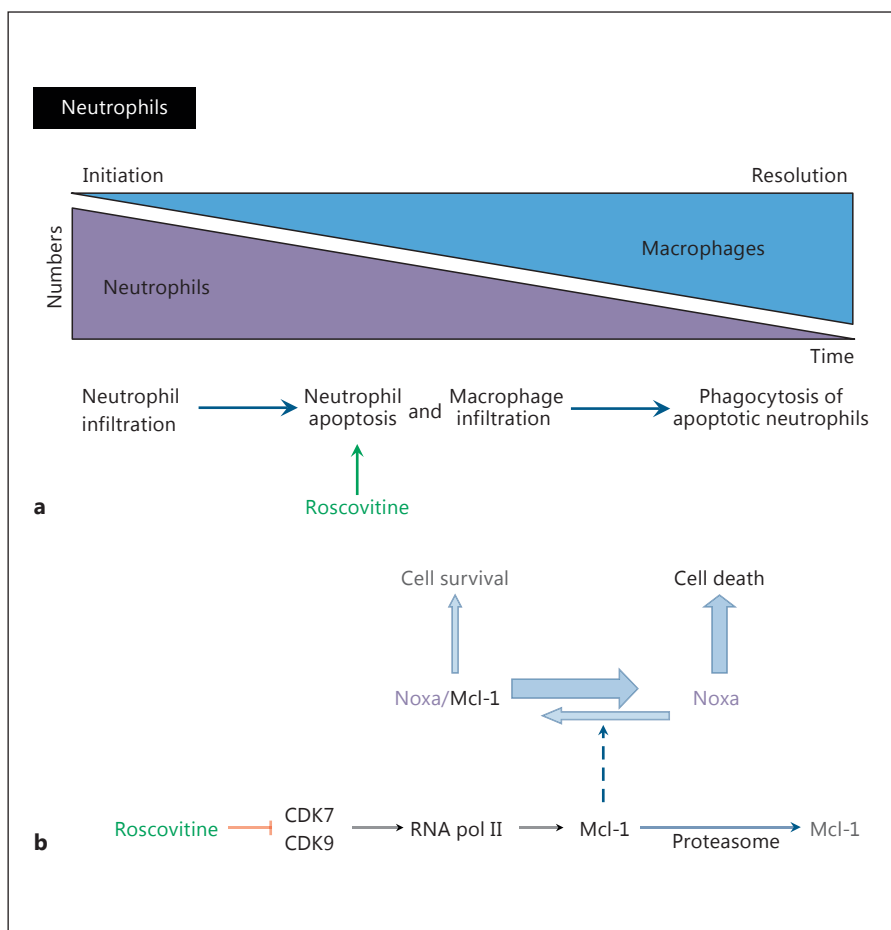
TRPC6 is expressed in cells implicated in inflammation and innate immunity, neutrophils [109–111] and macrophages [112, 113]. TRPC6 is highly expressed in the lungs. Its expression is increased in macrophages from patients with chronic obstructive pulmonary disease and pulmonary hypertension [112, 113]. Activation of TRPC6 is implicated in pulmonary edema (lung ischemia-reperfusion-induced edema) [114]. Deletion of TRPC6 in mice (*trpc6*^{-/-}) specifically inhibits pulmonary inflammatory reactions of allergic origin [115]. Few antagonists and agonists of TRPC6 have been described; they generally display low efficiency [116]. Hyperforin (from the St. John herb or St. John's wort) is an activator of TRPC6 [117]. A series of TRPC6 channel antagonists has been described by Sanofi [118]. Work by Antigny et al. [119, 120] suggested that TRPC6 activity is regulated by the CFTR channel. The physical interaction of the two channels leads to an inhibition of calcium entry through TRPC6. On the other hand, F508del-CFTR is unable to interact with TRPC6, and this would lead to excessive activation of

TRPC6 and abnormal entry of calcium. The influx of calcium can be normalized once the trafficking of F508del-CFTR is corrected (miglustat) or by anti-TRPC6 siRNA [119, 120]. Our results show that roscovitine acts as an indirect activator of the TRPC6 channel, independently of CFTR channel expression or mutation. Roscovitine induces the production of diacylglycerol, which activates the translocation of TRPC6 calcium channels to the plasma membrane. Following phagocytosis, TRPC6 channels are integrated in the phagosomal membrane and contribute to cation depletion inside the phagolysosomes, thus amplifying intraphagolysosomal acidification due to vacuolar-type (V)-ATPase (which, by hydrolyzing ATP, allows proton entry). This effect is responsible for the intraphagolysosomal acidification of macrophages (fig. 3c).

Roscovitine Displays Anti-Inflammatory Properties Effects on Neutrophils

Neutrophils represent the first line of defense against microbes but are also powerful proinflammatory cells

Fig. 5. Roscovitine and neutrophils. **a** The different steps of inflammation: initiation by neutrophil infiltration is followed by neutrophil apoptosis. Macrophage infiltration then allows phagocytosis of apoptotic neutrophils and resolution of inflammation [adapted from 159]. Neutrophils from CF patients appear to be partially protected from apoptosis. Roscovitine induces apoptosis of neutrophils, improving their elimination by macrophages and thus favoring the resolution of inflammation. **b** Proposed molecular mechanisms underlying induction of apoptosis by roscovitine [adapted from 36]. The cell survival/cell death balance is maintained by Mcl-1, a Bcl-2 family member survival factor that binds to and neutralizes proapoptotic proteins such as Noxa. Mcl-1 is a short-lived protein being constantly synthesized (through RNA polymerase 2, itself under the control of CDK7/cyclin H and CDK9/cyclin T) and constantly degraded (through Mcl-1 ubiquitin ligase and proteasome). Roscovitine inhibits CDK7 and CDK9, preventing phosphorylation of RNA polymerase 2 at Ser-2 and Ser-9, respectively. Consequently, mRNA synthesis is transiently inhibited, and short-lived mRNAs and proteins are down-regulated. This is the case for Mcl-1. Reduction in the Mcl-1 protein level results in a transient increase in free Noxa protein, which triggers Bax/Bak-dependent apoptotic cell death.



able to injure host tissue (fig. 5a). CF constitutes a representative example of a pathogenic condition in which the deleterious power of neutrophils is at work, with uncontrolled activated neutrophils unable to kill invading bacteria [121]. This particular picture has led to a still unresolved neutrophil conundrum in CF, i.e. whether neutrophils could be genetically modified to display such a proinflammatory phenotype [9]. Indeed, as in macrophages [89] and monocytes [122], CFTR is expressed in neutrophils and regulates bactericidal activity [101, 123–125].

Neutrophil extracellular traps (NETs) contribute to inflammation in a number of diseases, such as systemic lupus erythematosus [126] and inflammatory arthritis [127], and have been described in CF [128–130]. Furthermore, *P. aeruginosa* can induce NETosis [127, 131–134]. Oxidative burst and NADPH oxidase activity are central to the process of NET formation, with myeloperoxidase and neutrophil elastase acting as essential cofactors [135, 136]. The importance of NETs in the killing of

pathogens is a matter of concern and has recently been challenged by a report showing that neutrophils from patients with the Papillon-Lefèvre syndrome lacking serine proteinases and unable to produce NETs did not show any defect in bacterial killing [137]. Oxidative burst causes downstream activation of peptidyl dearginase (PAD4), which in turn translocates to the nucleus and hypercitrinulates histones, leading to nuclear decondensation [138]. NETosis is entirely distinct from apoptosis [139] but may involve the activation of autophagic pathways [140, 141]. The clearance of NETs, unlike that of apoptotic neutrophils, is poorly understood, with undegraded NET fragments promoting inflammation in systemic lupus erythematosus [142]. As such, the proinflammatory potential of NETs in the CF airway cannot be ignored.

A complex relationship exists between infections and inflammation in the lungs of CF patients [reviewed in 1–7, 143–146]. The persistence of neutrophils in CF lung that failed to clear bacterial infection and are not cleared

themselves by macrophages following apoptosis strongly points to the importance of innate immune cells in this process in CF. Furthermore, other forms of neutrophil death, such as NETosis, may be prevalent in the CF lung and contribute to lung damage and bacterial colonization [147, 148]. Of note is the modulation of immune responses to infections by proteases from neutrophils, especially through chemokines [149].

Through several structural and pharmacological properties, roscovitine targets innate immune cells via different mechanisms. The discovery that CDK inhibitors, such as roscovitine, could indeed favor the disposal of neutrophils by enhancing both their apoptosis and their phagocytosis by macrophages has opened a promising research field [74, reviews in 150, 151]. Roscovitine resolves the inflammatory response in various animal models [74, 150]. This activity is linked to the proapoptotic action of roscovitine on neutrophils (fig. 5a). The molecular mechanism is likely to implicate inhibition of CDK7 and CDK9 [152], which leads to reduced expression of the cell survival and anti-apoptotic factor Mcl-1 [153, 154], an effect we have also observed while analyzing the anticancer activity of roscovitine and its derivatives (fig. 5b) [155]. Roscovitine also inhibits the production of nitric oxide and inhibits the activation of NF κ B in macrophages [156, 157]. Inhibition of the NF κ B pathway by CR8, an analogue of roscovitine, was also observed in chronic lymphocytic leukemia cells [158]. The anti-inflammatory effect of roscovitine via enhanced apoptosis of neutrophils was confirmed in a zebrafish inflammatory model [159, 160], a mouse model with pulmonary inflammation induced by *S. pneumoniae* and lipoteichoic acid (a proinflammatory constituent of Gram-positive bacteria) [75], a mouse model of ventilator-induced lung injury [76] and an experimental mouse model of pneumococcal meningitis [161]. It was also shown that roscovitine, by inhibiting CDK2 and CDK5, blocks endothelial activation and leukocyte/endothelial cell interactions, contributing to the anti-inflammatory effect [162]. One of the specificities of CF is the persistence of an enormous burden of neutrophils in the airways [163, 164, reviewed in 165, 166]. In spite of the overabundance of neutrophils, the deficit in antimicrobial activity results in a chronic *P. aeruginosa* infection suggesting defective innate immunity [9, 10, 166–168]. In vitro studies show that the apoptotic death of neutrophils from CF patients is delayed, thus impairing their elimination by macrophages and promoting inflammation [169–171]. The in vitro kinetics of apoptosis is slowed down in neutrophils from CFTR mutation heterozygote parents, as seen with

neutrophils of their children (homozygotes) [171]. In vitro, roscovitine restores a normal level of apoptosis in neutrophils isolated from CF patients [171]. Roscovitine-induced apoptosis of neutrophils and their progenitors has been linked to the Noxa-dependent degradation of Mcl-1, which liberates Bim and Puma, two activators of the proapoptotic factor Bax [172], which, interestingly, has been demonstrated to be deficient in CF [173]. Furthermore, roscovitine inhibits the proliferation of those progenitor neutrophils which managed to escape apoptosis [161].

The spectrum of biological activities of roscovitine is wide and, importantly, appears to be cell specific. For instance, the proapoptotic effect in neutrophils is extremely effective, while no such effect is observed in macrophages. Roscovitine effects should, thus, be studied in each cell type and might also depend on the type of CFTR mutation.

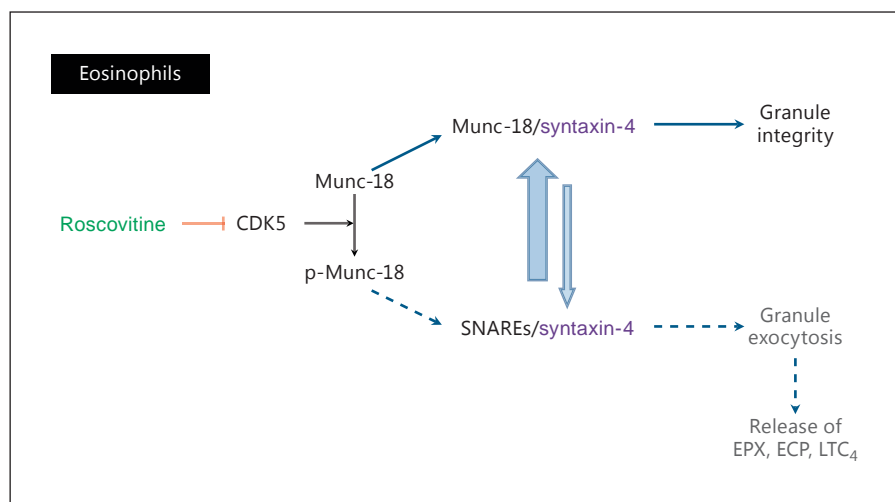
Effects on Eosinophils

In addition to neutrophils, eosinophils may participate to lung tissue injury in CF [174, 175]. The pathophysiological importance of eosinophils may be specifically relevant in CF patients showing allergic bronchopulmonary aspergillosis [176].

Upon stimulation, eosinophils release the content of their secretory granules, which comprise various toxic proteins, such as eosinophil cationic protein (ECP) and eosinophil peroxidase (EPX), and produce proinflammatory mediators, such as leukotrienes (LTC₄). Although their number remains stable in the peripheral blood and lung, eosinophils are activated in CF [177, 178], resulting in enhanced production of ECP and EPX [175–179] and LTC₄ [180] compared to healthy controls. Eosinophils isolated from CF patients release higher amounts of ECP than those of control, healthy patients [181]. ECP levels found in the sputum of CF patients reach levels similar to those able to induce pulmonary damage in vitro [179–181] and correlate with ions levels [182].

Eosinophils express various CDKs, CDK5 in particular [183, 184], and its activating partners p35 and p39 [185]. Upon eosinophil stimulation, CDK5 is phosphorylated on Ser-159, and its catalytic activity is increased [184]. This correlates with phosphorylation of one of its substrates, Munc-18 [184–186], release of syntaxin-4 and its binding to SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). The binding of syntaxin-4 to SNARE proteins allows interaction of vesicular R-SNAREs to plasma membrane Q-SNAREs, subsequent membrane fusion and

Fig. 6. Roscovitine and eosinophils. Proposed molecular mechanisms underlying the action of roscovitine on eosinophils. Under resting conditions, Munc-18 binds to syntaxin-4, preventing it from interacting with SNARE proteins. During inflammation, CDK5 catalytic activity is increased, leading to Munc-18 phosphorylation (p) and preventing the binding of syntaxin-4 to Munc-18. Syntaxin-4 is then free to bind SNAREs, allowing the fusion of intracellular granules to the plasma membrane and release of their contents (e.g. EPX, ECP and LTC₄) in the extracellular space. Roscovitine, by inhibiting CDK5, prevents the release of syntaxin-4 from Munc-18 and binding to SNAREs. Consequently, exocytosis is inhibited.



Color version available online

exocytotic degranulation [184]. Pharmacological inhibitors of CDKs, such as roscovitine and AT7519, or CDK5 siRNA, reduce EPX release by eosinophils activated by PMA (phorbol 12-myristate 13-acetate) or secretory IgA [184]. These results suggest that roscovitine, by inhibiting CDK5, may reduce degranulation of challenged eosinophils (fig. 6).

Finally, roscovitine also induces apoptosis (assessed by several techniques) of activated human eosinophils in vitro by reducing Mcl-1 expression [187], possibly by a mechanism involving inhibition of CDK7 and/or CDK9 [188]. Whether roscovitine, other CDK inhibitors or other agents that drive eosinophil apoptosis enhance the resolution of eosinophilic-dominant inflammation in vivo is under intense investigation [189, 190].

Altogether, these data suggest that roscovitine may reduce the number and secretory activity of eosinophils, an effect which is expected to be potentially beneficial to CF patients if seen in vivo.

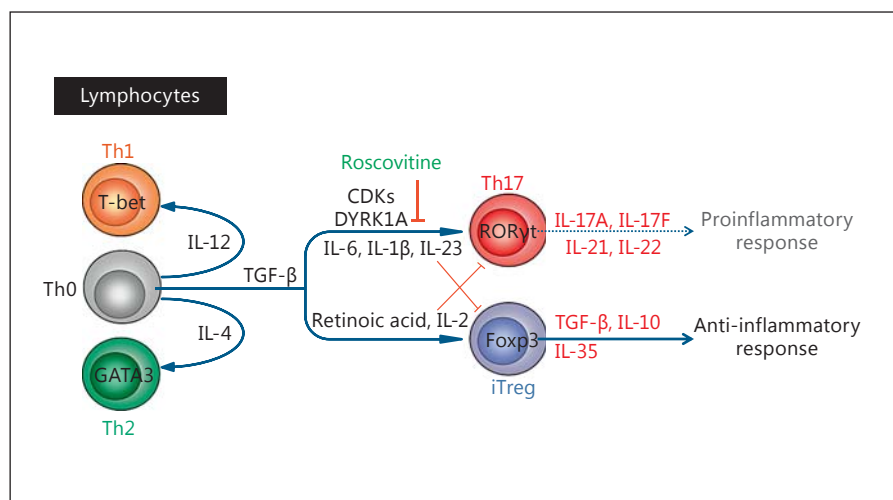
Effects on T Lymphocytes

CD4⁺ T-helper (Th) cells play a major role in immune responses. Once activated by antigens, these cells differentiate into different cell types, typically Th1 and Th2 lymphocytes, but also Th17 cells and iTregs (induced regulatory T cells; fig. 7). Proinflammatory Th17 cells are characterized by the production of IL-17A and play an important role in autoimmune diseases, cancer and elimination of extracellular bacteria. On the other hand, anti-inflammatory Treg cells play a key role in controlling immunological tolerance and in suppressing excessive immune responses deleterious to the host. There is an intricate link between iTreg and Th17 cell programs of dif-

ferentiation, which both require TGF- β (transforming growth factor- β). Upon activation in the presence of TGF- β , naive CD4⁺ T cells (Th0) can differentiate into either Th17 or iTreg cells, depending on the overall cytokine milieu [191]. Low or intermediate concentrations of TGF- β together with proinflammatory cytokines (IL-6, IL-1 β and IL-23) promote the differentiation of Th17 cells through expression of the nuclear receptor ROR γ t (retinoic acid-related orphan receptor). Such activation inhibits the expression and function of Foxp3, the transcription factor driving the Treg differentiation program. Conversely, in the absence of proinflammatory cytokines, high levels of TGF- β promote the expression of Foxp3 and differentiation of iTreg cells. This process is further enhanced by IL-2 and retinoic acid, and is associated with inhibition of ROR γ t expression and function. Th17 and iTreg cells thus reciprocally inhibit their differentiation (fig. 7) [192, 193].

Emerging evidence suggests that an imbalance of T-cell responses may contribute to CF pathophysiology. A role for the Th17 and Th2 T lymphocytes in chronic pulmonary inflammation in CF patients was recently proposed. Th0 cells from CF patients or mice show a predisposition to differentiate towards the proinflammatory Th17 phenotype, while normally having a propensity to differentiate into Th1 and Treg lineages [194]. High peripheral blood Th17 levels are associated with poor lung function in CF [195]. A specific profile of proinflammatory cytokines/chemokines (particularly IL-17A and IL-5) may be a risk factor for *P. aeruginosa* infection [196]. A link between the inflammatory background mediated by T cells and susceptibility to *P. aeruginosa* infection remains to be shown. IL-17A plays a major role in the

Fig. 7. Roscovitine and lymphocytes. Depending on external stimuli, CD4⁺ Th0 cells differentiate into Th1 (IL-12), Th2 (IL-4), Th17 (TGF- β , IL-6, IL-1 β and IL-23) and iTreg (TGF- β , IL-2 and retinoic acid) lymphocytes. The relative amounts of TGF- β , interleukins, retinoic acid and additional cytokines skew the differentiation of Th0 cells into either highly proinflammatory Th17 (through ROR γ t) or anti-inflammatory iTreg (through Foxp3) cells. Possibly through inhibition of CDKs and DYRK1A, roscovitine inhibits the differentiation into Th17 cells, lowering the production of proinflammatory interleukins (IL-17A, IL-17F, IL-21 and IL-22). Consequently, the balance of Th17/iTreg shifts towards the anti-inflammatory response.



recruitment, activation and migration of neutrophils in CF patients [197], its expression is increased in CF patients' sputum [198] and its overproduction has even been suggested as the cause of chronic lung inflammation in CF patients [199, 200]. Expression of IL-17 could serve as an early biomarker for *P. aeruginosa* infection [196]. A robust increase in Th17 lymphocytes (proinflammatory) together with enhanced Th2 responses and a decrease in Treg lymphocytes (anti-inflammatory), observed in *cfr-/-* mice, was coupled to susceptibility to infection by *Aspergillus fumigatus*. A reduction in the expression of indoleamine 2,3-dioxygenase (IDO), the first enzyme in the tryptophan degradation pathway, was observed in CF patients and in the *cfr-/-* mouse model. The imbalance of Th17 versus Treg is linked to the reduction in IDO activity. Inhibition of Th17 activation (IL-17A siRNA) or stimulation of the IDO pathways (kynurenines) restores protection against *A. fumigatus* [200]. Heightened Th2 responses in CF patients with allergic bronchopulmonary aspergillosis were associated with lower frequencies of Tregs compared with *A. fumigatus*-colonized CF patients without allergic bronchopulmonary aspergillosis [201]. A previous report suggested significantly lower percentages of circulating Tregs in children with CF, and a correlation between decreased frequencies of Tregs and lower FEV₁ [202]. A recent study further showed that patients with CF who have chronic *P. aeruginosa* infection show an age-dependent, quantitative and qualitative impairment in Tregs. Tregs isolated from CF patients or from *cfr-/-* mice showed reduced functional suppressive activity compared with Tregs from non-CF controls. Both 'extrinsic' *P. aeruginosa*-induced effects and 'intrinsic' CFTR-me-

diated Treg functional skewing contributed to Treg impairment in CF [203]. Th17 cells, through IL-17 production, may also be involved in CF-related diabetes [204]. The involvement of T lymphocytes in CF is presented in two brief reviews [205, 206].

Several articles have described the effects of roscovitine on T lymphocytes [207–209]. A screen of 256 inhibitors of intracellular signaling pathways has led to the identification of CDK inhibitors, and roscovitine in particular, as suppressors of Th17 differentiation and, thus, as activators of iTreg differentiation [207]. Induction of iTreg cell differentiation by CDK2 inhibition was recently confirmed with kenpaullone, another pharmacological inhibitor of CDKs [210]. Another essential kinase regulating the differentiation of Th17 and Tregs is DYRK1A [211]. Inhibition of DYRK1A enhances Treg differentiation, impairs Th17 differentiation and attenuates inflammation [211]. As roscovitine is also a DYRK1A inhibitor (IC₅₀ in the μ M range) [34, 212], its effect on DYRK1A may contribute to its effects on T-cell differentiation. In a mouse model, roscovitine ameliorates experimental autoimmune encephalomyelitis, an autoimmune disease mediated by Th17 cells [207]. Roscovitine suppresses CD4⁺ Th cells and has a beneficial effect on a uveitis mouse model, an autoimmune disease [208]. Roscovitine decreases the production of proinflammatory interferon and IL-17 [208], confirming previous results [209]. Roscovitine thus modifies the Th17/Treg balance in a favorable, anti-inflammatory direction (fig. 7). Whether roscovitine displays additional direct effects that may mitigate CFTR-dependent intrinsic functional skewing of Tregs remains to be determined.

Roscovitine Has Analgesic Properties

Pain is a common event in CF [213, 214]. Among several CDKs, roscovitine inhibits CDK5, a kinase activated by the binding of one of the regulatory subunits (p35 or p39) and their respective proteolytic fragments (p25 or p29). CDK5 is known to be involved in modulating pain signaling [review in 215]. CDK5 is mostly expressed in the nervous system, namely sensory neurons of dorsal root ganglia (DRG), spinal cord and trigeminal ganglia [216–218], and its expression as well as activity is significantly increased upon pain sensation. Roscovitine exhibits analgesic properties in various animal models of pain. Wang et al. [219] were the first to carry out behavioral studies based on the antinociceptive properties of roscovitine. Subsequent studies revealed the efficacy of roscovitine in attenuating peripheral inflammation induced by complete Freund's adjuvant (CFA). The subcutaneous injection of CFA evokes local inflammation, redness, swelling and hypersensitivity to noxious stimuli (hyperalgesia) that, subsequently, activate protein kinases like CDK5 in primary sensory neurons. Roscovitine treatment significantly reverses heat hyperalgesia induced by intraplantar CFA injection [218, 220–223]. The analgesic effects of roscovitine can occur through inhibition of CDK5 activity, decreased p35 expression [218] and/or reduced CDK5 phosphorylation at S159 by ERK MAP kinase, a posttranslational modification that promotes CDK5 activity [223]. Roscovitine can also affect CFA-induced inflammatory pain by suppressing TrkB (tropomyosin receptor kinase B) levels [222], reducing synaptophysin expression [221] and by preventing trafficking of TRPV1, an ion channel known to be involved in the detection of noxious heat, to the plasma membrane [224, 225]. The level of CDK5 activity can also affect heat hyperalgesia from acute inflammation induced by carrageenan [216], and inhibition of CDK5 by roscovitine in cultured DRG neurons attenuates calcium influx through TRPV1 [226].

Recent reports have also demonstrated the antinociceptive effects of roscovitine in neuropathic pain models. Significantly increased expression of CDK5 is observed in the dorsal horn of rats following chronic constriction injury of the sciatic nerve, and intrathecal delivery of roscovitine significantly attenuates mechanical allodynia in these rats [227]. Roscovitine can down-regulate expression of the NR2A receptor, which, in turn, can alleviate neuropathic pain caused by chronic DRG compression [228]. Additionally, roscovitine prevented remifentanyl-induced postoperative thermal and mechanical hyperalgesia by decreasing expression and activity of CDK5/p35 and phosphorylation of NR2A (S1232), NR2B (Y1472)

and mGlu5 (S1167) [229]. Roscovitine can also down-regulate NMDA (N-methyl-D-aspartate) receptors in animal models of cancer pain, where roscovitine treatment significantly reduced mechanical allodynia and thermal hyperalgesia by inhibiting the NR2B receptor [230]. Additional evidence indicates that roscovitine promotes analgesia through of DARPP-32 dephosphorylation (T75) in a formalin-induced model of nociception [231]. Interestingly, CDK5 is found to be involved in cross-organ reflex sensitization and colon irritation caused an increase in CDK5 expression in the spinal cord and DRG. Intrathecal injection of roscovitine attenuates cross-organ sensitivity and colon irritation by decreasing NR2B phosphorylation [232].

All of these studies indicate promising analgesic effects of roscovitine in different animal models of pain. The antinociceptive properties of roscovitine along with its anti-inflammatory effects may prove helpful in developing effective treatments of pain in CF patients.

The 'Weaknesses' of Roscovitine

Beside its properties in favor of its evaluation as a CF drug candidate, roscovitine has a few weaknesses, which can be summarized as follows. First of all, it was not optimized for this specific indication, in particular for its effects on macrophage intraphagolysosomal acidification. One can anticipate that identification of its molecular target(s) in macrophages – different from the usual kinase targets – should allow the optimization of much more potent and selective roscovitine analogues. Secondly, roscovitine was not optimized in terms of action on its anti-inflammatory targets. Analogues much more potent at inhibiting kinases are available, but, unfortunately, they are more toxic. Thirdly, roscovitine was not tested in animal models of CF. Despite their disputed predictive values, a positive effect would have been encouraging. Fourthly, roscovitine has a short half-life in human plasma. It remains, thus, to be seen whether sufficient lung biodistribution and exposure can be reached via the oral route. Possibly improved efficacy of roscovitine by administration through inhalation/nebulization has not been but should be evaluated. Despite these weaknesses, and considering its specific favorable properties and the benefits of drug repurposing in general, roscovitine has been favorably evaluated by official institutions to enter a first clinical trial which will both investigate tolerability in CF patients chronically infected with *P. aeruginosa* and possible beneficial effects [233].

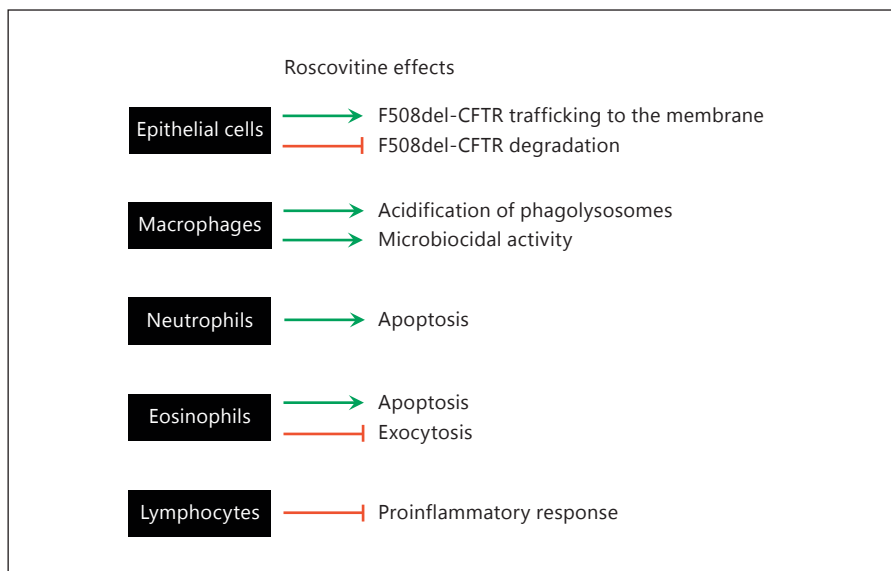


Fig. 8. Summary of cellular effects of roscovitine which may be beneficial for the treatment of CF. Roscovitine acts independently on epithelial cells, macrophages, neutrophils, eosinophils and lymphocytes. Arrows: induction or enhancement; lines: inhibition or reduction.

Conclusion and Perspectives

Evidence indicates that roscovitine has diverse biological properties that could potentially converge toward a novel treatment for CF (fig. 8): (1) roscovitine acts as a corrector of the F508del-CFTR channel by protecting it from proteolytic degradation, favoring its relocation in the plasma membrane; (2) roscovitine improves the bactericidal properties of macrophages from CF patients by translocating/activating the TRPC6 calcium channel (independently of the CFTR mutation) and by partially lowering the intraphagolysosomal pH, which is abnormally elevated in CF macrophages; (3) its main hepatic metabolite also shows a F508del-CFTR corrector effect and biological activity on macrophages, despite extremely reduced kinase-inhibitory effects; (4) roscovitine has an anti-inflammatory effect likely originating from its ability to promote apoptosis in neutrophils and their elimination by macrophages; (5) roscovitine reduces degranulation of eosinophils and promotes their apoptosis; (6) roscovitine suppresses the differentiation of CD4⁺ Th cells into Th17 (proinflammatory lymphocytes, thus reducing the production of proinflammatory interleukins such as IL-17) and promotes their differentiation into Tregs (anti-inflammatory lymphocytes); (7) roscovitine displays analgesic properties, which could contribute to the management of CF-associated pain, and (8) roscovitine is an orally available drug which has already undergone preclinical pharmacological and toxicological studies, and extensive phase I and II clinical trials, in particular against lung cancer. Repurposing this anticancer drug

candidate for CF should thus be a therapeutically valid proposal.

Besides direct clinical trials of roscovitine in CF patients, we foresee four main developments in this novel approach to CF. Firstly, the effects of roscovitine on CF models (organoids [234], ferret [235–237] and pig [95–98, 237, 238] models of CF and animal models of *P. aeruginosa*-induced infection [238, 239]) should be investigated. Secondly, given the host-directed antibacterial effects of roscovitine, its action on other pulmonary pathogens associated with CF, besides *P. aeruginosa*, [240–242], should be investigated. Thirdly, the expected long-term treatments required for CF and the multiple biological consequences of the disease call for serious consideration of combination treatment of roscovitine with other currently developed treatments and for alternative modes of administration. The recent combination therapy Orkambi[®] composed of the corrector lumacaftor (or VX809) and the potentiator ivacaftor (or VX770; www.vrtx.com) can now be prescribed to F508del homozygous CF patients [243]. It will thus be important to compare the combination of roscovitine/ivacaftor with lumacaftor/ivacaftor in a future study. Fourthly, the optimization and development of second-generation drugs derived from roscovitine, based on its CF-relevant molecular and cellular targets, should be envisaged. The chemistry and biology of purines in general [244, 245], and 2,6,9-trisubstituted purines in particular, have been extensively explored, providing a solid starting ground.

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Disclosure Statement

L.M. and H.G. are cofounders of ManRos Therapeutics, L.M. is coinventor in the roscovitine patent, and L.M. and F.B. are co-inventors in the 'roscovitine and CF' patent.

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