Anticipated classes of new medications and molecular targets for pulmonary arterial hypertension

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ABSTRACT

Pulmonary arterial hypertension (PAH) remains a life-limiting condition with a major impact on the ability to lead a normal life. Although existing therapies may improve the outlook in some patients there remains a major unmet need to develop more effective therapies in this condition. There have been significant advances in our understanding of the genetic, cell and molecular basis of PAH over the last few years. This research has identified important new targets that could be explored as potential therapies for PAH. In this review we discuss whether further exploitation of vasoactive agents could bring additional benefits over existing approaches. Approaches to enhance smooth muscle cell apotosis and the potential of receptor tyrosine kinase inhibition are summarised. We evaluate the role of inflammation, epigenetic changes and altered glycolytic metabolism as potential targets for therapy, and whether inherited genetic mutations in PAH have revealed druggable targets. The potential of cell based therapies and gene therapy are also discussed. Potential candidate pathways that could be explored in the context of experimental medicine are identified.

Key Words: pulmonary arterial hypertension, cellular mechanisms, inflammation, metabolism, tyrosine kinase inhibition, genetics, epigenetics, apoptosis

Despite the availability of prostacyclin analogs, endothelin receptor antagonists (ERAs) and phosphodiesterase-5 (PDE5) inhibitors, agents all developed on the basis of the "vasodilator" hypothesis of pulmonary hypertension (PH),[1] pharmacologic treatment of PH continues to have significant limitations. A meta-analysis of control trials in pulmonary arterial hypertension (PAH) reveals that mortality remains high (approximately 1.5% during an average study duration of 14.3 weeks) and that overall, specific therapies only moderately increased 6-Minute Walk Distance (6MWD) by only 11%.[2] The annual mortality from incident cases of idiopathic PAH (iPAH) is approximately 15%.[3] Furthermore, prostacyclin analogs, ERAs and PDE5 inhibitors have demonstrated efficacy in PAH only; in other forms of PAH, it has been suggested that their effects are unproven or may even be harmful. These observations demonstrate an urgent unmet need for improved pharmacologic treatments across PH subtypes. Although vasoconstriction is a major part of the

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pathophysiology of PAH, particularly early in the disease, pathologic studies also show obliteration of vessels by inflammation, smooth muscle proliferation and fibrosis in vessel walls. These processes have suggested physiological processes to target in an attempt to broaden the therapeutic options for treatment of PH.

Fortunately, in the past few years, there has been a remarkable increase in our knowledge of the cellular and molecular mechanisms potentially responsible for the pathobiology of PAH.[4-10] Based on this new appreciation of pathogenetic mechanisms, many new therapeutic approaches are currently being considered for patients. Encouraging results with many of these agents have been demonstrated in preclinical animal models of PH. Some have already been used in small human trials. The drugs are aimed at reversing sustained or abnormal vasoconstriction and/or at stopping or reversing abnormal cell growth and abnormal extracellular matrix protein deposition.

ANTICIPATED AND POTENTIAL CLASSES OF NEW MEDICATIONS

Novel approaches to reversing sustained vasoconstriction

Soluble guanylate cyclase stimulators. Riociguat (BAY 63-2521) is the most studied compound in this class of drugs. Riociguat has a dual mechanism of action: To stimulate sGC in an NO-dependent and-independent mode of action and thereby to enhance cGMP synthesis, producing vasodilatation.[11] In preclinical animal studies, Schermuly et al. investigated oral riociguat in two animal models of PH: Mice subjected to chronic hypoxia and rats injected with monocrotaline (MCT). In both models, riociguat improved pulmonary hemodynamics and prevented and even partially reversed features of adverse structural remodeling, such as right ventricular (RV) hypertrophy and muscularization of small pulmonary arteries.[12] Based on these and other similar observations, riociguat went into Phase I and Phase II clinical studies. A proof-of-concept study was conducted to investigate oral riociguat in patients with moderate-to-severe PH in a two-part, nonrandomized, open-label, single-center trial.[13] Importantly, this study aimed to recruit as broad a range of PH types as possible. This study demonstrated that riociguat was superior to NO with regard to reduction in mean pulmonary arterial pressure (mPAP) and pulmonary vascular resistance (PVR) and the increase in cardiac index. Thus, Phase III trials in PAH and chronic thromboembolic pulmonary hypertension (CTEPH) were embarked upon[14] with results expected by the end of 2012. Currently, it can be said that early clinical trials indicate that riociguat is well tolerated, easily administered and produces significant and long-lasting improvements in pulmonary hemodynamics and exercise capacity in PAH patients. Such improvements are evidenced in patients with CTEPH in addition to those with PAH.

Prostacyclin receptor agonists. A promising therapeutic approach under investigation is the use of a nonprostanoid agonist to directly activate the prostacyclin IP receptor. Selexipag is a first-in-class orally active prodrug metabolized to the highly selective prostacyclin receptor agonist,[15] which has a half-life of over six hours. Selexipag does not exhibit high affinity for the prostaglandin E receptor 3 (EP_3) and exerts similar vasodilatory activity on both large and small pulmonary arterial branches.[16] These properties are likely to account for the greater vasodilatory activity observed with selexipag than with beraprost and iloprost. Preclinical study results showed that twice-daily administration of selexipag attenuates RV hypertrophy, improves pulmonary hemodynamics and significantly increases survival in MCT-treated rats.[16] In a microdosing study using 100 μg of selexipag in healthy, white, male volunteers, headache was the most commonly reported adverse event.[15] A 2012 report from a Phase IIa study involving 43 patients with PAH showed that treatment with selexipag conferred significant improvements in PVR values compared with placebo.[17] An improvement in 6MWD was also observed. A Phase III randomized trial (GRIPHON) to examine the effect of selexipag on morbidity and mortality in PAH is underway.

Rho-kinase inhibitors. The role of calcium sensitization in contributing to vasoconstriction of the pulmonary artery (PA) is receiving increasing attention. Elevation of cytosolic calcium is also a critical step in promoting proliferation of PA smooth muscle cells (PASMCs). Activation of Rho-A/Rho-kinase signaling (which can occur in response to many ligands implicated in the pathogenesis of PAH, including reactive oxygen species (ROS), endothelin-1, thromboxane-A2 and serotonin) leads to inhibition of myosin-light chain phosphatase (MLCP), which mediates smooth muscle relaxation when in its dephosphorylated form by leading to actin–myosin cross-branch dissociation, through phosphorylation of the MYPT1 regulatory subunit of MLCP.[18-23] Inhibition of MLCP by Rho-kinase prolongs actin–myosin interaction and sustained smooth muscle contraction at any given level of intracellular calcium. This phenomenon is known as calcium sensitization. Accumulating evidence from several laboratories strongly suggests that Rho-A/Rho-kinase signaling plays a key role in the pathogenesis of various animal models of PH, including hypoxia-induced, MCT-induced, shunt-induced, bleomycin-induced, spontaneously hypertensive fawn-hooded rats and even the newly developed model of PH induced by treatment with the vascular endothelial growth factor (VEGF) receptor antagonist sugen5416 plus

hypoxia where occlusive lesions resembling those observed in humans are reported.[18-23] Importantly, in these studies, administration of the Rho-kinase inhibitor, fasudil, caused dramatic reductions in PA pressure even in animal models of PAH where traditional vasodilators had little or no effect.[20,23] Furthermore, a recent report indicates that there is high Rho-A/Rho-kinase activity in small hypertensive pulmonary arteries of human PAH lungs.[24] In contrast to the dramatic effects in rodent studies, low doses of intravenous fasudil have been administered acutely to small groups of patients with moderate PAH and have been found to cause only slight decreases in PA pressure.[25-27] It has therefore been suggested that higher doses of Rho-kinase inhibitors will have to be used in human PAH, although this may cause systemic vasodilatation which is not well tolerated in PAH. Based on animal studies performed by Oka et al., higher doses of drugs may have to be given by inhalation to avoid the systemic vasodilation, which is observed in response to systemic administration of the drug in all animal models of PH tested to date.[23] Recently, the new highly selective Rho-kinase inhibitor azaindole-1 has demonstrated efficacy in hypoxia-and MCT-induced PH,[28] and may represent a further development of fasudil for the treatment of PH. An alternative approach to specifically target the hypertensive pulmonary circulation might be the use of novel cyclic peptide, such as CARSKNKDC (CAR), which accumulates in damaged, but not healthy, pulmonary arteries following intravenous injection.[29]

Serotonin inhibitors. Serotonin promotes PASMC proliferation, PA vasoconstriction and local microthrombosis.[30] It also causes proliferation of PA fibroblasts (PAFs).[31] Exogenously administered serotonin potentiates the development of hypoxia-induced PAH in rats.[32] In rodents, inhibition of serotonin receptors or the serotonin transporter (SERT) has been shown to inhibit PAH secondary to hypoxia and MCT injection.[33,34] It has been shown that the proliferative and/or contractile effects of serotonin on PASMCs can involve the following:

1. *Uptake of serotonin via the SERT.* Serotonin causes proliferation of PASMCs and PAFs by entering the cell via the SERT with subsequent activation of phosphorylated extracellular-signal regulated kinase (pERK), inducing the production of ROS and the transcription factor GATA-4.[35-37] Mice deficient for the SERT are less susceptible to hypoxia-induced PAH.[38]
2. *Activation of serotonin receptors.* It is the $5HT_{1B}$-receptor that mediates constriction in human PAs[39,40] and the inhibition of $5HT_{1B}$ activity, either by genetic knockout or antagonism, reduces hypoxia-induced PAH.[34]
3. *Serotonin receptors.* Serotonin is required for the proliferative effect of the calcium binding protein S100A4/Mts1.Both the $5HT_{1B}$-receptor and the SERT are codependent in regulating S100A4/Mts1-induced human PASMC proliferation.[41]
4. *Cooperativity between the $5HT_{1B}$-receptor and SERT in mediating pulmonary vascular contraction.*[42] The $5HT_{2B}$ receptor may also play a role in experimental PAH,[43] and the serotonin 5HT2A/B receptor antagonist terguride, which is approved for hyperprolactinemia, showed efficacy in an animal model of PH.[44] A double-blinded Phase IIa study of terguride in PAH has recently been reported. This 16-week study showed no overall significant effect of terguride versus placebo on PVR or secondary endpoints.
5. *De novo synthesis of serotonin via tryptophan hydroxylase 1 (TPH1) activity.* TPH1 catalyzes the rate-limiting step in the synthesis of serotonin from tryptophan. There is evidence for local serotonin synthesis within the lung. Both hypoxia and mechanical stretch have been shown to increase TPH expression and serotonin release in rabbit lung.[45] Expression of the *TPH 1* gene is increased in lungs and the pulmonary endothelial cells of remodeled PAs from patients with iPAH.[46] Hypoxia-induced PAH/pulmonary remodeling is ablated in TPH 1-/- mice,[47] and hypoxia increases *TPH 1* expression in mouse PA endothelial cells. In an interesting intersection with metabolic therapies for PAH, mice overexpressing the serotonin transporter benefit by treatment with the pyruvate dehydrogenase kinase (PDK) inhibitor, dicholoroacetate.[48]

Vasoactive intestinal peptide. VIP is emerging as a critical regulator of tone and structural remodeling in the pulmonary circulation. Recently it was shown that male mice lacking the gene for VIP spontaneously developed features of moderately severe iPAH.[49] It was shown that administration of VIP to these animals attenuated the vascular remodeling and RV hypertrophy.[49] It is interesting that the VIP knockout mouse expresses spontaneous PAH during normoxic breathing, supporting a primary role for VIP in regulating tone and structure in the pulmonary circulation. VIP reduces hypoxic vasoconstriction in cats, newborn lambs, fawn-hooded rats and rabbits with MCT-induced PH.[50] It inhibits proliferation of pulmonary vascular smooth muscle cell (SMC) from patients with iPAH. VIP containing nerves, normally plentiful in the PA, were reported absent in the PAs of iPAH patients and, most importantly, inhalation of the peptide had a beneficial therapeutic effect on these patients.[51] Thus, there may be a subset of patients who have deficiency in the production of the peptide (for a variety of reasons) and in whom substitution of the hormone results in substantial improvement of hemodynamic parameters. Systemic dosing may be limited by reduced systemic vascular resistance.

Adrenomedullin. ADM is a potent vasodilator peptide originally isolated from human pheochromocytoma. Its vasodilatory effects are mediated through cAMP and nitric

oxide (NO) dependent mechanisms.[52,53] In addition, ADM has angiogenic, anti-inflammatory and positive inotropic activities and is also known to inhibit SMC proliferation and migration.[53] The actions of ADM are mediated by calcitonin receptor-like receptor (CRLR), which functions as a selective ADM receptor. Several studies have shown that plasma ADM levels are elevated in proportion to the severity of PH.[54] These findings suggest that ADM plays an important role in the regulation of pulmonary vascular tone. ADM reduced PH in rats, induced by both hypoxia and MCT.[55] Most importantly, it has been demonstrated that administration of ADM, either by intravenous or intratracheal routes, significantly decreases PA pressure and pulmonary vascular resistance in patients with PH.[52,56] Recently, *ADM* gene-modified endothelial progenitor cells (EPCs) have been shown to incorporate into the lung tissue and attenuate MCT-induced PH in rats.[57] Aerosolized ADM appears not to cause systemic vasodilatation.[58]

Apelin. Apelin is an endogenous peptide vasodilator. The acute vasodilatory effect of apelin on PAs[59,60] and pulmonary pressure is modest (10-17%) and is attenuated in PH. However, this does not exclude the possibility that vasodilatation might occur over a longer period with chronic administration. Apelin could potentially modify secretion of endothelial-derived vasoactive factors, as shown in cardiomyocytes.[61] The pressure-reducing effect seen in the long-term experimental models of PH with chronic administration of apelin[61-63] may possibly be attributed to the stabilizing effect of apelin on endothelial cells,[62] and the prevention of loss of microvasculature.[64] A link between the bone morphogenetic protein type 2 receptor (BMPR2) and apelin was recently recognized.[62] It was shown that disrupted BMPR2 signaling mediated through peroxisome proliferator-activated receptor gamma (PPARγ)/β-catenin resulted in decreased pulmonary vascular apelin expression and increased endothelial cell apoptosis. In addition, the authors showed that apelin secreted from pulmonary endothelial cells inhibited proliferation of PASMCs. The authors suggested that apelin is a downstream protein from the BMPR2 receptor signaling involved in pulmonary vascular homeostasis. Phase I clinical studies are now underway to determine whether infusion of apelin alters pulmonary hemodynamics in humans.

eNOS couplers. Endothelial dysfunction contributes to the pathogenesis of PAH. An important aspect of this dysfunction relates to impaired production/bioavailability and downstream activity of NO. Endothelial production of NO driven by endothelial nitric oxide synthase (eNOS) is critical to maintain normal tone in the vasculature. When eNOS is uncoupled, that is, not dimerized, production of NO is decreased and production of the ROS peroxynitrite and superoxide, both of which can act directly and indirectly to cause vasoconstriction, is increased.

Two potential eNOS couplers have been evaluated preclinically with mixed results. The pteridine cofactor tetrahydrobiopterin (BH4) is required for eNOS enzymatic activity and dimerization. Levels of BH4 are directly linked to eNOS activity and endothelial function,[65,66] making this an attractive agent to directly evaluate the applicability of enhanced eNOS coupling and activity to the treatment of PAH. The pharmaceutical formulation of BH4, sapropterin dihydrochloride, was studied as add-on to treatment with sildenafil and/or ERAs in a small open-label, 8 week dose escalation study of 18 patients with World Health Organization (WHO) Group 1 PAH or inoperable CTEPH.[67] Although small improvements in exercise tolerance were observed, no significant changes in measures of NO synthesis or oxidative stress could be established. These data would question the relevance of the preclinical findings in relation to eNOS coupling and improvements in exercise tolerance in clinical disease. However, it remains unclear what magnitude of NO enhancement in the pulmonary vasculature is required to promote an enduring, long-term therapeutic benefit.

Cicletanine hydrochloride is an antihypertensive with thiazide-like diuretic properties that is marketed for the treatment of systemic hypertension.[68] Prompted by preclinical studies conducted at supra-therapeutic concentrations that demonstrated eNOS enhancing activities, namely increases in NO production and reductions in circulating peroxynitrite and superoxide,[69] cicletanine has now also been evaluated as a PAH therapeutic.[70] Unlike the strong biochemical evidence that has accumulated for BH4 as an important regulator of eNOS function (coupling), no data exists to support the hypothesis that cicletanide functions similarly at clinically relevant concentrations. Consistent with this, data from a recently conducted placebo-controlled, dose-ranging study of cicletanine in 162 patients with WHO Group 1 PAH showed no improvements in exercise tolerance, symptoms, or cardiopulmonary hemodynamics when cicletanine was coadministered with an ERA, phosphodiesterase type 5 inhibitor (PDE5I), or IV/SC prostanoid, or any two-drug combination of these.[71]

Further evidence supports a potential role of the eNOS coupling agent cicletanine in the treatment of disorders associated with endothelial dysfunction.[68,69,72] Several preclinical studies have supported the view that cicletanine acts as a coupler of eNOS, documenting that it increases NO production and decreases the production of peroxynitrite or decreases superoxide in treated patients. Cicletanine demonstrated favorable effects in endothelial dysfunction associated disorders, including systemic hypertension.[68,69,72] It has recently been reported that cicletanine resulted in the improvement of an adult patient with PAH. Further studies demonstrate improvement in pulmonary hemodynamics in

patients taking cicletanine for PH associated with chronic hypoxia due to chronic obstructive pulmonary disease.[70]

Summary and guidance. Agents that act on predominantly vasodilator pathways have become the mainstay of our current treatment approaches in PAH. Although several of these agents show benefit in terms of symptom relief and increased exercise capacity, it remains doubtful whether these agents target the complex loss of vascularity, vessel remodeling, and inflammation observed in the hypertensive lung.[73] Most studies with currently available agents show a ceiling on the degree of benefit that can be obtained. Some may bring advantages over the existing agents with improved tolerability, bioavailability, oral route of administration, or local lung delivery. In addition, there may still be pathways with vasodilator activity that also regulate more complex aspects of the pathology of PAH (e.g., apelin). Further research in this area is indicated, but the most useful advances are likely to be with agents that have a broad mechanism of action on vascular tone, growth, and inflammation.

Agents that target proliferation, apoptosis and metabolic changes in the treatment of pulmonary hypertension

With the realization that traditional treatments directed at the vasoconstrictive component of PH are "successful" only in a minority of patients,[74,75] researchers began to investigate in more detail cellular and molecular aspects of the vascular remodeling component of the disease. Evidence has accumulated of cancer-like phenotypic changes in the cells and extracellular matrix (ECM) of the PAH vasculature. Following the recognition of exuberant endothelial growth with cells disobeying "the rule of the monolayer" within plexiform lesions,[76] "apoptosis resistance" emerged as perhaps the most convincingly reported quasi-malignant phenotypic change, shown in vitro[77] and in lung tissue[78,79] in patients and animal models of PH. An associated finding was a metabolic shift favoring glycolysis[77] and mitochondrial hyperpolarization in PASMCs.[80] Also, similar to cancer, cells in the PAH vascular wall overexpress the angiogenic proteins hypoxia-inducible factor 1-alpha (HIF-1α) and VEGF,[81] and exhibit receptor tyrosine kinase (RTK) activation in relation to additional growth factors, including epidermal growth factor (EGF),[82] fibroblast growth factor (FGF),[83] and platelet-derived growth factor (PDGF).[84] Additional cancer-like findings in the PH vasculature include microsatellite instability,[5] cytogenetic changes,[85] monoclonal expansion of endothelial cells,[86] influx of circulating hyperproliferative progenitor cells,[87] and increased expression of the ECM protein Tenascin C.[88] Changes in DNA methylation and histone acetylation are highly relevant to the proliferation/apoptosis imbalance in PAH. The hyperproliferative phenotype of both cancer cells and PAH PASMCs is related in part to epigenetic silencing of superoxide dismutase 2 (SOD2) (interestingly at the same CpG islands in both cancer and PAH).[89] Thus, an argument could be made that eight out of 10 "hallmarks of cancer" in PAH, defined by Hanahan and Weinberg,[90] are as follows: Genomic instability, sustained proliferative signaling, evasion of growth suppressors, resistance against cell death, induction of angiogenesis, tumor promoting inflammation, evasion of immune destruction and reprogramming of energy metabolism. However, unlike cancer, there is neither evidence of metastatic disease nor do PAH cells cross tissue barriers within the lung.

These observations have led to the idea that in limited ways, the mechanisms involved in vascular remodeling in PH are analogous to neoplasia. Thus, it has been hypothesized that agents which can either block cell proliferation and/or migration or induce apoptosis may lead to a regression of the remodeling and thus a lessening of pulmonary vascular resistance. Many agents have now been shown, at least in animal models, to reverse the established PH by inhibiting proliferation and inducing apoptosis in the vascular wall of remodeled PAs. These agents include growth factor receptor inhibitors, kinase inhibitors, elastase inhibitors, statins, dicholoroacetate, immunosuppressants and phosphodiesterase inhibitors.

Kinase inhibitors. Tyrosine kinases (TKs) can be inhibited pharmacologically by targeting multiple signaling proteins, including PDGF receptor (PDGFR), C-KIT, VEGF and Abl. Since blockade of these targets has been linked to the toxicities reported with tyrosine kinase inhibitors (TKIs), attempts have been made to increase selectivity and therefore improve their safety profile. The available TKIs have different but overlapping kinase inhibitory profiles.[91] Common toxicities that may be associated with their molecular targets include hematological, dermatological, cardiovascular disorders and fluid retention. Moreover, kinase inhibitors have a number of overlapping target specificities that determine their cellular activity and off-target effects.[91]

Imatinib. The agent that has prompted the most investigation to date with regard to targeting abnormal cell proliferation and survival in PH is the RTK inhibitor, imatinib mesylate (Gleevec). Imatinib was initially developed as an inhibitor of the kinase BCR-ABL for the treatment of chronic myeloid leukemia (CML), but has also been shown to act on other forms of cancer through the inhibition of PDGF receptor-beta (PDGFRB) and c-kit.[92] Imatinib reversed PH in two complementary chronic animal models of PH (hypoxia and MCT) and the therapeutic efficacy in these models was specifically attributed to the inhibition of the PDGFRB on vascular SMCs.[84] Furthermore, in the same and subsequent studies,[93] upregulation of the PDGFR in precapillary

resistance vessels of patients suffering from PAH was shown in comparison to healthy controls.

In 2010, Ghofrani et al. reported the results from a pilot study of imatinib treatment in 59 patients with PAH who had New York Heart Association (NYHA) Functional Class II–IV symptoms despite treatment with prostanoids, ERAs, PDE5 inhibitors, or combinations thereof.[94] Imatinib treatment failed to improve 6MWD compared with placebo, although patients in the treatment group showed marked hemodynamic improvements.[94] Because post hoc analyses in this study showed that patients with more marked hemodynamic impairment appeared to respond best to imatinib, enrollment for a Phase III trial (IMPRES) was limited to patients with PVR > 800 dynes s/cm^5. Patients who remained on imatinib during the trial and the subsequent extension had significant improvements in 6MWD, PVR, cardiac output and RV function,[95] but the side effects of imatinib were limiting in many patients. The efficacy of Gleevec in patient subsets raises the need for further studies to identify individual phenotypes and/or genotypes that predict a positive response to this drug.

Hitherto, the potential beneficial effects of TKIs in PH have been attributed primarily to inhibition of vascular SMC proliferation, induction of apoptosis and subsequent reversal of pulmonary arterial remodeling.[96,97] So far, there is only one study suggesting that this class of agents might additionally exert pulmonary vasodilatory activity.[98] In this regard, Abe et al. have demonstrated that intravenous bolus administration of a highly concentrated imatinib solution reduces RV systolic pressure in the model of SU5416/hypoxia-induced PH in rats. In addition, in preconstricted large PA rings, dose-dependant relaxation after administration of imatinib, sorafenib and nilotonib was shown. The investigators speculated that this effect might occur through inhibition of a kinase or signaling pathway that regulates Ca^2 + sensitivity, such as Rho-kinase (ROCK). The main limitations of this study were as follows: (1) That effects observed in central PAs are not reflective of the behavior of precapillary pulmonary resistance vessels; (2) vasorelaxation in isolated ring preparations started at dosages of imatinib that were 100 times higher than the IC50 value for PDGFR inhibition; and (3) in the animal preparations, intravenous bolus injections of imatinib were used at dosages 10 times higher than in PAH patients and interpretation of the hemodynamic effects are limited due to variations in heart rate, pretreatment of some animals with lower doses of imatinib and unspecific vasoactive effects by endothelial irritation.

Sorafenib. Sorafenib is a multi-kinase inhibitor with a wider spectrum of TK activity than imatinib that has been shown to attenuate pulmonary vascular remodeling and hemodynamic changes in rat models of PH.[99-101] In a 16-week, dose-finding, Phase Ib study involving 12 patients with PAH who were receiving parenteral prostanoids, with or without associated sildenafil, oral sorafenib conferred increases in exercise capacity and echocardiographically estimated RV ejection fraction.[102] A lesson from this study was that the maximum tolerated dose of sorafenib was significantly less in PAH patients than has been used in cancer patients. This reinforces the need for dose-ranging experiments in PAH trials of drugs approved for other indications. Of note, hemodynamic measurements indicated a reduction in cardiac output on therapy,[102] emphasizing possible cardiac effects of agents that block VEGF. Indeed, with the emergence of TKIs as a potential therapy in PAH, the benefit to risk ratio of this class of agent will need careful assessment. In particular, concerns have been raised about potential cardiac toxicity, especially in patients with pre-existing heart disease.[103,104]

Nilotinib. The second-generation RTK inhibitor, nilotinib, is a follow-up compound of imatinib and is used as an oral treatment for CML. In preclinical models of PAH, nilotinib showed efficacy on hemodynamics and pulmonary vascular remodeling.[105] Nilotinib is currently being assessed in a multicenter, proof-of-concept trial as a potential therapy for PAH. The investigators plan to compare three doses of nilotinib with placebo among 66 patients with NYHA Class II–III symptoms, using the change in PVR as the primary endpoint.

Dasatinib. This compound is a broad PDGF-R/BCL-ABL/c-kit/src kinase inhibitor that is currently approved for CML and some forms of imatinib-resistant cancers. While dasatinib blocks multiple growth factor induced proliferation of SMCs in vitro and experimental PH in vivo,[105] it caught attention by some clinical cases of patients with CML that developed PAH upon treatment.[106] The FDA released a warning letter in 2010 addressing this side effect. However, PAH is usually and very likely linked to the inhibition of src kinase family members.[107] Nevertheless, these important observations may provide mechanistic insights into PAH pathobiology and raise safety concerns over broad-spectrum TK inhibition.

EGF receptor blockers. Activated serine elastases within the PA wall can directly activate EGF receptors.[5,108,109] This could lead to a situation similar to the autophosphorylation of the EGF receptor, which is seen in many types of cancer. Therefore, it was reasoned that inhibition of EGF signaling might mimic inhibition of serine elastases, which was shown to both inhibit and reverse remodeling in the MCT model. This is important because at the moment elastase inhibitors are not yet clinically available.[109,110] Indeed PIC1166, which inhibits phosphorylation and activation of the EGF receptor, was shown to decrease PA pressure, reverse vascular remodeling, activate PASMC apoptosis

and improve survival in rats with established MCT-induced PH.[109] Similarly, the EGF receptor antagonists gefitinib, erlotinib and lapatinib reversed experimental PH in MCT rats.[111]

Antiapoptosis-directed therapies. Therapies targeted at inducing apoptosis in cells that are resistant to proapoptotic signaling mechanisms have been examined in animal models of PH. Among these are survivin inhibitors. Survivin is an "inhibitor of apoptosis protein," primarily thought to be expressed only in cancer cells.[112] However, survivin was also recently found to be expressed in the PAs of patients with PAH and in rats with MCT-induced PAH, but not in normal PAs from rats or humans.[78] Gene therapy with inhalation of an adenovirus carrying a dominant negative survivin reversed the established rat PAH.[78] Both in vivo and in vitro inhibition of survivin induced PASMC apoptosis and decreased cellular proliferation. Early phase clinical trials of survivin inhibitors are ongoing in the cancer field. It is possible that application of these therapeutic strategies to PAH will be forthcoming.[113]

Summary and guidance. The area of antiproliferative and proapoptotic treatments of PAH is promising. The role of growth factors like PDGF, insulin-like growth factor (IGF), FGF, or EGF in both experimental and clinical PAH is manifest. Growth factor signaling via RTK results in proliferation, migration and resistance to apoptosis of vascular cells. Based on convincing experiments in rodent models of PAH, the first clinical trials have been initiated addressing safety and efficacy of growth factor antagonists that already achieved approval for malignant diseases. Clinical Phase II data are available for the multikinase inhibitor sorafenib and the PDGF receptor blocker imatinib. Further, some less-specific RTK inhibitors like dasatinib may even induce reversible PAH via yet not known mechanisms limiting the use of this class to more selective compounds. In a recent randomized placebo-controlled Phase II trial, imatinib increased 6MWD and improved hemodynamics in severe PAH patients who received at least two available treatment options (prostanoids, endothelin antagonists, or PDE5 inhibitors). However, the side effect profile is comparable to the original indication CML and gastrointestinal stromal tumor (GIST) and may restrict clinical use to severe forms of PAH or responders to this therapy. Additional clinical trials with other RTK inhibitors such as nilotinib are currently underway.

Mitochondria-metabolic dysfunction in pulmonary arterial hypertension

PASMCs from the pulmonary hypertensive fawn-hooded rat and PASMCs[80] and endothelial cells[114] from PAH patients exhibit dysmorphic and hyperpolarized mitochondria and a glycolytic shift in metabolism. Such a shift to glycolysis, which occurs independent of pO_2, was first described in cancer cells (the Warburg phenotype) and is thought to confer resistance to apoptosis. In the pulmonary vasculature, key molecular contributors to this metabolic phenotype include activation of HIF-1α as a consequence of epigenetic silencing of SOD2[89] and/or changes in mitochondrial fission/fusion.[115] The net consequence of this transcriptional and mitochondrial derangement of activation of the mitochondrial enzyme is PDK. PDK phosphorylates and inhibits pyruvate dehydrogenase (PDH), which effectively inhibits Krebs' cycle and slows oxidative metabolism. The resulting glycolytic shift in metabolism occurs in the PASMC and endothelium, but not the airways.[115] This metabolic shift has pathophysiologic relevance since these glycolytic, HIF-1α positive cells are hyperproliferative and likely contribute to vascular obstruction. The cause of the metabolic change differs in the RV versus the pulmonary circulation. In the RV, metabolic remodeling may be initiated by ischemia (due to microvascular rarefaction and/or reduced coronary perfusion pressure), which activates transcription factors leading to increases in PDK expression. The glycolytic shift is bioenergetically disadvantageous and leads to RV hypokinesis.

In experimental models and in humans with PAH, the lung's glycolytic phenotype can be detected using ^{18}fluorodeoxyglucose positron emission tomography (FDG PET). It is noteworthy that the glycolytic shift in lung and heart metabolism can also be used to evaluate the development and resolution of PAH in humans and in rodent models.[114,116] The FDG PET's ability to detect that these glycolytic changes occur early in the course of PAH, when pressure is minimally elevated,[116] suggests this phenotype may be useful as a diagnostic biomarker. The FDG PET signal also is decreased with effective PAH therapy, suggesting it may be a useful parameter to monitor during PAH therapy.

Dicholoroacetate is an inhibitor of PDK. It inhibits all four isoforms thereby activating PDH and promoting glucose oxidation. It activates mitochondria-dependent apoptosis potentially by opening the mitochondrial transition pore allowing efflux of proapototic mediators.[117] It has been shown to inhibit growth and induce apoptosis in cancer cell lines and PASMCs from humans and rats with PAH.[117] Dicholoroacetate improves survival and regresses PH in most models of PAH, including those induced by hypoxia, MCT, or the spontaneous PAH in fawn-hooded rats.[80,118,119] In PAH PASMCs (but not normal PASMCs), dicholoroacetate depolarizes the mitochondria, which increases hydrogen peroxide production and restores the expression and function of the voltage-gated potassium channel, Kv1.5, an oxygen-sensitive ion channel that is downregulated in PAH and which is important to the mechanism to hypoxic pulmonary vasoconstriction (reviewed in[120]). The net effect of inhibiting PDK is an induction of apoptosis and a

decrease in proliferation. Interestingly, there is little effect of dicholoroacetate on normal cells because PDK is normally relatively inactive. An advantage in translating the use of dicholoroacetate from rats to humans is that it has been used safely as a treatment for lactic acidosis in children[121] and has been tested acutely in adults with heart failure. In addition, dicholoroacetate has parallel effects on the RV (which is also shifted metabolically toward glycolysis in PAH). Oral dichloroacetate enhances glucose oxidation and improves RV function in experimental PAH.[122,123] New isoform-selective PDK inhibitors are in development for diseases such as diabetes mellitus.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear receptor superfamily. On ligand activation, PPARs heterodimerize with the retinoid X receptor and bind to PPAR response elements in regulatory promoter regions of their target genes. A series of recent observations suggests that PPARγ could be a drug target in PAH.[124,125] PPARγ is a downstream target of bone morphogenetic protein 2 (BMP2) in human PASMCs.[125] PPARγ is important for BMP2-mediated inhibition of PDGF-induced vascular SMC proliferation.[124] Mice lacking SMC PPARγ develop PAH.[124] PPARγ activation stimulates apolipoprotein E expression. Recombinant apolipoprotein E inhibits PDGFR-γ–mediated SMC proliferation and migration.[126] PPARγ targets, independent of apolipoprotein E, may also be important in the suppression of pulmonary vascular remodeling, because male apolipoprotein $E^{-/}$ mice fed a high-fat diet develop PAH that is reversed by rosiglitazone, a PPARγ agonist.[125] PPARγ agonists have direct anti-inflammatory and proapoptotic effects. The iPAH patients have reduced lung expression of PPARγ and apolipoprotein E mRNA. Because the thiazolidinedione rosiglitazone is widely used in the treatment of type II diabetes mellitus, a trial in PAH would be feasible. Despite this promise, rosiglitazone failed to ameliorate PH in hypoxic-PH rats, although it did reduce right ventricular hypertrophy (RVH) and pulmonary vascular remodeling.[127]

Summary and guidance. Recently identified mitochondrial-metabolic abnormalities in PAH, notably PDK-mediated inhibition of PDH, result in aerobic glycolysis in both the lung vasculature and RV.

1. This glycolytic shift is detectable in both the lung and RV as increased uptake of FDG using PET
2. The metabolic shift has pathophysiologic and therapeutic relevance. The glycolytic switch reduces RV contractility and, in the PAs, promotes elevated rates of cell proliferation and favors apoptosis resistance
3. Reactivation of PDH increases glucose oxidation and improves RV function and regresses experimental PAH. This can be achieved directly by PDK inhibition (using dichloroacetate) or indirectly via activating Randle's cycle using inhibitors of fatty acid oxidation (FAO), trimetazidine and ranolazine.
4. Randomized controlled trials are required to assess whether the benefits of enhancing glucose oxidation on RV and pulmonary vascular function result in benefits to patients. These trials require endpoints that include assessment of the RV using magnetic resonance imaging and evaluation of metabolism using ^{18}FDG-PET.

Inflammatory and immune targets in pulmonary hypertension

Despite strong evidence that inflammation is heavily involved in PAH, this is the least studied aspect of the disease. However, there is increasingly strong evidence for an inflammatory and/or immune pathogenesis of PAH. Certainly PAH is associated with rheumatoid arthritis, systemic lupus erythematosis, collagen diseases (e.g., scleroderma and mixed connective tissue disease), hyperthyroidism, hypersensitivity pneumonitis and infection with HIV.[128-130] In addition, patients with idiopathic PH as well as infants and children with high flow congenital heart disease have been demonstrated to have pulmonary arterial lesions, which demonstrates both perivascular and vascular accumulation of inflammatory cells.[76] Both monocyte/macrophages and lymphocyte subsets are present in the lungs and PAs in animal models of PH.[131] In both the hypoxia and MCT models, therapies targeting inflammatory cells' accumulation have been shown to ameliorate and/or reverse the disease process.[132,133] Direct evidence has recently been presented demonstrating that the Th2 immune response is sufficient to cause severe pulmonary arterial muscularization.[134] Elevation of a range of serum cytokines is observed in PAH and increased levels may predict a worse outcome.[135]

Additional evidence supporting inflammatory mechanisms in PAH includes experiments demonstrating that *NFATc3* is expressed in PASMCs and is activated by chronic hypoxia in a calcineurin-dependent manner.[136] In these experiments, it was shown that chronic hypoxia-induced RV hypertrophy, upregulation of α-SM-actin and vascular remodeling were mediated by calcineurin/*NFATc3*.[136] These findings are consistent with recent observations in humans, which showed activation of *NFATc2* in lung and PASMCs. Inhibition of nuclear factor of activated T-cells (NFAT) signaling by either VIVIT or cyclosporine restored KV1.5 expression, leading to decreased proliferation and increased apoptosis.[137] In vivo, cyclosporine treatment reversed established MCT-induced PAH. Additionally, *NFATc2* levels were increased in circulating leukocytes from PAH patients versus healthy volunteers. CD3 + lymphocytes with activated *NFATc2* were seen in the arterial wall in PAH but not in normal lungs. It should be noted that many cytokines/chemokines known to be

upregulated in PAH [interleukin (IL)-6, tumor necrosis factor (TNF) α, regulated and normal T-cell expressed and secreted (RANTES) and fractalkine] are regulated by NFAT. Thus, targeting NFAT signaling in PH may lead to a reduction in inflammatory, remodeling and RV hypertrophic responses. Other approaches may include direct antibody targeting of chemokine/cytokine receptors such as CCR5, CCR2 and CXCR4.

IL-6 is emerging as a potential target in PAH, although it is not clear whether increased IL-6 expression is causative, or a reflection of the underlying inflammation. Higher levels of IL-6 are found in chronic obstructive pulmonary disease (COPD) patients with PH. Mice overexpressing IL-6 in the lung develop spontaneous PH.[138] In addition, there is an association of PAH with Castleman's disease in man, known to be associated with high circulating levels of IL-6.[139] Case reports of tocilizumab,[140] a humanized antihuman IL-6 receptor monoclonal antibody in connective tissue disease associated PAH, have shown benefit.

Autoimmunity. There is a longstanding association of autoimmunity in PAH.[128,129] This association has been supported in recent reports by the detection of autoantibodies in the serum of patients with PAH associated with systemic sclerosis (SSc-PAH) and iPAH. An important number of autoantibodies have been detected in the serum of patients with SSc-PAH, including scleroderma-specific antibodies such as anticentromere and antitopoisomerase-1 as well as nonspecific antibodies including antiendothelial antibodies (AECAs) and antifibroblast antibodies.[141] AECAs could play a role in the pathogenesis of SSc-PAH since these antibodies activate endothelial cells (ECs), in vitro, including the expression of adhesion molecules and can also cause endothelial cell apoptosis.[142] The presence of AECAs in patients with PAH is now well documented.[143] AECAs also activate ECs in patients with IPAH.[143] Antifibroblast antibodies are also detected in up to 40% of patients with iPAH and 30% with SSc-PAH.[141] Target antigens of the antifibroblasts antibodies have been detected and include proteins involved in the regulation of cytoskeletal function, cell contraction, oxidative stress and PAH cell energy and metabolism. Fibroblasts from patients with iPAH and those with SSc share many common target antigens; this suggests that common features take part in the pathogenesis of iPAH and SSc.[141] Of relevance to the chronic inflammation observed in PAH are observations that these antifibroblast antibodies induce acquisition of a proinflammatory and proadhesive phenotype. They also induce the production of ROS. Antifibroblasts antibodies also appear to be capable of inducing the production of profibrotic chemokines potentially through exploitation of toll-like receptor-4.[144] Recent studies from the Perros laboratory and the Yeager laboratory suggest that the source of this autoantibody production in PAH may be the inducible bronchial associated lymphoid tissue (BALT).[145] Increases in BALT are commonly observed in human PAH as well as in animal models including those associated with hypoxia and MCT.

Rapamycin has been used successfully in certain cancers and vascular diseases including restenosis of systemic vessels and lymphangio leiomyomatosis.[146] Rapamycin is a well-known immunosuppressive agent with antiproliferative activity, not only against lymphocytes, monocytes and EPCs, but also against resident vascular cells.[147] Rapamycin binds to the FK-binding protein 12 and this complex binds to the mammalian targets of rapamycin (mTOR) leading to inhibition of both DNA and protein synthesis and cell cycle arrest. Recently it has been shown to attenuate PH and neointimal formation in rat models of PAH, including MCT, MCT + pneumonectomy and hypoxia.[148-151] Rapamycin has been shown to inhibit expression of monocyte chemoattractant protein 1 (MCP1), a chemoattractant and cytokine/chemokine thought to be important in many inflammatory conditions, including PH.[147] Importantly, some of these effects may be mediated through direct induction by rapamycin of hemoxygenase-1 in pulmonary vascular cells, including SMCs.[150] Because many of the cell types demonstrating augmented growth properties and/or attenuated apoptotic responses show activation of the PI3 kinase and mTOR signaling pathways, it is possible that rapamycin, potentially in combination with other agents, could be beneficial in the setting of PH. It should be noted that like statins (which are not beneficial in PAH) there are studies showing no benefit from rapamycin in experimental PAH. A key point in the positive versus negative findings in many such studies is the duration of the experiment. Shorter studies favor finding benefit (as was the case in the statin rodent studies).[152] There are currently plans for a trial of rapamycin in human adult patients with PH.

Summary and guidance. There is excellent and accumulating data highlighting the role of inflammation and autoimmunity in the pathophysiology of PAH. Though presently not clear, it seems possible, if not likely, that inflammation and inflammatory-mediated pathways play a particularly important role early in the disease course. These observations point out the increasingly greater interest regarding immunotherapy to modality, the outcome of the disease, or even to treat specifically patients at risk, especially those with connective tissue disease, infectious diseases (HIV, schistosomiasis, etc.), or relatives of PAH patients. What is needed is focused attention on a better understanding of mechanisms involved in the initiation and ultimately the perpetuation of chronic inflammation. As discussed below, epigenetics could be important in this process. It is also important to better understand how autoimmunity participates in the development of PAH. This is difficult because at present very little attention has been

paid in animal models to the possibility that autoimmune mechanisms contribute to the development of longstanding PAH. These deficits need to be corrected in the future.

Epigenetic targets in pulmonary hypertension

Common usage today defines epigenetic traits as a stably heritable phenotype resulting from changes in a chromosome without alterations in DNA sequence.[153] Epigenetic changes are thought to be at the root of cellular reprogramming, the process by which a differentiated cell type can be induced to adopt an alternate cell fate. This idea appears to be consistent with observations in PH, where endothelial cells, SMCs and adventitial fibroblasts have all been demonstrated to acquire significantly altered characteristics including stable increases in proliferation, resistance to apoptosis, metabolic switching and proinflammatory gene expression. Recent studies have documented changes in the methylation status of important genes, such as superoxide dismutase in cells from the pulmonary hypertensive vasculature.[89] A key finding was lung-specific upregulation of DNA methyltransferase (DNAMT) 3b, which allows de novo methylation of CpG islands and inhibition of transcription of a nonmutated gene. This points to DNAMT as a potential therapeutic target. One of the more exciting opportunities for epigenetic cell therapy is the fact that these changes may be reversible and new agents are being developed. Among the possibilities are demethylating agents such as 5-azacytidine, 5-aza-2′-deoxycytidine and numerous classes of histone deacetylase (HDAC) inhibitors. HDAC inhibitors (HDACi) have shown potential for reducing proliferation in cancer cells and have also been demonstrated to significantly attenuate inflammatory signaling in a wide variety of chronic inflammatory diseases in vivo and in stromal and marrow-derived cells in vitro.

HDACs catalyze removal of acetyl groups from lysine residues in a variety of proteins. They have mainly been studied in the context of chromatin, where they regulate gene transcription by deacetylating nucleosomal histones. The 18 mammalian HDACs are grouped into four classes.[154] Dysregulation of HDACs is associated with a variety of pathophysiological processes, including cancer and inflammatory signaling in rheumatoid arthritis. As such, there is intense focus in the pharmaceutical industry on development of novel small molecule inhibitors of HDACs, particularly since the first HDACi reached the market in 2006 with the FDA approval of vorinostat (SAHA) for the treatment of cutaneous T-cell lymphoma.[155] SAHA is a pan-HDACi that contains a zinc-binding hydroxamic acid warhead. Surprisingly, SAHA and other pan-HDACi have been shown to be efficacious in rodent models of left ventricular (LV) dysfunction, reducing pathological hypertrophy and fibrosis and improving pump function, suggesting a novel application for HDACi for the treatment of human heart failure.[156] However, given the toxicities associated with pan-HDAC inhibition (e.g., thrombocytopenia), concerns remain regarding the translational potential of these findings. Medicinal chemistry efforts have led to the discovery of compounds that selectively inhibit specific HDAC isoforms.[157] It is believed that these compounds will be safer and more efficacious than pan-HDACi in the setting of heart failure.

Two reports have addressed the effects of HDACi in models of RV remodeling. Valproic acid was shown to block RV cardiac hypertrophy in response to pulmonary artery banding (PAB), as well as in the setting of PH caused by MCT-induced lung injury.[158] In contrast, trichostatin A (TSA) failed to block hypertrophy in response to PAB and actually appeared to worsen RV function.[159] Valproic acid is a weak HDACi with many additional pharmacological activities,[160] and thus it is unclear whether effects of this compound on the RV were a direct consequence of HDAC inhibition. TSA is a potent pan-HDACi. The deleterious effects of this compound on the RV could be a reflection of a protective role for an HDAC (s) in this chamber of the heart. Additional investigation is needed to elucidate the roles of specific HDACs in RV remodeling.

Expression of Class I HDACs, particularly HDAC1, is dramatically elevated in PAs of humans with PH and in lungs and vessels from pulmonary hypertensive models. Based on these findings, recent studies have begun to address the role of Class I HDACs in the pathogenesis of PH. In a three-week rat model of hypobaric hypoxia, the Class I HDAC-selective inhibitor, MGCD0103, reduced PA pressure through a mechanism involving suppression of PASMC proliferation.[161] The antiproliferative effect of MGCD0103 was due, in part, to upregulation of the FoxO3a transcription factor and induction of a downstream target gene encoding the p27 cyclin-dependent kinase inhibitor. Importantly, RV function was maintained in animals receiving MGCD0103 and the Class I HDACi blocked cellular and molecular processes that contribute to RV failure, supporting the hypothesis that isoform-selective HDACi will have improved safety profiles compared to pan inhibitors. These data, together with the recent data demonstrating that Class I HDACi block the stable, proinflammatory phenotype of pulmonary adventitial fibroblasts from hypoxic calves,[162] strongly support a role for these epigenetic regulators in the pathogenesis of PH.

Importantly, recent studies have demonstrated that Class I specific HDACi can prevent hypoxia-induced remodeling and preserve RV function.[161] These observations are consistent with those in the LV where it has become increasingly clear that HDACi can be utilized to reduce cardiac hypertrophy and fibrosis.[163]

Summary and guidance. Pharmacogenetics has failed to completely explain the variability and individual response

to medical treatments. This is true for PH as well as for a large number of other systemic inflammatory diseases and for cancer. It is now clear that epigenomics seems to contribute to the inter-and intra-personal drug response variation.[164] It is becoming increasingly clear that the drugs contained in our current medical armamentarium have failed to provide sufficient options to prevent the onset or modify the progression of many chronic inflammatory diseases, including autoimmune disease. This is of particular relevance to PH since many patients appear to exhibit evidence for circulating autoantibodies. What is particularly exciting about HDACi as epigenetic treatment for PH is that they have actions, which go far beyond their traditional mechanism of action (hyperacetylation of nuclear histones).[165] HDACi are now known to enhance the level of acetylation of nonhistone proteins as well. This function seems to be involved in the potent anti-inflammatory properties of HDACi and may explain why low doses are effective in a wide spectrum of diseases not related to cancer.[166,167] In fact, in chronic inflammatory disease, the effects of HDACi are consistently observed at much lower concentrations than those required for killing tumor cells. Thus, at present, an attractive aspect of HDACi is that they are orally active and at low concentrations are most effective in reducing inflammation in both humans and animal models.[168,169]

The success of HDACIs in the treatment of inflammatory diseases including PH will ultimately depend on two factors: lack of organ toxicity and tolerability, and specificity of the inhibitor for the relevant HDAC involved in the disease process. Future development of HDACs will be focused on selective inhibitors since there are 18 distinct HDACs. Inhibition of HDACs will offer optimal efficacy depending on the dominant cell type in a particular disease. In PH, much work will need to be done to determine the relative role of different cell types at different stages of the disease and the HDACs involved. For instance, targeting inflammatory cells with HDACi may require different treatment regimens than targeting SMCs. Ultimately, whether HDACs will become a specific treatment choice for PH will need much further experimentation. However, we are convinced that the study of epigenetics may provide new therapeutic targets for the chronic inflammation and the autoimmunity involved in PH.

Drugs targeting the BMP/TGFβ pathway

The identification of heterozygous germline mutations in the *BMPR2* gene in 2000 was a step change in our understanding of the pathobiology of PAH.[170,171] *BMPR2* is a receptor for the transforming growth factor-beta (TGF-β) superfamily and specifically for members of the large subfamily of BMP ligands. Mutations have been identified in at least 70% of familial PAH and 15%-26% of sporadic cases of PAH.[172] Furthermore, studies in animal models and PAH patients have shown that PAH of diverse etiologies is associated with reduced expression of *BMPR2*.[173,174] Although *BMPR2* downregulation is found in many animal models, there is controversy as to whether increasing *BMPR2* expression (by adenovirus) is beneficial.[175] Evidence supporting causality comes from studies demonstrating that lung endothelial targeting of *BMPR2* expression by adenovirus rescues endothelial *BMPR2* expression in rat models and prevents and reverses the course of PAH.[176,177] The majority of *BMPR2* mutations are nonsense or frameshift mutations, which lead to nonsense-mediated mRNA decay of the mutant transcripts, leading to a state of haploinsufficiency. Thus, approaches that increase either mRNA expression or protein levels of wild-type *BMPR2* might offer benefit in PAH. For example, *BMPR2* is removed from the cell surface by ubiquitination and degradation via the lysosome.[178] Inhibition of this pathway could maintain *BMPR2* function. Moreover, it was shown that specific mutations in the ligand-binding domain of *BMPR2* are retained within the endoplasmic reticulum due to protein misfolding.[179] Correction of misfolding offers the opportunity for intervention in these cases. Agents that promote transcriptional readthrough may alleviate mutations that lead to premature stop codons.

Since the identification of mutations in *BMPR2*, mutations have also been identified in activin-like receptor kinase-1 (*ALK-1*) in PAH patients,[180] as well as mutations in genes encoding the canonical downstream BMP signaling intermediaries, Smad 1 and 8. An important advancement over the last 3 years is the observation that *BMPR2* and *ALK-1* form a receptor complex, which signals specifically in response to BMP9 and 10.[181] *ALK-1* is almost exclusively expressed on the endothelium, which suggests that the major impact of *BMPR2* mutation is to disrupt endothelial BMP9/BMPR2/*ALK*-1 signaling as a major initiating event in PAH pathogenesis. Of note, a proportion of mice heterozygous for a null mutation in *ALK*-1 also develop PAH. The cellular model that is emerging involves disruption of endothelial *BMPR2* signaling leading to increased endothelial apoptosis[182] and increased vascular permeability,[183] which drives underlying mesenchymal cell proliferation[184] and vascular obliteration. Thus, approaches that enhance BMP signaling, potentially those based on the BMP9/10 ligand or peptide analogues, are worth exploring in PAH.[185] A further approach, which has shown benefit in animal models, is to block the effect of endogenous BMP inhibitors, such as gremlin.[186]

Summary and guidance. A full understanding of the mechanisms by which mutations in *BMPR2* mutation cause PAH remains unclear, although a number of major advances have been made since the original identification of these mutations. Since this pathway is centrally and directly implicated in the pathobiology of PAH, it makes sense that investigators now attempt to target the BMP pathway therapeutically to determine whether this

offers a new approach for PAH. There are a number of ways by which signaling can be enhanced, as described above (e.g., correcting the effects of individual mutations on *BMPR2* mRNA or protein, or by enhancing signaling using BMP receptor agonists, or blockers of endogenous inhibitors of BMP signaling).

Stem and progenitor cells as therapy for pulmonary hypertension

The use of regenerative cells to promote lung vascular repair and regeneration is an attractive approach for the therapy of advanced PAH, which has been explored in a number of preclinical studies. EPCs, a population of circulating proangiogenic cells that repair and regenerate blood vessels, were shown to be effective in the MCT rat model, reducing RV hypertrophy, elevations in right ventricular systolic pressure (RVSP) and mPAP and pulmonary vascular remodeling.[187-189] Although there was some evidence of EPC persistence and direct endothelial transdifferentiation, it is likely that paracrine effects account for much of the efficacy. As well, early clinic studies have suggested modest but favorable effects on exercise capacity and pulmonary hemodynamics following autologous EPC infusion in patients with PAH.[190]

Similarly, mesenchymal stem cells (MSCs) have been reported to reduce RVSP and RV remodeling in preclinical models.[191,192] MSCs represent another bone marrow-derived population which has the advantage of being relatively immune-privileged, thus allowing them to be transplanted allogeneically. There is also evidence that MSCs secrete soluble factors that are immunomodulatory or immunosuppressive. Intravenous delivery of MSCs 1 week after MCT resulted in a significant reduction of RVSP and RV remodeling. Similarly, others demonstrated prevention of PAH with intratracheal administration of MSCs in the rat MCT model.[193] However, unmodified stem and progenitor cell transplantation does not appear to be effective in reversing established PAH, which would be a requirement for the successful translation to a clinical therapy.

Gene-enhanced cell therapy. Cells can be readily manipulated during cell culture and processing and thus provide a convenient opportunity to combine cell and gene therapy by using transfected cells. Nagaya et al. first demonstrated the potential of gene transfer as a strategy to enhance EPC therapeutic potential by transplanting EPCs overexpressing ADM into immunodeficient MCT-treated rats.[187] Similarly, EPCs overexpressing calcitonin gene-related peptide were more effective in preventing PH in a left-to-right shunt model.[57] The use of EPCs overexpressing eNOS may represent an innovative approach to improving endothelial dysfunction through restoration of damaged pulmonary microvasculature. Indeed, early outgrowth EPCs overexpressing eNOS were shown not only to prevent, but also to completely reverse, established PAH in the MCT model.[194] The ability for cell-based gene therapy to reverse established PAH holds great promise for a clinically applicable therapeutic strategy for the treatment of PAH. Based on the success of these preclinical studies, the first clinical trial using autologous EPC-based eNOS gene therapy for PAH, the PH and eNOS Cell Therapy Trial (PHACeT), has been initiated in Toronto and Montreal to establish safety and appropriate dosing.[195]

In many ways, MSCs may represent a more convenient platform for cell-based gene therapy, given their greater ability to expand in culture and their potential for use for allogeneic transplantation. MSCs overexpressing eNOS, prostacyclin (PGI2) synthase and heme oxygenase-1 (HO-1)[191,192,196] have shown efficacy for the reversal of established PAH. The results of these studies suggest that MSCs may serve as a suitable vehicle for cell-based gene therapy to treat experimental PAH and that gene transfer can enhance the effectiveness of stem cell therapy. However, as yet, the efficacy of gene-enhanced MSCs for PAH has not been studied in a clinical trial.

Summary and guidance. Cell therapy holds tremendous promise as a treatment that may be able to address the fundamental structural abnormalities underlying advanced PAH. The preclinical studies to date are encouraging and, although limited, early clinical experience supports the safety and potential efficacy of this approach. However, the field of cell therapy for PAH is only in its infancy and many questions need to be addressed, including determining the best cell product. To date, only adult stem and progenitor cells have been studied and the potential of truly pluripotent cells, such as embryonic or inducible pluripotent stem cells to repair and regenerate the lung vasculature, remains to be evaluated. As well, the present literature would suggest that an enhancement strategy such as gene engineering is necessary to unlock the full potential of cell therapy. Much more research is required to establish which cell products will be optimal for the treatment of PAH and what enhancement strategies are needed to provide a truly effective therapy.

Gene therapy for pulmonary hypertension

The lung provides a unique opportunity as a target for gene therapy since it can be selectively accessed directly by the airways, allowing the potential for selective overexpression of transgene in it.[175,197] Selective targeting of the endothelium of the lung via the venous circulation is another promising approach for PAH.[176,177,198] Delivery of an adenovirus encoding the *eNOS* gene via the airways was successful in preventing PH in the rat chronic hypoxia model.[199] Another NOS isoform, inducible NOS (iNOS), was also shown to reduce PAP as well as arterial and RV remodeling in this model.[200] To avoid the use of viral vector, Liu et al. employed the Sleeping Beauty (SB)

transposon-mediated gene transfer approach to provide effective, long-term transgene expression of eNOS in MCT rats[201] with a significant improvement in pulmonary hemodynamics and remodeling. Although this approach avoids the potential concerns associated with the use of viral gene vectors, SB transposons integrate into the host genome thus limiting its translation to human therapy.

Prostaglandins currently represent the gold standard for therapy of patients with severe PAH. However, a major limitation of PGI2 pharmacotherapy is the medication's short half-life (~three minutes), necessitating a continuous intravenous or subcutaneous infusion. Using hemagglutinating virus of Japan (HVJ)-liposomes as vectors delivered intratracheally, Nagaya et al. reported that selective pulmonary overexpression of prostacyclin synthase (PGIS) prevented the development of PAH in the rat MCT model[202] and improved long-term survival upon administration of the vector every two weeks. Similarly, the delivery of an HVJ-liposomal complex of human PGIS into the liver of rats with established MCT-induced PAH produced a significant decrease in PAP, but with no improvement in arterial remodeling.[187]

Since "loss-of-function" mutations on one morphogenetic protein receptor type 2 gene (*BMPR2*) are the major causes of hereditary and sporadic PAH, gene therapy aimed at replacing the defective *BMPR2* allele with a fully functional form may be an attractive therapeutic strategy for some patients with PAH. Using an adenoviral construct specially designed to target the pulmonary vasculature, Reynolds et al. demonstrated attenuation of hypoxia-induced PAH in rats receiving *BMPR2* gene therapy.[203] In contrast, McMurtry et al. could not demonstrate rescue of MCT-induced PAH using nebulized Ad-BMPR2 delivered 14 days post-MCT treatment.[175] More recently, vascular-targeted *BMPR2* gene therapy was shown to be effective in reversing MCT-induced PAH.[204] Angiogenic factors such as VEGF protect against endothelial apoptosis and can repair endothelial injury. Intratracheal instillation of adeno-VEGF decreased PAP and RV hypertrophy and normalized arteriolar wall thickness and muscularization.[205] Interestingly, this report also described an increase in eNOS activity, suggesting that VEGF overexpression may have functioned in part by preserving endothelial function.

Summary and guidance. Gene therapy for PAH represents another promising therapeutic avenue. The lung can be accessed either by the airways or the venous circulation, both of which may provide a direct and convenient route for selective pulmonary gene therapy. Despite the relative abundance of preclinical studies demonstrating the efficacy of various therapeutic transgenes delivered using a variety of vectors and routes, there have as yet been no attempts to translate a stand-alone gene therapy into a clinical strategy for PAH. This may, in part, be due to the disappointing results of gene therapy for cardiac diseases in a number of rigorous clinical trials undertaken in the last couple of decades. However, cell-based gene therapy may have a number of advantages for the treatment of pulmonary vascular diseases and has already entered into early phase clinical testing (the PHACeT trial) and it will be of great interest going forward to learn to what extent any benefits of this approach may relate to the cell or the transgene or both.

PRECLINICAL ASSESSMENT OF NEW DRUGS

Overview of existing animal models

A wide range of animal models of PAH has been described in PH literature. The most widely used are the chronically hypoxic rat model[206,207] and the MCT model,[208,209] though various other small and large animal models have been characterized that approximate various forms of PH. Unfortunately, no single model recapitulates all the features of human disease. The central features of human disease include the following: (1) Obliteration of the lung arterioles; (2) nonreversibility of PAH; and (3) development of RV failure. Recent efforts have generated animal models that recapitulate more, but not all of these features, for example, the SU-5416 (a VEGF receptor inhibitor)/hypoxia model in the rat[210] which develops a severe progressive form of PH accompanied by RV dysfunction. The latter has been modified for mice,[211] though in this species the model is reversible. In general, RV failure does not occur in the mouse models, or has been poorly characterized and RV hypertrophy is modest.[212] The specific features and utility of animal models of PH have been reviewed extensively recently.[212-215]

Summary and guidance. The use of clinically relevant endpoints in animal models is important. These will include the following: (1) Reversal of an established disease rather than prevention; (2) complete hemodynamic assessment, including assessment of cardiac output and function; (3) assessment of the functional capacity of the treated animals, for example, exercise capacity; (4) survival studies are important but consideration needs to be given to what the animals are dying of, for example, MCT may cause death by mechanisms other than right heart failure; and (5) dose-dependent effects should be evaluated. Therefore, drug investigators and developers should have a clear understanding of their animal model and rationalize their use. Usually multiple rather than single or simple models should be used by drug developers to cover several aspects of the pathophysiology of PH and PAH in man that are not covered by the study of a single animal model.

OVERALL SUMMARY AND CONCLUSIONS

The last 20 years have witnessed major advances in the treatment of PH and the cellular and molecular mechanisms of disease. Several classes of new agents have been licensed for the treatment of this condition, moving the field from a position of therapeutic nihilism to one of cautious optimism. However, almost all of these have been licensed for the rarer forms of PH, leaving a large unmet need for the treatment of more common forms of the disease. In this article, we highlighted some of the major pathways and molecular targets that might inform the development of novel therapeutic agents over the next few years. Many of these will be limited by factors such as toxicity or nonefficacy in man, but are based on the best information we have that are available from preclinical studies. The focus now should be on novel approaches that target the proliferative angiopathy, metabolic dysfunction and inflammation that have emerged as central to the pathobiology. Raising the bar in preclinical studies in animals by the use of more sophisticated models and a more comprehensive assessment of efficacy and biomarker validation in these models will help to reduce the range of potential targets to move forward into the clinic. Further, valuable information remains to be learned from genetic studies which may yet indicate the critical pathways involved in disease. It remains to be seen whether targeting those pathways (e.g., the BMP pathway) will reverse established disease in man. The molecular landscape in PH is complex, likely involving dysregulation of several key pathways. Thus, approaches that target wide-ranging biological effects such as epigenetic modifications or microRNAs provide a means to influence interrelated networks. The use of new technologies such as patient-derived stem cells may also provide valuable new information for target validation and drug discovery. Ultimately, pulling through discoveries in basic science to the clinic remains a major challenge and will require careful and informative experimental medicine studies that efficiently assess efficacy, safety and mode of action.

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