Review

Integration of Global Analyses of Host Molecular Responses with Clinical Data To Evaluate Pathogenesis and Advance Therapies for Emerging and Re-emerging Viral Infections

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ABSTRACT: Outbreaks associated with emerging and re-emerging viral pathogens continue to increase in frequency and are associated with an increasing burden to global health. In light of this, there is a need to integrate basic and clinical research for investigating the connections between molecular and clinical pathogenesis and for therapeutic development strategies. Here, we will discuss this approach with a focus on the emerging viral pathogens Middle East respiratory syndrome coronavirus (MERS-CoV), Ebola virus (EBOV), and monkeypox virus (MPXV) from the context of clinical presentation, immunological and molecular features of the diseases, and OMICS-based analyses of pathogenesis. Furthermore, we will highlight the role of global investigations of host kinases, the kinome, for investigating emerging and re-emerging viral pathogens from the context of characterizing cellular responses and identifying novel therapeutic targets. Lastly, we will address how increased integration of clinical and basic research will assist treatment and prevention efforts for emerging pathogens.

KEYWORDS: emerging pathogens, kinomics, cell signaling, virology, kinases, high-consequence pathogens
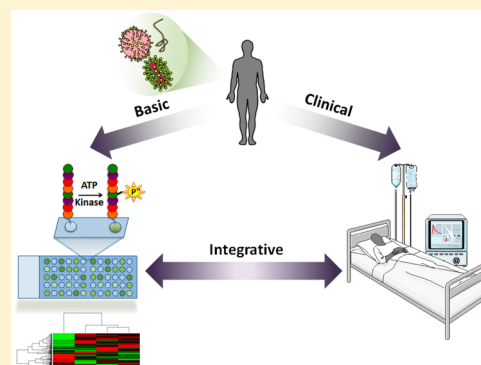
Emerging and re-emerging viruses pose a significant threat to public health and global economies. Moreover, outbreaks caused by emerging and re-emerging viruses continue to increase in frequency as a result of changing socio-economic, environmental, and ecological factors.[1] Notably, the zoonotic viral pathogens, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), Ebola virus, chikungunya virus, and Zika virus, have emerged on a global scale in recent years; although less widely publicized, other emerging viral pathogens such as monkeypox virus and Andes virus have led to smaller recurrent outbreaks. A critical challenge for combating these outbreaks is often the discordant relationship between the economic status of outbreak "hotspots" and resource distribution or control capacity within these regions. In addition, the development and delivery of therapeutics for combating such outbreaks have been complicated by both the associated costs in design and development for novel anti-infective therapeutics and the requirements for regulatory approval and licensure.[2] Importantly, emerging infectious diseases present the additional inherent challenge that they are only "emerging", and thus limited resources are made available for research until they present a significant risk. For many emerging viral pathogens, the requirement for high-containment facilities has further impeded widespread research. From the perspective of drug development, the limited knowledge and understanding of molecular pathogenesis for these agents is a daunting challenge to overcome when outbreaks emerge.

In the face of an increasing burden of emerging and re-emerging pathogens, it is necessary to overcome the barriers imposed by a paucity of information regarding molecular pathogenesis for these agents. OMICS-based approaches present a mechanism to rapidly generate large amounts of data in regard to host responses and assist in target identification for drug development efforts. In addition, OMICS-based analyses allow for the characterization of molecular events that mitigate cellular responses to viral pathogens from a global perspective across multiple levels of cellular complexity (individual cell types < tissues < organs). High-throughput global analyses of host gene expression, including microarrays and RNA-Seq, provide important information regarding transcriptional responses during infection. Although these validated approaches are among the most widespread of the OMICS-based technologies for infectious disease investigations, they do not provide a direct measure of the activation status of the cell signaling pathways that regulate underlying cellular responses. In contrast, global investigations of cellular kinase activities (the kinome) are able to provide insight into the activation status of cell signaling networks (including those that mediate pathogen recognition and innate immune activation, cell cycle activities, metabolic status, wound healing and repair, and cell death) at the level of
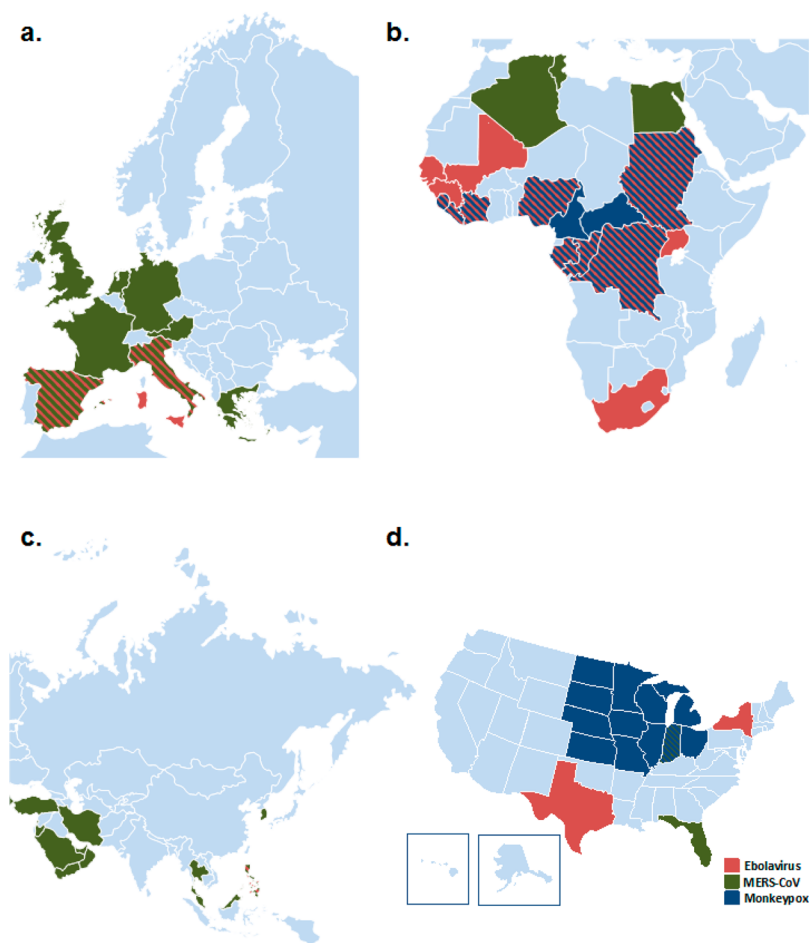
Figure 1. Global outbreaks of Ebola virus, MERS-CoV, and monkeypox: (a) Europe; (b) Africa; (c) Asia; (d) United States. Data include all transmission-related infections that have been documented. Laboratory accident-related infections and medical evacuations are not included.

individual kinase-mediated phosphorylation events. In addition, kinome investigations allow for potential identification of kinase drug targets. Kinases are currently one of the top targets for drug design and development, and there is potential for repurposing of kinase inhibitors with existing regulatory approval.

As emerging viral infections often result in severe illness including respiratory failure [severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and influenza] and multiorgan failure [Ebola virus disease (EVD)], understanding complex pathogenesis of these infections is required for effective vaccine and therapeutic design and for improved patient care. Healthcare providers caring for patients with severe emerging viral infections are generally focused on clinical care and biosafety as compared to the complex molecular events that underlie pathogenesis. In contrast, basic researchers typically focus on discrete aspects of pathogenesis through a variety of in vitro and in vivo analyses rather than the complex interplay between these events and the clinical, physiologic, and pathologic abnormalities observed by the clinician. Integrating basic and clinical research is needed to accelerate the translation of knowledge for emerging infections toward vaccine development and therapeutic discovery. Specifically, detailed natural history studies merging multiple data streams including OMICS approaches (high-throughput gene expression and kinomics) and focused translational investigations utilizing relevant models that can be validated

to human disease are needed to clarify disease pathogenesis, advance therapeutic discovery, and facilitate regulatory approval.

Although an integrated approach between basic and clinical research is ideal for investigating the connections between molecular and clinical pathogenesis, there has been a paucity of investigations for which this has been undertaken. Here, we will discuss emerging pathogens for which there is available information regarding the clinical course of disease, host immune responses during natural infection, and molecular information regarding the global cellular responses to infection, with particular attention on host kinome investigations. In this regard we will focus on the emerging viral pathogens MERS-CoV, Ebola virus (EBOV), and monkeypox virus (MPXV) (Figure 1). We will review the clinical presentation and immunological and molecular features of the diseases and summarize available OMICS data informing pathogenesis of these pathogens. Lastly, we will discuss the benefit of improved integration of available clinical knowledge or data regarding the pathologic manifestations of disease with basic research investigations to advance treatment and prevention of severe emerging viral infections.

■ INVESTIGATING MOLECULAR PATHOGENESIS THROUGH KINOME ANALYSIS

Global gene expression investigations have provided information regarding host response to emerging and re-emerging
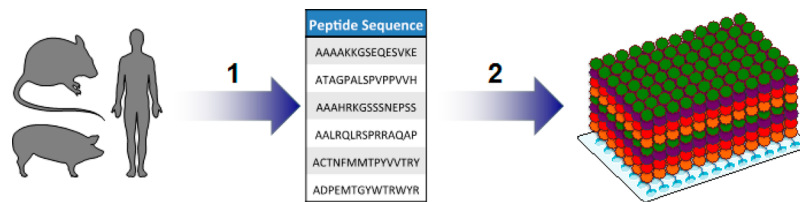
Figure 2. Generation of kinome peptide array targets and kinome peptide arrays: (1) species-specific proteomic or genomic information from a diverse range of species can be used to identify kinase recognition motifs that are composed of a central phosphorylation target and the surrounding amino acids (normally +4 and −4 amino acids from the central phosphorylated residue); (2) peptides that comprise the kinase recognition motifs identified in (1) are synthesized and covalently linked to a glass surface. Peptide targets are spotted in replicates of three to nine spots on each array to account for intra-array variability. Individual amino acids of the peptides are represented by orange, red, purple, and green spheres.
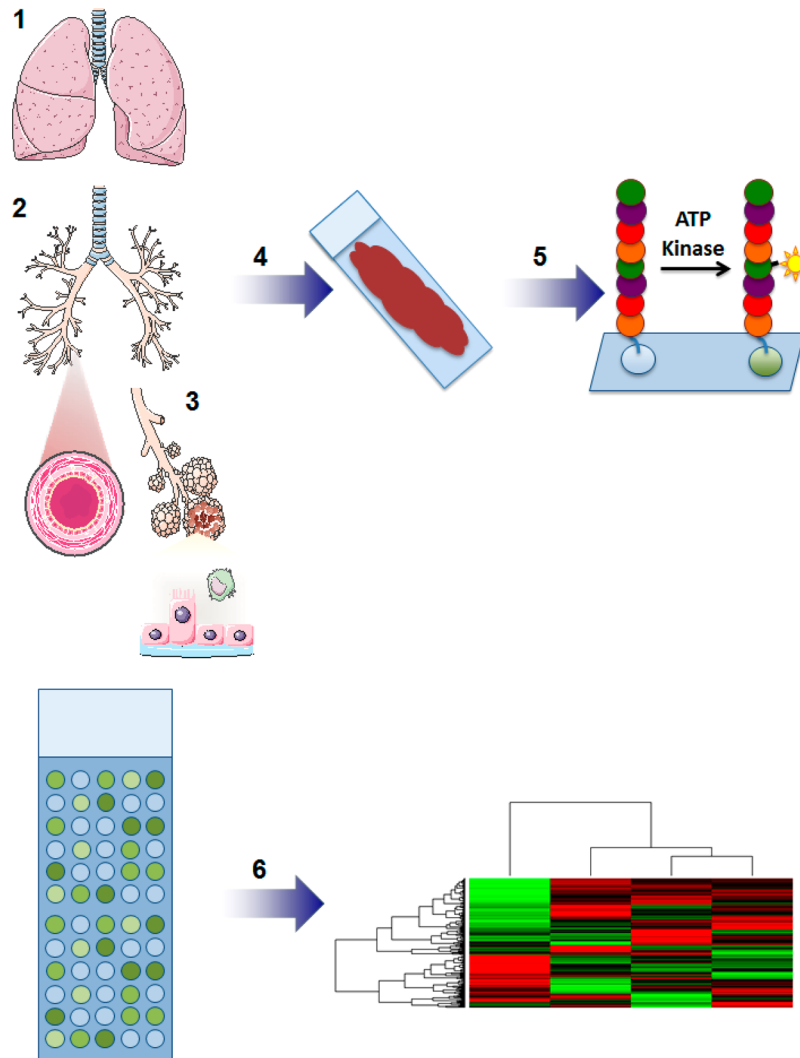

Figure 3. Kinome analysis of biological samples. Biological samples for kinome analysis can encompass (1) complex biological tissues (lung), (2) focused tissue sections (bronchioles), or (3) individual cell types associated with a particular tissue (alveolar epithelial cells or alveolar macrophages). (4) Biological samples are processed to generate cell lysates that are activated with ATP and applied to the kinome peptide array. (5) Following the application of the cell lysate, activated kinases in the cell lysate will recognize their respective kinase recognition motifs and phosphorylate the central phosphorylated residue of the peptide. (6) Kinome peptide arrays are subsequently stained with a phospho-specific fluorescent stain and imaged followed by comparative bioinformatics analyses. Tissue and cell images were derived and/or modified from Servier Medical Arts under a Creative Commons Attribution 3.0 Unported License.

pathogens at the level of individual genes or gene clusters. However, there is a paucity of information regarding the relationship of cell signaling networks, and in particular their activation status, with the biological/pathological events that occur throughout infection.

It has been well established that many biological processes can be regulated independent of transcriptional or translational changes through post-translational modification (PTM) events. Indeed, kinase-mediated phosphorylation of proteins, in which kinases catalyze the transfer of the γ phosphate group from ATP to the hydroxyl group of a specific Ser, Thr, Tyr residue, is

among the most thoroughly characterized PTM. Virtually all cell signal transduction events are regulated by kinases independent of biological complexity of the host (i.e., prokaryotes and eukaryotes).[3] Lending further credence to the biological importance of kinases, >500 kinases have been identified in the human genome, and ~30% of the human proteome is modulated by kinase-mediated phosphorylation events.[4,5] Thus, considering the central role of kinases in a broad range of cellular processes (including growth and development, metabolism, and immune responses), it has been postulated that the activities of individual kinases may represent more reliable predictors of cellular phenotypes than transcriptional or translational changes.[6] Indeed, transcriptional or translational-based OMICS approaches are often unable to account for regulatory events including gene silencing, mRNA stability, translational efficiencies, protein turnover, enzyme/substrate subcellular sequestration, or protein activation/repression PTMs.[7] Given the central role of kinases in the regulation of biological processes, kinases are a logical drug target. As a testament to this, 33 kinase inhibitors have been granted licensure by the U.S. Food and Drug Administration (FDA) for a broad range of malignancies, and there are a continually increasing number of kinase inhibitors that are in various stages of preclinical trials. Furthermore, kinases are the second most frequently targeted gene class in cancer therapy after the G protein coupled receptors.[8,9] The recent prioritization for the repurposing of approved therapeutics for alternative malignancies by the National Institutes of Health Center for Advancing Translational Sciences (NCATS)[10,11] also provides considerable impetus for the investigation of licensed kinase inhibitors as infectious disease therapeutics. Concerns exist regarding the therapeutic application of kinase inhibitors as novel therapeutics for infectious disease, in particular to the potential immunosuppressive effects following prolonged treatment. However, it should be appreciated that the application of kinase inhibitors in such cases would need to be targeted in terms of timing and dose, with appropriate molecular biomarkers guiding initiation and cessation. It should also be appreciated that the clinical symptoms associated with many emerging and re-emerging pathogens have been associated with dysregulated host immune responses (in particular pro-inflammatory responses).

Thus, the global analysis of the activation state of host kinases (the kinome) can provide critical insight into the specific activation state of individual kinases, cell signaling pathways, or larger biological networks. In addition, kinome investigations may offer important, and predictive, insight into the cellular mechanisms that regulate phenotypic changes within cells.[7] As many kinases recognize a particular phosphorylation motif composed of the central phosphoacceptor site and the amino acids +4 and −4 residues from the central phosphorylation site,[12] peptides representing this kinase target motif can be synthesized with relatively high efficiency and low expense. Indeed, kinase target motif peptides have been shown to be appropriate substrates for their respective kinases with V_{max} and K_m values approaching those of the full-length protein.[13] Thus, peptide kinome arrays can be constructed in an analogous manner to traditional DNA microarrays where kinase target motif peptides are spotted onto a glass slide representing hundreds to thousands of unique peptide targets for kinases (Figure 2). Following this, samples in the form of cellular lysates from whole organs, tissues, or individual cell types can be applied to the kinome peptide arrays, allowing for the phosphorylation of specific peptide targets by kinases within the lysate (Figure 3). The development of kinome-specific bioinformatics analysis software, including the Platform for Intelligent, Integrated Kinome Analysis (PIIKA), has provided a mechanism to identify the complex patterns of kinase-mediated phosphorylation events and quantitate the differences between compared conditions.[14,15]

INTEGRATING KINOMICS WITH CLINICAL AND MOLECULAR PATHOGENESIS INVESTIGATIONS FOR EMERGING AND RE-EMERGING PATHOGENS

Middle East Respiratory Syndrome Coronavirus (MERS-CoV). Human coronaviruses are the causative agents of an estimated 30% of upper and lower respiratory tract infections in humans resulting in rhinitis, pharyngitis, sinusitis, bronchiolitis, and pneumonia.[16,17] Coronaviruses are members of the Coronavirinae subfamily of viruses and together with the Torovirinae subfamily comprise the Coronoviridae virus family (order Nidovirales). Multiple coronavirus family members, including OC43 and 229E, can be found across the globe and largely result in mild illness with more-severe illness limited to children and the elderly.[18−20] Since the emergence of SARS in 2003 and MERS in 2012, there is increased interest in coronaviruses as global public health threats.

SARS-CoV was first identified in China before spreading to 37 countries resulting in more than 8000 confirmed cases and 775 deaths. No additional cases have been reported since 2004.[21] In 2012, MERS emerged in Saudi Arabia as a severe respiratory disease with gastrointestinal and renal complications.[22] MERS-CoV has subsequently spread to 26 countries (WHO), resulting in 1733 confirmed cases and 628 deaths as of June 2016 (http://www.who.int/emergencies/mers-cov/en/). MERS may have emerged from a bat reservoir, likely spilling into humans via dromedary intermediate hosts. Human-to-human spread occurs primarily within healthcare settings, often leading to severe disease. No licensed vaccines or targeted therapies are available.

Clinical Findings during MERS-CoV Infection. Syndromic case-definition for MERS infection requires a compatible clinical syndrome and an epidemiologic risk factor including travel to an affected region or contact with a known or suspected case. Initial symptoms of MERS-CoV infection include fever, chills, cough, shortness of breath, myalgia, and malaise following a mean incubation period of 5 days, which can range from 2 to 14 days.[23] Mildly symptomatic or possibly asymptomatic infections have been reported, and progression to severe disease is associated with pre-existing medical conditions including cardiopulmonary disease, obesity, and diabetes. Most (98%) of reported MERS cases are among adults, with a median age of 50 years. In severe cases respiratory failure requiring mechanical ventilation typically occurs within 7 days of symptom onset.[22] Laboratory abnormalities include lymphopenia, leukopenia, thrombocytopenia, elevated serum creatinine levels consistent with acute kidney injury, and elevated liver enzymes.[23−27] High lactate levels and consumptive coagulopathy have also been reported.[24,28] Chest radiographic abnormalities are observed in most cases consistent with viral pneumonitis, secondary bacterial pneumonia, or acute respiratory distress syndrome.[22−24,27,29]

Soluble Immune Mediators Associated with MERS-CoV Infections. Data characterizing immune responses during MERS-CoV infection are limited. Faure et al. evaluated

cytokine levels in serum and bronchoalveolar lavage (BAL) from two MERS patients, one with fatal disease and one who survived.[30] Higher levels of retinoic acid-inducible gene 1 (RIG-1), melanoma differentiation-associated protein 5 (MDA5), interferon regulatory factor (IRF)-3 and -7, interleukin (IL) 17A, and IL-23 and lower levels of IL-12 and IFNγ were observed in the fatal case compared with the survivor. More recently, Min et al. performed a temporal analysis of cytokine, chemokine, and growth factor blood levels from 14 patients during the recent outbreak of MERS in South Korea.[31] The patients were subcategorized into four groups on the basis of disease severity: Group I patients developed fever and recovered. Group II patients developed mild pneumonia without hypoxemia. Group III patients had prolonged and severe pneumonia. Group IV patients had severe pneumonia and acute respiratory distress syndrome. Group IV patients included five fatal cases of MERS; all patients in groups I−III fully recovered from illness. IFNα was elevated in all groups and largely peaked during the second week of illness. Granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage (GM)-CSF were similarly elevated across all patient groups; however, patients with fatal disease had reduced GM-CSF responses following antiviral treatment as compared to patients that recovered. Patients with pneumonia had relative elevations of IL-1, tumor necrosis factor (TNF)-α, IL-6, and IL-10 during the second and third weeks of illness. Elevated IL-6 and IL-10 appeared to trend positively with the severity of illness. A robust induction of multiple chemokines was found in most patients. Notably, eotaxin and regulated on activation, normal T expressed and secreted (RANTES) was elevated in all patients. In contrast, IL-8, monocyte chemotactic protein (MCP)-3 and macrophage inflammatory protein (MIP)-1β were more prominent in groups II and III as compared to groups I and IV. Furthermore, elevated interferon gamma induced protein (IP)-10 correlated with the development of pneumonia (groups I−III). Multiple growth factors, including epidermal growth factor (EGF), fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), and TGF-α, were significantly elevated across all patients; however, EGF was significantly higher in patients that recovered from disease as compared to the fatal cases. Further evaluations are needed to characterize the natural history of immune response during acute MERS-CoV infection and recovery.

Transcriptome Analyses of MERS-CoV. In an effort to better characterize MERS-CoV pathogenesis in the absence of available samples from human patients and, in particular, address pathologic changes associated with infection, multiple animal species have been employed in MERS-CoV investigations. While multiple small animals are not susceptible to MERS-CoV infection,[32] rhesus macaques and marmosets develop mild to severe lung pathology following experimental infection. Transcriptome analysis in MERS-CoV-infected rhesus macaques revealed that genes related to antiviral immunity, chemotaxis, and inflammation were overexpressed in lesional versus grossly normal lung tissue at 3 days postinfection.[33] A significantly smaller number of differentially expressed genes was found on day 6 postinfection with no obvious trends following pathway enrichment analysis. Significant changes in the transcriptome profiles of peripheral blood mononuclear cells (PBMCs) were observed at only day 1 postinfection. This global analysis suggests a key role for an initial rapid innate immune and inflammatory response (through pattern recognition receptors) followed by rapid

resolution.[33] A study of MERS-CoV-infected marmosets evaluated lung lesions by RNaseq at days 3−6 postinfection.[34] Pathway analyses demonstrated that chemotaxis and cell migration, cell cycle progression, and cell proliferation and fibrogenesis were highly over-represented relative to uninfected controls. To a lesser degree, pathways associated with inflammation, vascularization, endothelial activation, proliferation of smooth muscle, and tissue repair were also over-represented in infected animals. Differences were most significant on days 4 and 6 postinfection during illness progression relative to day 3.

Recently, Menachery and colleagues examined the interaction between MERS-CoV EMC/2012 and the host IFN-stimulated gene (ISG) response by transcriptomics.[35] ISG responses in MERS-CoV infected Calu3 cells, a lung adenocarcinoma cell line, had no discernible induction initially upon infection but were up-regulated by 12 h postinfection.[35] Down-regulation of a subset of ISGs resulted in altered histone modifications, a potential epigenetic contributor to early impairment of antiviral cellular defenses. In a separate analysis genetically distinct MERS-CoV strains, MERS-CoV SA1 and MERS-CoV Eng1, produced distinct gene expression profiles in Calu-3 cells.[36] These analyses may better inform early host-cell antiviral responses and the impact of viral evolution on these and other complex biological responses. Proteomics analysis corroborated these transcriptional data with induction of ISGs observed 18 h postinfection. Significantly reduced levels of STAT1 and PKR compared with uninfected controls were also noted. Differential host transcriptome responses to MERS-CoV SA1 and MERS-CoV Eng1 highlight both the propensity of emerging viral pathogens to evolve rapidly and the importance of additional host response analyses for augmenting and clarifying such complex biological responses.

Kinome Analyses of MERS-CoV Infection. Host responses to MERS-CoV infection through kinome analysis were recently assessed using Huh-7 cells, an immortalized human hepatocyte cell line, that are highly permissive to MERS-CoV infection.[37] Temporal analysis of kinome responses by peptide arrays revealed selective modulation of extracellular signal-regulated kinases (ERK)/mitogen-activated protein kinases (MAPK) and phosphatidylinositol-3-kinases (PI3K)/AKT (also known as protein kinase B)/mechanistic target of rapamycin (mTOR) signaling responses. Over-representation analysis (ORA) revealed ERK/MAPK and PI3K/AKT/mTOR signaling responses were consistently up-regulated during infection. Multiple ERK/MAPK family members formed central components of functional networks and signaling pathways throughout infection. Similar results were observed for intermediates of the PI3K/AKT/mTOR signaling pathway at 1 and 24 h postinfection, suggesting that modulation of ERK/MAPK and PI3K/AKT/mTOR signaling may be important for productive MERS-CoV infection.

Downstream analysis of the phosphorylation patterns of pathway intermediates from the ERK/MAPK and PI3K/AKT/mTOR signaling supported observations from the kinome analysis. Both investigations demonstrated that nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB)-regulated family members were important mediators of MERS-CoV infection.[38] IL8- and IFN-mediated signalings were also modulated during MERS-CoV infection, consistent with prior analyses.[39,40] These results were also in agreement with in vitro transcriptional analysis of MERS-CoV infection.[38] Prophylactic or therapeutic addition of FDA-licensed kinase

inhibitors targeting activated kinases in MERS-CoV infection impaired viral replication. These hypothesis-generating data may inform directed investigations into MERS-CoV pathogenesis and, importantly, demonstrate the potential to identify novel host-centric therapeutic targets.

Ebolaviruses. The Filoviridae family of viruses consists of three genera: Ebolavirus, Marburgvirus, and the newly identified Cuevavirus. Structurally, filoviruses have a pleomorphic enveloped, filamentous virion particle that encapsulates a negative-sense single-stranded RNA genome. Ebolaviruses were first described in 1976 following disease outbreaks in the Democratic Republic of Congo and Sudan and are composed of five viral species, including Ebola virus (EBOV), Sudan virus (SUDV), Bundibugyo virus (BDBV), Taï Forest virus (TAFV), and Reston virus (RESTV). Sporadic outbreaks of EBOV, SUDV, BDBV, and TAFV have occurred throughout central Africa for more than three decades, resulting in thousands of infections. Case fatality rates during these outbreaks have routinely exceeded 50%.[41] Isolated outbreaks of RESTV have occurred outside Africa in nonhuman primate facilities in the United States, Italy, and the Phillipines, and infection results in high morbidity and mortality in nonhuman primates; however, RESTV has only been associated with asymptomatic infections in humans.[42] Although ebolaviruses have been historically associated with isolated outbreaks involving small cohorts of infected patients (<500), an outbreak of EVD in West Africa beginning in 2014 has resulted in 28,616 cases and 11,310 deaths (40% CFR) as of June 2016 (http://www.who.int/csr/disease/ebola/en/). Although virus transmission has greatly decreased in West Africa, surveillance for sporadic infections continues.

Clinical Findings in EVD. EBOV transmission occurs through exposure of infected body fluids or tissues to mucous membranes or nonintact skin.[43] The mean incubation period is 6−10 days, ranging from 2 to 21 days.[43] Initial signs and symptoms are nonspecific including fever, myalgia, and malaise and cannot be reliably distinguished from other endemic illnesses in Africa including malaria and enteric infections.[43] Whereas mild illness has been described, most patients develop severe disease within days of symptom onset. Massive gastrointestinal fluid losses of up to 5−10 L per day due to vomiting and watery diarrhea may result in progressive dehydration and hypovolemic shock. Even in the setting of adequate fluid and electrolyte replacement, sequential multiorgan failure may occur. EBOV infects multiple organs and cell types throughout the body with the notable exception of lymphocytes that are indirectly depleted early during infection. Organ injury due to direct viral or indirect host-mediated responses results in severe complications including meningoencephalitis, uveitis, respiratory failure, secretory diarrhea, disordered coagulation, renal failure, hepatic necrosis, and myositis. The clinical presentation, laboratory values, viral kinetics, and clinical management of EVD patients in West Africa, Europe, and the United States during the 2014−2015 outbreak have been recently well-characterized.[44]

Soluble Immune Mediators Associated with EBOV Infections. There is a paucity of information regarding EBOV pathogenesis in humans primarily due to the limited frequency of EVD outbreaks prior to 2014 and limitations presented by sample acquisition from infected patients in the field as well as the overall size of patient cohorts. Largely contradictory findings regarding the immune responses in those who survive or succumb to EVD have further confounded the understanding of EBOV pathogenesis in human patients. For example, Villinger et al. reported that serum cytokine concentrations (including IFNα, IFNγ, TNF-α, IL-2, and IL-4) were elevated in patients with fatal infections in comparison to survivors.[45] In contrast, additional studies have suggested that fatal infections were instead related to general immunosuppression including IFNγ, IL-2, and IL-4.[46−49] An investigation of SUDV infection in humans by Sanchez et al. demonstrated limited changes in the expression levels of cytokines, Fas antigen, and Fas ligand in PBMCs from infected patients relative to those found for uninfected patients.[50] Furthermore, an investigation of 42 fatally infected EVD patients by Wauquier et al. has further confounded the role of host immune responses in fatal EVD as hypersecretion of multiple cytokines and growth factors and decreased secretion of T lymphocyte-derived cytokines were associated with fatal disease.[51]

Transcriptome Analyses of Ebola Virus Infection. To date, no investigations of host gene expression in EBOV-infected patients have been reported, although limited data are available from animal models of infection or from in vitro investigations.

In a study of PBMCs from EBOV-infected crab-eating macaques, Rubins et al. found few notable changes in the early stages of infection (1−2 days); however, broad changes were observed over days 4−6 post-infection. Pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) and chemokines (MIP-1α and MCP1−4) were up-regulated at days 4−6 postinfection relative to healthy controls.[52] Multiple genes related to apoptosis including Bcl-2 family members, multiple caspases, Fas-associated death domain protein, and TNF superfamily member 10 were also up-regulated at late time points. IFN-regulated genes were up-regulated by day 2 postinfection and remained so through study day 6.

Yan et al. investigated PBMC gene expression in EBOV-infected rhesus macaques with or without anticoagulant administration. Untreated animals displayed up-regulation of immune response genes, B cell receptor signaling intermediates, NK cell mediated cytotoxicity, leukocyte activation, and lymphocyte activation compared with anticoagulant-treated animals during the early stages of infection. The expression levels of these gene clusters fell to pre-infection levels at the late-stage of infection. In contrast, genes related to defense responses, apoptosis, wounding, inflammation, coagulation, and leukocyte activation remained elevated during early- and late-stage infection.

Following the isolation of RESTV from pigs,[53] subsequent investigations have demonstrated that pigs were susceptible to both RESTV and EBOV infection with preferential targeting of macrophages in the lungs. Recently, Nfon et al. demonstrated that EBOV infection in pigs resulted in up-regulation of chemokine expression beginning on day 3 postinfection as compared to mock-infected pigs.[54] The most pronounced changes in gene expression were found on days 5 and 7 postinfection and included the up-regulation of a broad set of cytokines (IL-5, IL-6, IL-8, IL-10, IL-22, IL-26, IL-27, resistin), chemokines (CCL2, CCL10, CCL19, CCL20, AMCF-II, CCL3L1, CCL4), cell adhesion protein (selectin), antimicrobial protein, palate, lung, and nasal epithelium clone proteins, and pro-apoptotic molecules (multiple caspases, caspase recruitment domain-containing protein 6 (CARD), apoptosis-associated tyrosine kinase (AATK), Fas, Fas-associated protein with death domain (FADD), TNF receptor-associated factor 3 (TRAF3), TNFα-induced protein 3-interacting protein 1

(TNIP1)). In addition, expression of multiple genes related to microbial sensing (pattern recognition receptors) or antiviral responses (ISGs) was up-regulated in the lungs of infected animals. Although the localization of the cytokine response of pigs and humans or NHPs differs during the course of EBOV infection (localized responses in the lungs of pigs versus a predominantly systemic response in humans and NHPs), the cytokine profiles of pigs, humans, and NHPs were quite similar. For example, comparison of NHP[52] and porcine responses[54] during EBOV infection demonstrated multiple gene expression similarities between the two species (i.e., IL-6, IL-8, caspase family members). It is also likely that direct comparison of both data sets would likely yield many common gene signatures that are conserved in their identity as well as their directionality (up-regulation vs down-regulation).

Macrophages are an early target of EBOV infection and support high-level viral replication. EBOV attachment and entry into human macrophages in vitro induces pro-inflammatory mediators including IL-6, IL-8, and TNF-α as early as 1 h postinfection.[55] Noncardiogenic pulmonary edema is a recognized complication of EVD, and human autopsy data support that alveolar macrophages are a target of EBOV infection. EBOV infection of alveolar macrophages in vitro resulted in an early, transient increase in cytokine and chemokine expression,[56] supporting that paracrine-soluble mediators of inflammation may contribute to vascular leakage in the lungs. Gene expression responses of EBOV- and MARV-infected Huh7 cells resulted in the global suppression of antiviral responses, including Toll-like receptor (TLR), IRF3, and protein kinase R (PKR)-mediated pathways.[57] However, signal transducers and activators of transcription (STAT) phosphorylation in EBOV- and MARV-infected cells were differentially modulated. EBOV-mediated IFN inhibition has been well characterized and is thought to be attributable to EBOV proteins VP24 and VP35.[58] Interestingly, RESTV infection, which does not induce clinical illness in humans, resulted in the activation of >20% of the IFN-stimulated genes (ISGs).

Kinome Analysis of Ebola Virus. Hepatocytes are an early target of EBOV infection, directly contributing to diffuse hepatic necrosis observed in fatal cases. EBOV infection of Huh7 cells has been evaluated by kinome analyses, shedding light on liver pathogenesis in EVD.[59] EBOV infection of Huh-7 cells resulted in temporal modulation of the TGF-β signaling pathway as compared to mock-infected cells. Pathway ORA demonstrated that multiple TGF-β-mediated signaling pathways were up-regulated at 1 and 24 h post EBOV infection. Furthermore, these responses were associated with changes in the expression patterns of multiple cellular proteins associated with a mesenchyme-like transition. These included the up-regulation of matrix metalloproteinase 9, N-cadherin, and fibronectin and down-regulation of E-cadhering and claudin 1. In this process cells lose polarity and cell-to-cell adhesion transforming into mesenchymal stem cells that contribute to wound healing or organ fibrosis; however, the role of these events in EBOV infection remains to be elucidated. Additional analysis demonstrated that inhibition of PI3K/AKT, ERK/MAPK, or PKC pathways with kinase inhibitors reduced EBOV replication when administered prophylactically or therapeutically. Supporting this observation, a subset of kinase inhibitors administered to EBOV-infected mice reduced lethality. Defining mechanisms by which kinase inhibitors show benefit

in these models will better clarify their role as potential therapeutics.

Monkeypox Virus. MPXV, a member of the genus *Orthopoxvirus*, causes zoonotic infections with a case fatality rate of ~11%.[60] MPXV, vaccinia virus (VACV), cowpox virus (CPXV), ectromelia virus, and variola virus (VARV), the etiologic agent of human smallpox, comprise the Orthopoxviridae family of viruses. MPXV was first isolated in 1958 from cynomolgus macaques in Denmark; however, human MPXV infections were not recognized until 1970 following the isolation of the virus from a suspected case of smallpox infection in the Democratic Republic of Congo.[61] MPXV is composed of two distinct clades that are genetically, clinically, and geographically distinct. The Congo Basin MPXV (Central African MPXV) clade is considered to have both higher lethality and morbidity than the West African MPXV clade as demonstrated from comparative infection models in various animal species (including nonhuman primates, mice, prairie dogs, and ground squirrels) and as well natural infection in humans.[61−63] Fifty-four cases of human MPXV disease were recorded in West and Central Africa from 1970 to 1979. Although no fatalities were reported in West African cases (including Liberia, Nigeria, Ivory Coast, and Sierra Leone), 21% of cases from the Democratic Republic of Congo resulted in fatal disease.[61] Furthermore, West African MPXV was responsible for the 2003 MPXV outbreak in the United States that resulted in 69 diagnosed cases of MPXV and no associated fatalities.[64] Although human MPXV infections have been recorded in West Africa, the majority of human MPXV infections have occurred in the Congo Basin region of Central Africa, largely in the Democratic Republic of Congo.[60]

Clinical Findings in MPXV Infections. Clinical and epidemiological information regarding human MPXV disease has been derived from enhanced surveillance campaigns in the Congo Basin.[61] From this work, it has been demonstrated that human MPXV infection and illness largely mirror those of discrete, ordinary smallpox.[61] The incubation period for both viruses (VARV and MPXV) is 7−17 days with an initial febrile prodromal period of 1−4 days. This prodromal period is normally accompanied by fever, headache, backache, malaise, and prostration.[61] The rash period for both smallpox and MPXV (including lesion appearance and desquamation) normally occurs 14−28 days postinfection with highly similar appearance, distribution, and progression of lesions.[60,61] As with smallpox, MPXV-associated rash progresses through macular, papular, vesicular, and pustular phases. A second febrile period occurs when the lesions become pustular and is often associated with deteriorating conditions in the patient. Lymphadenopathy (maxillay, cervical, or inguinal) is often associated with MPXV infections prior to, or concomitant with, rash development but is absent in VARV infections. It has been postulated that this reflects the effective generation of host immune responses during MPXV infection as compared to VARV; however, this has yet to be validated.[60,61] Severe complications have been noted late in the course of MPXV infection, including pulmonary distress or bronchopneumonia, corneal scarring and permanent vision loss, and encephalitis.[60] Severe dehydration due to excessive vomiting or diarrhea may also occur. Long-term sequelae in survivors are most commonly associated with pitted scarring.

Soluble Immune Mediators Associated with MPXV Infections. Although MPXV infections in humans have been recorded for over four decades, there has been little information

regarding host immune responses during the course of natural infection. As disease presentation is highly similar during MPXV and VARV infections, it has been postulated that immune responses would likely be highly conserved. Recently, Johnston et al. provided the first empirical evidence for a relationship between cytokine responses and disease severity during MPXV infection.[65] Serum cytokines were analyzed from 19 patients with confirmed MPXV infections ranging from mild to severe as assessed by the WHO smallpox lesion scoring system.[66,67] Serum concentrations of IL-1β, IL-1RA, IL-2R, IL-4, IL-5, IL-6, IL-8, IL-13, IL-15, IL-17, MCP-1, and RANTES were elevated in all disease groups (mild to severe) as compared to normal serum concentrations. IL-10 concentrations were also elevated in all disease groups and were proportional to disease severity. However, patients with serious MPXV disease had significantly higher concentrations of IL-10 compared to all other disease groups. MPXV infection resulted in elevated MIP-1α and MIP-1β; mild cases had significantly elevated levels above the moderate or severe disease groups. Serum concentrations of IL-2R were elevated across all disease groups; however, patients with serious disease had significantly higher IL-2R serum levels than those with mild to severe MPXV disease. GM-CSF levels were significantly elevated only in those with serious MPXV disease as compared to normal serum ranges. On the basis of these observations, MPXV infection resulted in prominent T helper 2 (Th2) and dampened Th1 responses.

Transcriptome Analyses in MPXV Infection. Transcriptome analyses have largely been employed for the in vitro investigation of the molecular pathogenesis of MPXV infection. Alkhalil et al. investigated the host transcriptome responses to MPXV infection during the first cycle of viral replication (3 and 7 h postinfection) in rhesus macaque kidney epithelial cells.[68] Interestingly, MPXV infection resulted in a strong down-regulation of host transcriptional responses. Of the transcripts that met the authors' criteria for significance, 89% of the transcripts were found to be down-regulated at both post-infection time points. Comparative functional analysis from both time points suggested that the primary biological functions associated with these down-regulated transcriptional responses were largely related to cell morphology, cell development, metabolic responses, and post-translational modifications. Canonical pathway analysis demonstrated a general conservation in the identities of over-represented pathways at both time points including multiple growth factor signaling pathways, p53 signaling, and cell cycle-related pathways. More recently, Bourquain et al. investigated host transcriptome responses in MPXV-infected HeLa cells, a cervical epithelial cell line.[69] At 6 h post-MPXV infection, only 1.1% of the transcripts analyzed were found to have >2-fold changes in gene expression. In contrast to Alkhalil et al., the majority of these transcripts (~68%) were found to be up-regulated as compared to mock-infected controls. Functional analysis of all transcripts with >2-fold changes in gene expression demonstrated a strong over-representation of genes involved in the negative regulation of MAPK signaling and the intracellular protein cascade. Positive regulation of pathways related to Toll-like receptor signaling, chemotaxis, and regulation of leukocyte migration was also predicted from the data. An investigation by Rubins et al. compared the temporal host transcriptome response to MPXV in multiple human cells targeted by MPXV including primary macrophages, primary fibroblasts, and HeLa cells.[70] The trasncriptome of

MPXV-infected fibroblasts was found to have the most significant changes where MPXV infection resulted in the depletion of ~2000 genes by a factor of ≥ 3. Interestingly, MPXV infection resulted in the broad repression of many transcripts related to innate immune responses in all cell types tested. In contrast, inactivated MPXV resulted in strong up-regulation of innate immune responses in all of the cell types. It was also noted that MPXV infection resulted in strong cytopathic effects across all of the cell types in contrast to an almost universal repression of innate immune responses.

Kinome Analyses in MPXV Infection. Human MPXV infections and infection models of MPXV in various animal species have demonstrated that the Congo Basin MPXV clade is more virulent than the West African MPXV clade. However, there has been a paucity of information regarding the underlying molecular mechanisms mitigating these virulence differences. Furthermore, previous investigations focusing on gene expression or proteomic changes during MPXV infection have focused solely on Congo Basin MPXV. To address this, host kinome analysis was performed on Congo Basin and West African MPXV-infected human monocytes, a host cell targeted by orthopoxviruses.[71] As the genomes of both MPXV clades demonstrate considerable diversity in the regions coding host response modifier proteins, and in particular in genes associated with anti-apoptotic activities, it was postulated that the virulence differences of the two MPXV clades may be related to differential modulation of host cellular responses. Hierarchical clustering of the kinome data sets suggested limited similarities at the level of host kinase modulation between the two MPXV clades. The Congo Basin MPXV kinome data set clustered most strongly with the kinome data set from CPXV-infected monocytes and moderately with the VACV-infected monocyte data set. Both CPXV and VACV can cause serious disease in humans. The pathway ORA of the kinome data demonstrated that Congo Basin MPXV infection resulted in strong down-regulation of a large proportion of host cell responses, most notably apoptosis, in comparison to West African MPXV. Biological validation through fluorescence-activated cell sorting (FACS) and caspase 3 activity analyses confirmed this phenomenon. From the perspective of individual phosphorylation events, the kinome data also suggested that AKT phosphorylation at Ser473 was increased in Congo Basin MPXV-infected cells as compared to West African MPXV-infected cells. Pharmacologic inhibition of AKT phosphorylation at Ser473 resulted in a >250-fold inhibition of Congo Basin MPXV virus yields, whereas those for West African MPXV were unaffected. Prior investigations with CPXV and VACV demonstrated that pharmacological inhibition of AKT resulted in decreased viral yields for both viruses.[72] Overall, this investigation provided significant insight into the host cellular response differences between the two MPXV clades.

■ CONCLUSIONS

Emerging and re-emerging pathogens are a continual threat to global health. In recent years, disease outbreaks associated with SARS and the 2009 influenza pandemic have also demonstrated that these pathogens can have considerable effects on local, national, and international economies. As a consequence, regional outbreaks of emerging and re-emerging pathogens can have deleterious effects on global stability. Thus, it is prudent that a concerted effort is employed to assimilate data that bridge both clinical and molecular information in investigations

Table 1. Kinase Inhibitors Tested against EBOV, MERS-CoV, or MPXV

kinase inhibitor	host target	impact of inhibitor on viral replication (reduction in viral replication considered to be >40% inhibition)	impact of inhibitor on animal survival	reference
MERS-CoV				
Rapamycin	mTOR	in vitro reduction in viral replication with prophylatic and therapeutic treatment	no data available	37
GF109203X	PKC	in vitro reduction in viral replication with prophylatic and therapeutic treatment	no data available	37
Ro-31-8220	PKC	in vitro reduction in viral replication with prophylatic and therapeutic treatment	no data available	37
U0126	MEK1, MEK2	in vitro reduction in viral replication with prophylatic treatment	no data available	37
Wortmannin	PI3K	in vitro reduction in viral replication with prophylatic treatment	no data available	37
GW5074	c-Raf1	in vitro reduction in viral replication with prophylatic treatment	no data available	37
Imatinib	c-Abl1 family	in vitro reduction in viral replication with prophylatic treatment	no data available	79
SB203580	p38 MAPK	in vitro reduction in viral replication with prophylatic treatment	no data available	37, 38
Dasatinib (BMS-354825)	Src, Abl family kinases	in vitro reduction in viral replication with prophylatic treatment	no data available	79
PP2	Src family kinases	no significant inhibition of viral replication	no data available	37
Bay 11-7082	IKBα	no significant inhibition of viral replication	no data available	37
PKC-412	PKC	no significant inhibition of viral replication	no data available	37
AG490	EGFR; ERBB2	no significant inhibition of viral replication	no data available	37
L-NAME	nitric oxide synthase	no significant inhibition of viral replication	no data available	37
EBOV				
AG879	ErbB2 and FLK-1 (VEGF receptor)	in vitro reduction in viral replication with prophylatic and therapeutic treatment	improvement in mouse survival	59
LY294002	PI3K	in vitro reduction in viral replication with prophylatic and therapeutic treatment	improvement in mouse survival	59, 80
SB431542	activin receptor-like kinase receptors, ALK5, ALK4 and ALK7	in vitro reduction in viral replication with prophylatic and therapeutic treatment	improvement in mouse survival	59
SU1498	VEGF receptor 2	no significant inhibition of viral replication	improvement in mouse survival	59
Rottlerin	PKC	in vitro reduction in viral replication with prophylatic and therapeutic treatment	improvement in mouse survival	59
Wortmannin	PI3K	in vitro reduction in viral replication with prophylatic and therapeutic treatment	no data available	59
Indirubin-3-monoxamine	glycogen synthase kinase 3β	in vitro reduction in viral replication with prophylatic and therapeutic treatment	no data available	59
SP600125	JNK	in vitro reduction in viral replication with prophylatic and therapeutic treatment	no data available	59
GF109203X	PKC	in vitro reduction in viral replication with prophylatic and therapeutic treatment	no data available	59
Genistein	EGFR	in vitro inhibition of vsv-ebov pseudotype transduction with prophylatic treatment	no data available	81
Tyrphostin	tyrosine kinases	in vitro inhibition of vsv-ebov pseudotype transduction with prophylatic treatment	no data available	81
KN-93	CAMK2	in vitro reduction in infectivity	no data available	80
U0126	MEK1, MEK2	in vitro reduction in viral replication with prophylatic treatment	no data available	59
Nilotinib	c-Abl1 family	in vitro reduction in viral replication with therapeutic treatment	no data available	82
Imatinib	c-Abl1 family	in vitro reduction in viral replication with therapeutic treatment	no data available	82
p38inK II	p38 MAPK	in vitro reduction of viral entry	no data available	83
SB202190	p38 MAPK	In vitro reduction of viral entry	no data available	83
AG1024	insulin-like growth factor 1 receptor	no significant inhibition of viral replication	no data available	59
Tricirbine	Akt	no significant inhibition of viral replication	no data available	59
GW5074	c-Raf1	no significant inhibition of viral replication	no data available	59
ZM336372	c-Raf1	no significant inhibition of viral replication	no data available	59
HBDDE	PKC	no significant inhibition of viral replication	no data available	59
MPXV				
Dasatinib (BMS-354825)	Src, Abl family kinases	in vitro reduction in viral replication with prophylatic and therapeutic treatment (Congo Basin and West African clades)	no data available	84
Staurosporine	nonspecific kinase inhibitor	in vitro reduction in viral replication with prophylatic treatment (Congo Basin and West African clades)	no data available	71
SB202190	p38 MAPK	in vitro reduction in viral replication with prophylatic treatment (Congo Basin and West African clades)	no data available	71
BML-257	Akt	in vitro reduction in viral replication with prophylatic treatment (Congo Basin and West African clades)	no data available	71
LY294002	PI3K	in vitro reduction in viral replication with prophylatic treatment (Congo Basin clade only)	no data available	71

Table 1. continued

kinase inhibitor	host target	impact of inhibitor on viral replication (reduction in viral replication considered to be >40% inhibition)	impact of inhibitor on animal survival	reference
		MPXV		
Akt-X	Akt	in vitro reduction in viral replication with prophylatic treatment (Congo Basin clade only)	no data available	71
Nutlin 3	MDM-2	no significant inhibition of viral replication	no data available	71

of these pathogens. These efforts will not only provide considerable context in regard to the molecular events that potentiate clinical manifestations of pathogenesis but also better inform the design and implementation of novel therapeutics. To this end, global analyses of host molecular responses can provide considerable insight into the complex molecular events that underlie cellular responses. Indeed, transcriptome analyses have provided important information regarding host transcriptional responses during emerging and re-emerging pathogen infection. These investigations often provide critical insight into the kinetics of host immune responses during the course of infection as well as mechanistic information regarding the cellular intermediates involved in these processes. However, the role of PTMs in the regulation of these events cannot be captured by traditional transcriptome technologies. In particular, the role of kinase-mediated regulation of cell signaling pathways has remained poorly understood. Given the central role of kinases in the regulation of cellular processes (e.g., homeostasis, metabolism, proliferation, and stress responses), it is of inherent importance that future investigations also address the role of the kinome in the cellular response to pathogen insult. Furthermore, kinomics also provides a mechanism for the identification of novel therapeutic targets based on the direct assessment of the activation state of cell signaling pathways. For example, proinflammatory responses during early stages of infection, and in particular the dysregulation of specific cytokines or cell signaling events that contribute to these, may represent potential therapeutic targets in the early stages of high-consequence viral pathogen infection. However, the selection of immunomodulatory therapeutics that target these dysregulated host responses is complicated by the regulatory events (i.e., kinase-mediated cell signaling events) that occur upstream of changes in gene expression. In addition, mRNA is subject to a variety of regulatory processes (including gene silencing, mRNA stability, translational efficiencies, protein turnover, enzyme/substrate subcellular sequestration, and/or protein activation/repression PTMs). Thus, from the standpoint of therapeutic discovery, the sole reliance on technologies for the global investigations of host responses that do not account for these regulatory processes or the role of PTMs in the modulation of cellular responses could impede the identification of efficacious therapeutics.

To this end, kinome analysis may also facilitate the identification of immunomodulatory therapeutics that have gained licensure through analysis of a quantifiable biological event (kinase-mediated phosphorylation) or for identifying novel host therapeutic targets for which therapeutics could be designed/developed. Furthermore, kinase inhibitors may serve as primary or adjunctive therapies for emerging infectious diseases. In addition, preclinical data and the increasing number of kinase inhibitors that have gained regulatory approval for cancer and other maladies suggest this approach is feasible and efficacious. From the perspective of this review, kinome investigations have identified several therapeutic targets and licensed kinase inhibitors that have impaired viral replication in vitro and reduced the severity of disease in vivo (Table 1). For example, it has been demonstrated that the ERK/MAPK and PI3K/AKT/mTOR signaling pathways have a role in viral propagation during MERS-CoV infection.[37] Indeed, licensed kinase inhibitors that targeted these pathways (i.e., everolimus, selumetinib, and trametinib) resulted in decreased viral replication in vitro when added prior to, or following, infection. Furthermore, the pharmacologic inhibition of PI3K and PKC following EBOV infection provided partial protection in a lethal model of EVD in mice.[59] It should be noted that although the modulation of an individual kinase may have suppressive effects on infection (i.e., viral replication), this might not provide the level of inhibition required to completely negate viral escape. In addition, given the ability of many cell signaling pathways to signal through both canonical and noncanonical mechanisms, inhibition at a single intermediary point within a pathway may not provide the overall level of inhibition required to negate a deleterious response (i.e., viral replication, changes in cellular phenotypes, etc.). Thus, although previous investigations have demonstrated that individual kinases or cell signaling pathways may represent novel targets for anti-infective therapies, it is prudent that future investigations also examine combinations of inhibitors for efficacy and anti-infective activities. Furthermore, the targeting of cell signaling pathways at or near the origin point for the cell signaling cascade should also be examined as these likely represent stronger inhibitory targets given the generally reduced branching of cell signaling networks at or near the cell receptor.

In addition to host-directed therapeutic targeting, kinomics also confers the ability to identify novel inhibitors of pathogens through detailed characterization of the viral life cycle. Host-mediated PTMs, and in particular kinase-mediated phosphorylation, have been implicated in the viral life cycle and pathogenesis for several members of the order Mononegavirales, including EBOV.[73−75] Thus, therapeutic targeting of kinases may represent a novel therapeutic strategy that can be employed to modulate host-centric or pathogen-centric molecular events during infection. For example, in silico prediction of viral protein phosphorylation sites provides a mechanism for the construction and, ultimately, the annotation of viral protein PTMs that are critical to the viral life cycle. Furthermore, the use of kinome peptide arrays has extended beyond the human kinome and now extends to a variety of animal species.[76−78] It has been suggested that the interspecies phenotypic variability may reflect differences in phosphorylation sites found within the proteome.[78] Thus, the development of species-specific kinome peptide arrays provides additional utility for kinome analysis as peptide arrays representing traditional laboratory animal species (mouse, guinea pig, nonhuman primate) can be employed to detail the species-specific host response. The results from such analyses, and the overlap between these and those described previously from the analysis of human infections, may inform

the selection of appropriate animal models that meet regulatory approval through the FDA Animal Efficacy Rule.[29]

Taken together, it is of inherent importance that future investigations of emerging and re-emerging pathogens address the complex nature of biological responses. Thus, molecular investigations of pathogenesis should be guided by available knowledge regarding the clinical and pathologic manifestations of disease. Indeed, technologies that provide further granularity into the precise molecular events that potentiate cellular responses during the course of infection will assist investigations of emerging and re-emerging pathogens and the identification of novel therapeutic targets. To this end, kinomics-based analyses of host responses provide a mechanism to directly address the cellular events at the level of specific cell signaling phenomena that underlie the biological responses and, ultimately, the clinical presentation of disease for emerging infectious pathogens.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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