The enhanced permeability retention effect: a new paradigm for drug targeting in infection

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Multidrug-resistant, Gram-negative infection is a major global determinant of morbidity, mortality and cost of care. The advent of nanomedicine has enabled tailored engineering of macromolecular constructs, permitting increasingly selective targeting, alteration of volume of distribution and activity/toxicity. Macromolecules tend to passively and preferentially accumulate at sites of enhanced vascular permeability and are then retained. This enhanced permeability and retention (EPR) effect, whilst recognized as a major breakthrough in anti-tumoral targeting, has not yet been fully exploited in infection. Shared pathophysiological pathways in both cancer and infection are evident and a number of novel nanomedicines have shown promise in selective, passive, size-mediated targeting to infection. This review describes the similarities and parallels in pathophysiological pathways at molecular, cellular and circulatory levels between inflammation/infection and cancer therapy, where use of this principle has been established.

Keywords: multiple drug resistance, nanomedicine, drug delivery systems, Acinetobacter, Pseudomonas

Introduction

The last decade has witnessed a continued increase in microbial resistance, a decreased antimicrobial armamentarium, reduced funding and a decline in apparent pharmaceutical interest in anti-infective research.1,2 Multidrug-resistant (MDR) infection has steadily increased in the past decade and extended or even pan-drug resistant bacterial species have now emerged.3–5 MDR infection is clearly a universal health concern, accounting for 25 000 deaths and healthcare costs of >€1.5 billion in Europe6 and $14–22 billion in the USA.7 Scientific interest in MDR infection has increased exponentially.8

A major part of this disease burden has been attributed to the ‘ESKAPE’ pathogens, namely Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp., due to their virulence, ability to evade current antimicrobial therapy and ability to rapidly develop resistance.5,9,10 Modest success has been reported in drug development against Gram-positive infections, but current therapeutic options for Gram-negative bacteria are limited.9 The virtual absence of antimicrobial drugs in advanced stages of development is an imminent global health threat.5,11–13 With the increasing incidence of MDR, recourse to older antibiotics has become necessary.14,15 Clinical use of these antibiotics (e.g. colistin) has been limited by their extensive toxicity.9 Moreover, conventional ‘small molecule’ antibiotics display an indiscriminate volume of distribution, with no targeting to the site of infection. Distribution to unintended areas could facilitate toxicity.5,16–20

The search for improved antibiotic treatments is ongoing and research largely falls into two categories: the identification of novel molecular targets following genomic/proteomic research, and improved drug delivery and targeting. This situation bears close parallels to anti-cancer therapy,21 where the enhanced permeability and retention (EPR) effect has significantly contributed to cancer-targeting drug design.22–24 The EPR effect relates to the propensity of macromolecules to preferentially accumulate at sites of increased vascular permeability such as solid tumour tissue (Figure 1). EPR has afforded an efficient strategy for anti-cancer drug design, allowing high selectivity, improved therapeutic efficacy and decreased toxicity.21–23 Evidence suggests that the EPR phenomenon is not exclusive to solid neoplasms and is evident where vascular permeability is increased, e.g. infection.24 Since its conception, some 30 years ago, the EPR effect has delivered a paradigm shift in anti-cancer drug design.24–27 Harnessing such an EPR effect for antimicrobial delivery could facilitate targeted antibiotic delivery.

Small molecular weight antimicrobials in use today are indiscriminately delivered to normal and infected tissue alike. Over the last decade, nanomedicine has been increasingly employed as a means of targeting anti-infective drugs and improving their delivery to reduce toxic side effects (reviewed in Schiffelers et al.28 and Huh and Kwon29). Figure 2 illustrates the complexity of the interaction between bacteria, host and antibiotic. The passive targeting of macromolecules due to the EPR effect unlocks the potential of nanomedicines, such as polymer therapeutics, to selectively, safely and effectively target antibiotic delivery in human disease.
Since the pioneering work of Ringsdorf, over 50,000 publications bear witness to the versatility of bioconjugation, bestowing stability in circulation, reduction of toxic side effects, stability in circulation and accumulation at the intended site of action. As a result, the field of nanomedicine has rapidly emerged as a unique opportunity for development of new classes of drugs with highly enhanced therapeutic value. In this review, the hypothesis of a ‘harnessable’, clinically significant EPR effect in infection is reviewed and a comparison of pathophysiological pathways at the molecular, cellular and circulatory levels to cancer is discussed.

**Mechanisms contributing to an EPR effect**

EPR in cancer has been attributed to vascular permeability enhancement (VPE) and decreased efflux of macromolecules from the pathological locus. Several features of infection-induced inflammation resemble these processes. Following an initial insult, vasodilatation occurs rapidly, recruiting additional capillaries whose permeability is subsequently enhanced. Vascular permeability has been categorized into an immediate stage (contraction of endothelial cells), a transient response (endothelial injury) and transcytosis. Each of these processes has a potential for microbial protease-induced VPE. The ensuing macromolecular extravasation contributes to swelling. Furthermore, the chronicity of local inflammation, with continuous angiogenesis and ongoing remodelling, provides the opportunity for macromolecular accumulation (Figure 2).

**Abnormal circulation**

The unique characteristics of the tumour vasculature that contribute to the creation of an EPR effect have been identified. Angiogenesis and high vascular density characterize inflammation and infection, much like the hypervasculature classically present in solid tumours. Moreover, VPE has been attributed to tumour vascular abnormalities, including endothelial fenestrations and lack of smooth muscle. The majority of the VPE contribution to the EPR effect in infection is found to occur at the post-capillary venule region, which resembles the tumoral circulatory compartment demonstrated by Maeda and co-authors in terms of
Inter-endothelial junctions (IEJs) and the caveolar pathways.

Maeda et al. hypothesized that retention is the main difference between infection and cancer in the sustenance of an EPR effect, reasoning that a dysfunctional lymphatic system is essential and unique to EPR in cancer. They proposed that, in infection, an EPR effect may not be tenable owing to rapid lymphatic clearance. In contrast to this notion, workers have demonstrated early and significant macromolecular accumulation at sites of infection. Indeed, dysfunctional lymphatic drainage is a feature of infection, by way of increased interstitial pressure and tissue destruction. Moreover, a close relationship exists between microbial VPE mediator production and virulence.

**Indirect mechanisms governing the EPR effect**

Toll-like receptors (TLRs)

The 11-member TLR family plays a key role in the innate immune recognition of pathogen-associated molecular patterns. TLR downstream signalling occurs in both inflammation and cancer. Lipopolysaccharide (LPS), ubiquitously present in Gram-negative bacterial cell walls, binds to TLR-4 and triggers the release of inflammatory mediators, which stimulate vascular permeability. Lipoteichoic acid (LTA), ubiquitously present in the Gram-positive bacterial cell wall, activates TLR-2. Both LTA and LPS stimulate the kallikrein–kinin cascade, increasing vascular permeability by providing a suitable negative surface for Hageman factor (HF) activation. LPS also has been shown to strongly up-regulate the bradykinin-1 receptor (BKR-1) and enhance production of vascular endothelial growth factor (VEGF). These observations suggest that enhanced vascular permeability is a common feature across human bacterial pathogens.

**Direct mechanisms effecting the EPR effect in infection**

Kallikrein–kinin

Bradykinin (BK) plays a central role in directly activating VPE and receptor up-regulation in target cell populations. Bradykinin (BK) binds to two distinct, but complementary, receptors: BKR-1 and BKR-2. BK has a half-life of only 27 s, since it is rapidly degraded by the peptidases angiotensin-converting enzyme (ACE) and dipeptidyl carboxypeptidase kininase II, on the endothelial cell surface. Despite its short half-life, the effects of BK and the kallikrein–kinin cascade extend into each stage of enhanced vascular permeability by the resulting products—des-Arg9-BK, Lys-des-Arg9-BK, Lys-BK, and des-Arg10-kallidin—which preferentially activate BKR-1. While BK is ubiquitous, constitutively expressed and rapidly sequestered and internalized, BKR-1 is only induced during an infective insult, and is less susceptible to internalization and desensitization. A study on mice infected with *Trypanosoma cruzi*, a protozoan, determined that early-phase oedema was dependent on BKR-2 activation, whereas later-phase oedema is dependent on stimulation of BKR-1. BKR-1 has also been implicated in models of persistent inflammation (including infection) in animals. These findings support BKR-2 mediation of the initial response and BKR-1 mediation of the

**Immune system**

Enhanced vascular permeability may be induced by the immune system itself, as summarized in Figure 3. Neutrophils predominate in acute inflammation. They promote vascular permeability by releasing neutrophil elastase, which cleaves high molecular weight kinogen (HMWK) to E-kinin. Secretory leucocyte protease inhibitor (SLPI), which normally suppresses neutrophil elastase activity, may also be inactivated by bacterial proteases, leading to potentiated, elastase-induced permeability. Peroxynitrite (ONOO−) and nitric oxide (NO) radicals may activate matrix metalloproteinases (MMPs) secreted by macrophages which, alongside neutrophil-produced elastase, increases vascular permeability. MMPs are involved in the proteolytic degradation and inactivation of α1-proteinase inhibitor, facilitating kallikrein–kinin-mediated permeability as well as neutrophil elastase activity. Neutrophil lysis releases myeloperoxidase, elastase and MMP-9, which contribute to enhanced vascular permeability and nitration of albumin. Additionally, non-specific humoral immune components augment this vascular permeability. BKR-1-induced endothelial inducible nitric oxide synthase (iNOS) may be augmented by the inflammatory cytokines interleukin-1 (IL-1) and interferon-γ (IFN-γ), resulting in prolonged NO release.

**Figure 2.** Complexity of the interaction between bacteria, host and antibiotic. (1) Antibiotic size (macromolecular nanomedicine) versus conventional ‘small molecule’. (2) Effects of population dynamics, biofilm and inter-pathogen synergy. (3) Direct enhancement of vascular permeability. (4) Indirect mechanisms for VPE. (5) Enhanced vascular permeability to macromolecules and decreased drainage. (6) Toxicity.
Figure 3. Role of the immune system in the enhancement of vascular permeability: contributory functions of macrophages and neutrophils.

Table 1. Factors responsible for enhanced vascular permeability in bacterial infection identified in the literature to date

<table>
<thead>
<tr>
<th>Organism</th>
<th>Active protease or VPE inducer</th>
<th>Substrate</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><em>P. aeruginosa</em></td>
<td>protease elastase</td>
<td>HF</td>
<td>42,67,70,153,176–182,185,219</td>
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<td><em>Escherichia coli</em> and <em>Salmonella</em></td>
<td>curli fibres</td>
<td>pre-kallikrein</td>
<td>64,170–172</td>
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<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>SpeB</td>
<td>HMWK</td>
<td></td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>cysteine protease, Metalloproteinase</td>
<td>HMWK</td>
<td>195,196,198,199</td>
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<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>gingipains R &amp; (K) (cysteine protease)</td>
<td>HMWK (RGP releases from pre-kallikrein)</td>
<td>73,128,129,174,202–207,213,250–255</td>
</tr>
<tr>
<td><em>Vibrio vulnificus</em></td>
<td>V. vulnificus protease</td>
<td>HF</td>
<td>41,70,181,256–259</td>
</tr>
<tr>
<td><em>Acinetobacter</em></td>
<td>VPE-like factor</td>
<td>pre-kallikrein</td>
<td></td>
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<tr>
<td><em>S. aureus</em></td>
<td>staphopain (ScpA) (ScpB)</td>
<td>HMWK</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>NA</td>
<td>NA</td>
<td>65</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>subtilisin (serine protease)</td>
<td>LMWK</td>
<td>199</td>
</tr>
<tr>
<td><em>Clostridium histolyticum</em></td>
<td>clostripain</td>
<td>pre-kallikrein</td>
<td>65</td>
</tr>
<tr>
<td><em>Bacillus stearothermophilus</em></td>
<td>NA</td>
<td>HF, pre-kallikrein</td>
<td>181</td>
</tr>
</tbody>
</table>

LMWK, low molecular weight kininogen; NA, not applicable.
delayed, but more prolonged, reaction. A prolonged effect for BK-mediated VPE is also suggested by other recently reported mechanisms (Figures 4 and 5). Activation of HF or HMWK by microbial proteases results in the production of HFa (a kinin-free derivative of HMWK). Formation of HFa results in exposure of domain 5 (D5) of HMWK. Both HKa and D5 inhibit in vitro endothelial adhesion to vitronectin and fibrinogen, leading to anoikis and apoptosis.83 Endothelial injury, cell necrosis, cell detachment and anoikis have been proposed as mechanisms for prolonged vascular leakage.96

Recent evidence suggests that BK contributes to VPE through both a transcellular and a paracellular route (Figure 6). Jungmann et al.46 showed that BK is more likely to promote passage of interstitial fluid and labelled dextran through the transcellular rather than through the paracellular route. Conversely, Baffert et al.44 have suggested that BK's enhancement of vascular permeability is predominantly via the paracellular route. The latter's findings are supported by BK-induced IEJ formation through actin stress fibres, which is probably mediated through filamin control.97 Interestingly, BK-induced VPE is apparently independent of the guanine triphosphatases (GTPases) Rho and Rac, two key regulators of endothelial barrier function.98,99 BK may influence adherens junctions directly via production of NO15 and indirectly through filamin-mediated Rho-induced adherens junction control.15 Therefore, the exact mechanism by which BK increases vascular permeability is still unclear, although there is little doubt about its facilitatory role in EPR. An ability to widen endothelial gaps may explain why its presence significantly increases cancer metastasis100 and infection dissemination in pathogenic species such as Vibrio spp., Pseudomonas spp., Streptococcus spp. and Porphyromonas spp.104,105 Continuous production of first-generation BKs by bacteria is further enhanced by downstream activation/release of second-generation products and simultaneous BKR-1 up-regulation.106,107 BKR also contributes to delayed processes such as nuclear transcription and translation via nuclear factor-kB (NF-kB) and caveolins, and in cell death via anoikis and apoptosis.

**NO**

NO is synthesized from l-arginine by nitric oxide synthase (NOS), which exists in three different forms: endothelial (eNOS), inducible (iNOS) and neuronal (nNOS). eNOS and nNOS are constitutively expressed at low levels but may be rapidly activated by increased cytoplasmic calcium. iNOS is induced when macrophages and other inflammatory cells are activated by cytokines.32 Prolonged generation of NO by BKR-1 may contribute to sustained VPE. Early studies reported an association between the NO pathway, BK and VPE.23,108 It has since been shown that BK, despite its ability to activate the Gi/Gq pathway, cannot directly activate myosin light chain kinase (MLCK) or Rho.45 Gi/Gq activation, however, increases NO synthesis, as well as phosphatidylinositol (PI), cytosolic calcium and prostaglandins (PGs).109,110 Ignjatovic et al.79 demonstrated that BKR-1 may stimulate human endothelial iNOS, with prolonged NO release.79 This NO release is important in IEJ integrity.111 NO production, via protease-activated BK release, induces vascular permeability. NO and its highly reactive downstream products result in nitration of albumin.112 In cancer, generation of NO through NOS activation significantly increases vascular permeability in a tumour size-dependent manner.113–115 Within solid

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**Figure 4.** Action of specific bacterial proteases and mediators on the kallikrein–kinin cascade and receptors, in the mediation of enhanced vascular permeability. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
tumours, and in parallel with infection, -NO and $O_2^{-}$ induce extremely reactive $\text{ONOO}^-$.
-NO and $\text{ONOO}^-$ may also activate pro-MMP (1, 8 and 9). $^74$ Downstream targets of $\text{ONOO}^-$ in infection and cancer include activation of MMPs and nitration of tyrosine residues in albumin.$^24,74,112$

NO-releasing polymers for use in wound healing and ischaemic stroke have been described in the literature, including cross-linked polyethylenimine,$^{116,117}$ ethylene-vinyl acetate$^{118}$ and poly(vinyl alcohol).$^{119}$ NO-releasing monofilament polypropylene$^{120}$ has been investigated for use in infection. NO is a promising antimicrobial alternative, since reactive oxygen species (ROS) possess significant antimicrobial activity.$^{25}$ Since NO is a mediator of VPE in infection, such polymers would theoretically benefit from NO-promoted EPR, whilst exerting an antimicrobial effect.

**MMPs**

The endothelium is supported by an extracellular matrix (ECM), which contributes to the endothelium’s size and charge barrier.$^{121-124}$ Permeabilization of this barrier may be enhanced by BK-mediated induction of constitutively expressed host MMP,$^{125}$ in part mediated through induction of pro-MMP-9 expression via mitogen-activated protein kinase (MAPK) and the NF-$\kappa$B and ERK/Elk-1 pathways.$^{116}$

Bacterial proteases capable of inducing human MMP include the arginine-specific cysteine proteinase RGP-1 (in a MAPK- and NF-$\kappa$B-dependent manner$^{127-129}$), the lysine-specific cysteine proteinase KGP (MMP-1, -3 and -8) and thermolysin (MMP-1, -8 and -9). Furthermore, LPS induces the formation of BK (through HMWK)$^{69}$ while up-regulating BKRs,$^{130}$ simultaneously inducing cytokine formation and up-regulating MMP-9 expression.$^{69}$ Moreover, activation of MMP may increase VPE in normal skin$^{131}$ through a variety of pathways, including $\text{ONOO}^-$ generation.$^{74}$ This evidence suggests that during infection, MMPs contribute to VPE directly and by acting as downstream effectors of the kallikrein–kinin system, prolonging and amplifying its effect.

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**Figure 5.** Subcellular pathways involved in kallikrein–kinin enhanced vascular permeability. PKC, protein kinase C. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*. 
VEGF

VEGF may potently enhance vascular permeability in bacterial infection, as it does in solid tumours. Activated VEGF receptor-2 (VEGFR-2) may uncouple vascular endothelial (VE) cadherin–β-catenin, which are essential for endothelial junction integrity. Activated small GTPase (sGTPase) Rac promotes intercellular junction (ICJ) disassembly. BK activation of VEGF may, therefore, provide an indirect route for BK induction of Rho/Rac and ICJ control. Furthermore, endothelial NO, which determines IEJ integrity, is up-regulated by VEGF through Src. Since a similar VPE effect is observed by picomolar VEGF concentrations and nanomolar BK concentrations, significant and sustained modification of the paracellular route may result from BK–VEGF–NO crosstalk.

Eicosanoid pathways

The eicosanoid pathway in inflammation may contribute to the infection-induced VPE effect, in a similar manner to cancer. It is dependent on various regulatory elements, which may be BK dependent or BK independent, and these responses may be organ or tissue specific.

BK-dependent eicosanoid stimulation

BK induces cyclo-oxygenase (COX) expression in various human cell types, including endothelial cells, cerebral arterioles, human airway epithelial cells, smooth muscle cells and fibroblasts, which are responsible for up-regulating PG synthesis in solid tumours. PGJ2 and PGE1 may induce VPE in a similar fashion to NO, and this has been recently applied in vivo in Beraprost, a synthetic prostacyclin analogue. BK results in endothelial production of PGE2 and activation of phospholipase A2 (PLA2) and phospholipase C (PLC), both of which metabolize arachidonic acid for PGE2 synthesis. BK receptors play distinct roles within this pathway. Within human umbilical vein endothelial cells (HUVECs) BK-2, but not BK-1, induces increased synthesis and activation of cytosolic PLA2 in a Ca2+-dependent manner, which, in turn, induces the synthesis of PGJ2. In these models, BK stimulation of COX-1 is the main pathway by which PGE is released. COX-2 stimulation has an initial pro-VPE effect (production of PGE2), later exerting an anti-VPE effect through 15-deoxy-12,14-PGJ2.

BK-independent eicosanoid stimulation

Microbial-induced VPE may also be induced by BK-independent mechanisms. One such model is P. aeruginosa exotoxin U (ExoU), a recently characterized powerful intracellular toxin with PLA2 characteristics. ExoU is a marker for virulent pseudomonal strains. Machado et al. have recently demonstrated significantly increased vascular permeability induced by wild-type P. aeruginosa, compared with strains with ExoU knockout genes, supporting the notion that...
PGs may contribute to VPE directly and by acting as downstream effectors of the kallikrein–kinin system (Figure 5).

**Effects of population dynamics, biofilm and inter-pathogen synergy**

Biofilm formation may be associated with chronicity of infection and antimicrobial resistance. Specific infections (e.g. *P. aeruginosa* and anaerobic cocci) have been reported to delay or prevent wound healing. Quorum sensing by bacteria has been implicated in protease expression by *P. aeruginosa*. Pseudomonas elastase is known to be regulated via this mechanism. Secreted elastase and *Pseudomonas* alkaline phosphatase (PAP) in the biofilm may enhance macromolecular permeability and, hence, selective accumulation of macromolecular drugs.

Inhibition of co-transcribed proteases to exoproteases, such as BK, appears to play a key role in tight microbial control of the latter’s permeability-enhancing properties. This occurs not only within the same strain, but also across species. Staphostatin A and B selectively target the co-transcribed proteases staphopain A and B, respectively. Interestingly, however, an inhibitor from one staphylococcal strain may inhibit the corresponding orthologue from another species. The decreased virulence seen in staphostatin knockout strains suggests an important role for the regulation of permeability-enhancing exoproteases. This type of protease–inhibitor coupling has also been illustrated in *S. pyogenes*. Streptheroceptin and *Bacteroides* spp. Pathogen-induced enhanced vascular permeability has also been shown to significantly increase yield and infectivity of co-infecting organisms at the supra-species level, including strong potentiation of *V. vulnificus* and *Pseudomonas* spp. dissemination rates in the presence of BK.

**Bacterial enhancement of vascular permeability**

The existence of common pathways for VPE and retention in infection, as well as cancer, suggests the feasibility of an EPR effect in infection. Utilization of this phenomenon for targeted antimicrobial delivery will, however, depend on the extent of vascular permeability, whether it occurs commonly in pathogenic bacteria and the magnitude of this effect. Several major pathogens, including the ESKAPE organisms, are known to enhance vascular permeability. This may occur via direct protease activation or through inactivation of natural inhibitors for major effectors (Tables 1 and 2).

**Gram-positive pathogens: Streptococcus spp. and Staphylococcus spp.**

Streptococcal pyogenic exotoxin B (SpeB) is secreted by most *S. pyogenes* isolates. It is implicated in mediating increased streptococcal virulence and a staphylococcal toxic shock-like syndrome in group A streptococci. SpeB is a cysteine protease (widely produced in groups A and B streptococci), which, after maturation through autocatalysis, efficiently cleaves circulating HMWK to kinin. Assembly of contact factors, and subsequent BK release, by *S. pyogenes* has been reported, affecting SpeB a significant role in BK production. Activation of the kallikrein–kinin cascade is also prominent in *S. aureus* pathogenesis through production of heat-labile cysteine protease exotoxins: staphopains A (ScpA) and B (SspB). BK requires cleavage of two peptide bonds for release from kinogens, one at each primary chain terminus. ScpA cleaves both HMWK and low molecular weight kininogen (LMWK) at the carboxy-terminus, conferring vascular permeability, while SspB adds two amino acids at the amino-terminus, thereby enhancing the activity of ScpA. Alone, however, SspB does not confer any vascular permeability. *S. aureus* infection results in additional events at the BKR level, through modulation of BKR-1 and BKR-2 expression.

**Gram-negative pathogens**

*P. aeruginosa*

*P. aeruginosa* stimulates BK production by releasing MMPs (alkaline protease and elastase). These MMPs were among the first proteases to be specifically attributed to disruption of the kallikrein–kinin system. Both proteases target zymogen HF and are implicated in mediating increased vascular permeability, including strong potentiation of *V. vulnificus* and *Pseudomonas* spp.

**Table 2. Bacterial inactivation of specific protease inhibitors**

<table>
<thead>
<tr>
<th>Protease inhibitor</th>
<th>Species/protease</th>
<th>References</th>
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<tr>
<td>SERPINS</td>
<td><em>P. aeruginosa</em></td>
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<td><em>S. marcescens</em></td>
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</tr>
<tr>
<td></td>
<td><em>S. aureus</em></td>
<td></td>
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<tr>
<td>α1-Protease inhibitor</td>
<td><em>P. aeruginosa</em></td>
<td>217–219, 223, 224</td>
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<td><em>S. marcescens</em></td>
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<td></td>
<td><em>P. gingivalis</em></td>
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<tr>
<td>α2-Antiplasmin</td>
<td><em>P. aeruginosa</em></td>
<td>220, 221</td>
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<td></td>
<td><em>S. marcescens</em></td>
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*Acinetobacter* spp. and *Klebsiella* spp.

*Acinetobacter* spp. have emerged as a major cause of MDR, especially in military personnel and following burns...
surgery. In a comparative study of sepsis patients and healthy control subjects, Soares et al. showed that components of the kallikrein–kinin system, including HMWK, are significantly depleted in infection. Acinetobacter spp. secrete vascular permeability factor (VPF), which significantly increases vascular permeability in vitro. However, Acinetobacter spp. and Klebsiella spp. also significantly activate the kallikrein–kinin cascade via LPS and, in the case of Klebsiella spp., secretion of MMPs.

**E. coli**

A study performed in healthy volunteers has demonstrated that E. coli induces significant VPE by LPS induction of VEGF. Evans et al. have also investigated the VPE properties of E. coli enterotoxin, and these studies demonstrated that accumulation of Evans blue dye in rabbit models was linearly related to dermal concentrations of enterotoxin. They hypothesized that Evans blue dye binds tightly to albumin, creating a macromolecule that facilitates permeability and retention. Furthermore, curli fibres expressed by E. coli can bind and assemble contact factors, resulting in BK release.

**Enterobacter spp.**

The enzymatic activity of Enterobacter spp. includes several MMPs, including gelatinase (secreted by Enterobacter aerogenes, Enterobacter cloacae and Citrobacter spp.) and elastase (secreted by E. cloacae), which may contribute to an EPR effect through increasing vascular permeability.

**P. gingivalis**

Synergic cleavage of kininogens has been well characterized in P. gingivalis, a major pathogen in human periodontal disease. P. gingivalis produces two thiol-dependent proteases, RGP and KGP. RGP gingipains also exist as two forms: high molecular weight RGPA and low molecular weight RGPB. The essential VPE-enhancing role of RGP and KGP for P. gingivalis survival in periodontitis is well described; however, the specific role of the proteases in this process remains unclear. Recently, Kadowaki et al. reported that a specific RGP inhibitor, KYT-36, inhibited 95% of Evans blue dye leakage in an experimental model, suggesting a larger role for KGP-mediated VPE than previously expected. It is believed that KGP may cleave the N-terminal end synergistically with a putative circulatory E-kinin-converting enzyme. Furthermore, gingipains have been shown to produce Lys-BK, which preferentially binds to BKR-1 and plays a greater role in VPE than BKR-2.

**Inactivation of regulatory protease inhibitors**

Inhibition of regulatory host protease inhibitors may also play a significant contributory role in microbial VPE (Table 2). The human kallikrein–kinin cascade is normally controlled by a well-characterized system of inhibitors, which may be inactivated. Pseudomonal proteases (PAP and elastase) and serratial proteases may effectively inactivate serine protease inhibitors (SERPINS), such as α1-protease inhibitor, α2-antiplasmin and α2-macroglobulin. A separate function is the ability of the inhibitor–α2-macroglobulin complex to be internalized via α2-macroglobulin receptors. After internalization, regeneration of the free protease may cause cell death. However, unless α2-macroglobulin were significantly depleted systemically, the effect would most likely be localized. P. gingivalis may cleave and inactivate α2-antiplasmin under physiological conditions, and periodontal, a 75k cysteine protease, may inactivate α1-protease inhibitor (facilitating BK production). SspA also efficiently inactivates α1-protease inhibitor in vivo.

**Relevance across human pathogenic bacteria**

EPR-enhancing mechanisms appear to be widespread and closely evolutionarily conserved among human bacterial pathogens. In addition to ubiquitous bacterial components capable of low-level VPE activation (e.g. LPS and LTA), there are several salient examples illustrating the evolutionary conservation. SpeB is a descendant of a closely evolutionarily conserved, common pre-eukaryotic ancestor of the papain family. Staphopain-encoding genes are tightly conserved and widely distributed in pathogenic staphylococci. The staphopain–staphostatin gene arrangement is preserved in all known S. aureus strains. Gingipain R and K active sites are well conserved and their catalytic dyad is common to caspases, clostripain and mammalian legumain, suggesting that they are probably evolutionarily related. Some of these VPE-generating proteases have been reported as members of a core of key factors responsible for colonization and infection by many infectious strains. These findings all suggest a common evolved system for increasing VPE at sites of bacterial infection.

Although this review principally addressed the main pathogenic bacteria in human disease, EPR may be valuable in infections in which micro-environments are achieved, e.g. the intracellular environment including Salmonella and Mycobacterium infection (reviewed in Ranjan et al.). Antimycobacterial drugs loaded in intravenously administered nanoparticles effectively target extrapulmonary infected macrophages in the disseminated form of the diseases. Pegylated lysine-based copolymeric dendrimer–artemether has been reported to be effective against Plasmodium falciparum.
Targeting potential of nanomedicines using the EPR effect in infection

The significance of EPR in infection has not yet been fully exploited in the passive targeted delivery of antibiotics since the phenomenon was first observed. The successful clinical exploitation of the EPR effect in the treatment of infection is dependent upon an early onset. EPR occurs relatively early in the acute phase of infection: gallium-transferrin can clearly delineate intra-abdominal abscesses within 4 h of injection, while 99Tc-labelled poly(ethylene glycol) (PEG)-coated liposomes accumulate around an infected locus within 2 h. Infected patients are prone to rapid deterioration. This evidence that an early EPR effect is compatible with harnessing of the EPR concept in drug design for infection, even without a long-circulating formulation approach. This situation is different from anti-cancer drug design, where a long-circulating formulation approach is suited to this biological rationale.

Radiolabelling studies have demonstrated that radiolabelled PEG-coated liposomes preferentially permeate into, and are specifically and selectively retained at, sites of infection. Sikkink et al. later demonstrated a significant qualitative correlation between uptake of these particles and the size of intra-abdominal abscesses. The presence of an EPR effect in infection may rationalize these observations of selective accumulation at sites of infection.

Clinical development of nanomedicines for infection

A number of studies have investigated various nanomedicine approaches to the safe and effective delivery of antibiotic drugs. This growing class of nanosized antimicrobial therapies, termed ‘nanoantibiotics’, includes antimicrobial nanomaterials (e.g. nanoparticles) and antibiotic delivery systems (e.g. liposomes). Table 3 provides a summary of nanomedicines that are undergoing clinical evaluation for the treatment of infection. While most of these have not yet progressed beyond the pre-clinical stage, significant evidence of reduced toxicity has been demonstrated and many studies have revealed targeting to infected tissues—presumably by the EPR effect, even if circulating time is not as prolonged as in anti-cancer drugs.

Polymer therapeutics is a class of nano-sized therapeutic agents comprising at least two components: a water-soluble polymer covalently attached to an active constituent, such as a drug, protein, gene or peptide. The term ‘polymer therapeutic’ encompasses many structures, including polymeric therapies (e.g. oligosaccharide nanomedicines), polymer–drug conjugates (e.g. PEG–ciprofloxacin) and polymer–peptide conjugates (e.g. dextrin–colistin). They are among the most successful classes of nanomedicines. Several polymer therapeutic approaches have been investigated for effective treatment of infection.

Non-biodegradable polymeric carriers have traditionally been successfully employed in clinically and commercially viable anti-cancer products. PEG has found favour among polymer–protein and polymer–aptamer conjugates, while N-(2-hydroxypropyl)methacrylamide (HPMA), PEG and poly(glu-tamic acid) (PGA) have been used in anti-cancer agents. Indeed, HPMA copolymer–doxorubicin was the first polymer anti-cancer drug to undergo human trials. Conjugates of non-biodegradable polymers rely on degradation of the ‘cleavable’ linker to control the rate of drug release. However, a biopersistent backbone may present a safety challenge, especially if repeat administration is required. The need to ensure renal elimination limits the size of non-biodegradable conjugates to below renal threshold, otherwise, non-biodegradable polymers carry a risk of toxic accumulation and lysosomal storage or other metabolic aberrations. A further example of flexibility in conceptual design is the loading of multiple drug copies onto dendrimers, as illustrated by Fréchet and Tomalia; such a design applied to infection would potentially increase the delivery of the effective payload to infection by EPR.

Table 3. Nanomedicines that have undergone/are in clinical evaluation for the treatment of infection

<table>
<thead>
<tr>
<th>Delivery vehicle</th>
<th>Drug</th>
<th>Carrier</th>
<th>Clinical status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoparticle</td>
<td>rifampicin</td>
<td>poly(lactide)-co-glycolide</td>
<td>in vitro, pre-clinical</td>
<td>260</td>
</tr>
<tr>
<td>Core–shell nanostructure</td>
<td>gentamicin</td>
<td>Pluronic™ PEO–PPO black copolymer</td>
<td>in vivo, pre-clinical</td>
<td>261</td>
</tr>
<tr>
<td>Dendrimer</td>
<td>erythromycin</td>
<td>PAMAM G4</td>
<td>in vitro, pre-clinical</td>
<td>262</td>
</tr>
<tr>
<td>Liposome</td>
<td>amikacin</td>
<td>PC:cholesterol (2:1)</td>
<td>Phase I</td>
<td>263</td>
</tr>
<tr>
<td>Liposome</td>
<td>gentamicin</td>
<td>PC:1,2–DSPE–N–[PEG–2000] (PEG–DSPE) (2.85:0.15)</td>
<td>Phase I</td>
<td>264</td>
</tr>
<tr>
<td>Liposome</td>
<td>gentamicin and ceftazidime</td>
<td>PC:cholesterol:PEG–DSPE (1.85:1:0.15)</td>
<td>in vivo, pre-clinical</td>
<td>265</td>
</tr>
<tr>
<td>Liposome</td>
<td>isoniazid and rifampicin</td>
<td>PC:cholesterol:diethylphosphate:PEG–DSPE (2:1.5:0.2:0.2)</td>
<td>in vivo, pre-clinical</td>
<td>266</td>
</tr>
<tr>
<td>Liposome</td>
<td>vancomycin and ciprofloxacin</td>
<td>PC:stearylamine:cholesterol (7:2:1)</td>
<td>in vivo, pre-clinical</td>
<td>267</td>
</tr>
<tr>
<td>Liposome</td>
<td>ciprofloxacin</td>
<td>PEG–DSPE:PC:cholesterol (5:50:45)</td>
<td>in vivo, pre-clinical</td>
<td>268</td>
</tr>
<tr>
<td>Liposome</td>
<td>colistin</td>
<td>PC:cholesterol (2:1)</td>
<td>in vivo, pre-clinical</td>
<td>269</td>
</tr>
<tr>
<td>Liposome</td>
<td>streptomycin</td>
<td>PC:cholesterol:PEG (2:1:0.1)</td>
<td>in vivo, pre-clinical</td>
<td>270</td>
</tr>
<tr>
<td>Polymer–drug conjugate</td>
<td>ciprofloxacin and norfloxacin</td>
<td>PEG</td>
<td>in vitro, pre-clinical</td>
<td>271</td>
</tr>
<tr>
<td>Polymer–drug conjugate</td>
<td>peptoid 7</td>
<td>PEG, PGA</td>
<td>in vivo, pre-clinical</td>
<td>272</td>
</tr>
<tr>
<td>Polymer–peptide conjugate</td>
<td>colistin</td>
<td>dextrin</td>
<td>in vitro, pre-clinical</td>
<td>273, 274</td>
</tr>
<tr>
<td>Polymeric drug</td>
<td>oligoG fragments</td>
<td>alginate oligomer</td>
<td>Phase I</td>
<td>273</td>
</tr>
</tbody>
</table>

PC, phosphatidylcholine; PEO, poly(ethylene oxide); PPO, poly(propylene oxide); PAMAM, poly(amidoamine); G4, fourth generation; DSPE, distearoyl-sn-glycero-3-phosphoethanolamine.

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More recently, increasing interest in biodegradable polymers has been registered, including PGA, hydroxyethyl starch and dextrin. Moreover our own experience with dextrin–colistin conjugates suggests that biodegradable polymer constructs offer advantageous design concepts, such as bioresponsive masking and unmasking illustrated by the polymer masking–unmasking–protein concept. These studies illustrate the flexibility of conceptual design that may be afforded by polymer therapeutics in harnessing the potential value of the EPR effect in infection. They suggest that EPR is essential for accumulation of macromolecular nanoantibiotics in infection using various constructs. Additionally, conjugation to long-circulating carriers may offer additional advantages. It is well known that conjugation of a polymer to a protein can significantly improve biological efficacy in vivo, by reducing proteolytic degradation, extending plasma circulation time and reducing protein immunogenicity, thereby facilitating EPR. Clinical translation of bioresponsive polymer–peptide conjugates that harness the EPR effect in infection are a possibility in the near future.

Conclusions

This review suggests that the presence of a clinically significant EPR effect in infection across the major pathogenic bacterial species is supported by the literature. This is maintained by several parallels between closely evolutionarily conserved mechanisms in infection and cancer in the pathogenesis of an EPR effect, as well as in vivo evidence. An EPR effect may be mediated by direct and indirect mechanisms. VPE may be non-specifically enhanced through a variety of pathways: TLRs. LPS and LTA exemplify ubiquitously present structural components which give rise to low-grade VPE. More significantly, specific bacterial exoproteases and their corresponding protease inhibitors may enhance vascular permeability via activation of several enzymatic steps in major amplificatory effector cascades. In infection, as in solid tumours, the kallikrein–kinin cascade is a major protagonist, and its downstream effects are amplified and extended through crosstalk with the NO, VEGF, MMP and PG pathways. Consistent evidence also supports parallels between the vascular changes in infection to the vasculature of solid tumours, which is essential for enhanced macromolecular permeability and retention. Disease models in which the ESKAPE pathogens play a significant role, including burns and aphthomal, respiratory and periodontal disease, are valuable, clinically significant, models for future investigation.

In conclusion, the EPR of macromolecules is a common, consistent and significant feature of Gram-negative and Gram-positive infections that significantly contributes to the burden of human disease. The application of a nanomedicine approach to harness an EPR effect may, in the near future, offer new therapeutically approaches to the management of human infectious diseases direly required by clinicians and patients alike. The successful development and progression towards clinical application of novel nanomedicine-based antimicrobial therapies, to exploit the EPR effect in infection, will require considerable interdisciplinary collaborations. Only through the formation and cooperation of cohorts of expert scientists (including polymer chemists, clinicians, biologists, analytical chemists, pharmacists and regulatory authorities) can the potential of nanomedicine in the diagnosis and treatment of infection be truly realized.

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Transparency declarations

None to declare.

References


