Table 3. Selected parameters affecting plasma residence times of different nanoparticles

Type of nanoparticle	Test animal	ζ potential (mV)	Mean particle size (nm)	Plasma residence time		Remarks	Ref
				$T_{1/2}$	$T_{1/10}$		
· Liposome (nonpegylated)	Mouse	-7.31	124	9.08 h	>24 h	Doxorubicin loaded, DPPC:Chol = 1:1	[97]
Liposome, weakly cationic	Mouse	+5.58	131	4.51 h	15 h (mean)	Doxorubicin loaded, DPPC:Chol:DC-Chol = 5:4:1 slightly positive	
Liposome, strongly cationic	Mouse	+24.25	95	<30 min	<1 h	Doxorubicin loaded, DPPC:DC-Chol = 5:5 strongly positive	
· pLL-DNA complex	Mouse	Positive	-	<5 min	30 min	^{32}P-labeled 8-kbp DNA	[49]
· Chitosan nanoparticle weakly anionic	Mouse	-13.2	149.2	-	12 h (mean)	CMC/MMA = 1:2 slightly negative	[51]
Chitosan nanoparticle strongly anionic	Mouse	-38.4	156.0	-	3 h (mean)	CMC/MMA = 2:1 strongly negative	
Chitosan nanoparticle weakly cationic	Mouse	+14.8	150.1	-	<1 h	CH/MMA = 1:1 slightly positive	
Chitosan nanoparticle strongly cationic	Mouse	+34.6	152.7	-	<1 h	CH/MMA = 2:1 strongly positive	

Abbrebiations: DPPC: L-α-dipalmitoylphosphatidylcholine

Chol: cholesterol

DC-Chol: 3β-[*N*-(*N*′,*N*′-dimethylaminoethyl)carbamoyl]cholesterol

pLL: poly(L-lysine)

CMC: carboxymethyl chitosan

MMA: methyl methacrylate

CH: chitosan hydrochloride

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Preface

EPR effect based drug design and clinical outlook for enhanced cancer chemotherapy☆

Twenty-five years have already passed since our discovery of the enhanced permeability and retention (EPR) effect [1]. The concept of the EPR effect has prevailed in a wide range of applications including antibody delivery, gene delivery, and other nanomedicine-based delivery systems such as micellar, liposomal and polymer conjugates. After a rather quiet initial two decades, its citations now reached more than 6000 and have been increasing logarithmically, as shown by J. Fang et al. [2] in this issue. It is an ideal time to assess the past and present state in terms of limitations and further development, because the EPR effect is a more general principle for tumor-selective drug delivery compared to "molecular target" drugs. In this connection, the limitations of macromolecular target drugs are discussed by Fang et al. [2] and the impact of the EPR effect in drug delivery is well documented by Torchilin in the Commentary [3].

This issue provides some historical background and covers a number of topics ranging from basic principles to clinical outlook, to advantages and limitations, to key factors involved and nanomedicines under clinical development. Based on the concept of the EPR effect, a more sophisticated drug design is presented by Harashima et al. and Murayama et al., and the circumvention of barriers in drug targeting is also discussed by Fang et al. in this issue [2].

One problem that has caused some concern is the heterogeneity of the EPR effect. When the tumor nodule is very small or at an early stage of cancer development, there is no heterogeneity. However, in larger or later-stage tumors, the EPR effect becomes heterogeneous. In the case of a mouse tumor nodule greater than 500 mg (>1.7 mg in rats), we found less production of nitric oxide, which is one of the major factors for EPR effect (see Fang et al. in this issue [2]). This would incidentally lower the extravasation of macromolecules into the interstitial tissue space. This concept may be applicable to the tumor implanted at a non-orthotropic site, which may exhibit a heterogeneous EPR effect of lower vascular density such as metastatic tumors in the liver, as clinically observed in CT scan images of primary vs. metastatic human liver cancer after intra-arterial injection of SMANCS/Lipiodol. The former was more uniform and exhibited denser vasculature, and thus higher EPR effect, while the latter exhibited a hypovasculature pattern in the central part and thus lower EPR effect (heterogeneity). We (H.M.) recently published basic findings and clinical demonstrations to augment the EPR effect, and circumvention of this heterogeneity is discussed elsewhere [2,4–7].

Whatever minor issues might remain, nanomedicine drug development has been increasing during the past 10 years (for instance, see Ref. [8]). Along this line, the reviews by Sahoo et al., Maruyama et al., and Matsumura et al. give us the present situation of nanomedicine development and future prospects. Recent trends in this area of polymeric drugs are reported by many authors more than ever, yet await for clinical breakthrough (see for instance ref.[9]).

Molecular targeting agents showed promise in the case of imatinib (Gleevec®). However, other cases appear disappointing, including antibody conjugates; some have unremarkable therapeutic effects and also occasionally exhibit serious side effects. The simple reason for this is that we have not yet found cancer-specific molecules that are universally effective for all or specific classes of cancer. Acquisition of information on the molecular biology of cancer is an ongoing process and molecular targeting agents are developed accordingly; however, it is still insufficient for cancer eradication. Consequently, there is an obvious need to clinically introduce the concept of the EPR effect as well as to improve conventional anticancer agents on this basis.

Under these circumstances, clinical application of nanomedicine based on the EPR effect would definitely benefit the patients, as discussed by Matsumura et al. in this issue. Furthermore, development of a method for augmenting the EPR effect, for instance, by using nitroglycerin, a nontoxic nitric oxide generator with proven safety if not overdosed, warrants future investigation and extension to the bedside.

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References

[1] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS, Cancer Res. 46 (1986) 6387–6392.
[2] J. Fang, H. Nakamura, H. Maeda, The EPR effect: unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect, Adv. Drug Deliv. Rev. 63 (2011) 136–151.
[3] V. Torchilin, Tumor delivery of macromolecular drugs based on the EPR effect, Adv. Drug Deliv. Rev. 131 (2010) 131–135.
[4] T. Seki, J. Fang, H. Maeda, Enhanced delivery of macromolecular antitumor drugs to tumors by nitroglycerin application, Cancer Sci. 100 (2009) 2426–2430.

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doi:10.1016/j.addr.2010.05.001

[5] H. Maeda, Nitroglycerin enhances vascular blood flow and drug delivery in hypoxic tumor tissues: Analogy between angina pectoris and solid tumors and enhancement of the EPR effect, J. Cont. Release 142 (2010) 296–298.
[6] H. Maeda, Tumor-selective delivery of macromolecular drugs via the EPR effect: Background and future prospects, Bioconj. Chem. 21 (2010) 797–802.
[7] A. Nagamitsu, K. Greish, H. Maeda, Elevating blood pressure as a strategy to increase tumor targeted delivery of macromolecular drug SMANCS: Cases of advanced solid tumors, Jpn. J. Clinical Oncol. 39 (2009) 756–766.
[8] M.J. Vicent, H. Ringsdorf, R. Duncan, Polymer therapeutics: Clinical applications and challenges for development, Adv. Drug Deliv. Rev. 61 (2009) 1117–1120.
[9] R. Duncan, Polymer conjugates as anticancer nanomedicines, Nature Rev. Cancer 6 (2006) 688–701.

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The EPR effect: Unique features of tumor blood vessels for drug delivery, factors involved, and limitations and augmentation of the effect ☆

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ABSTRACT

The enhanced permeability and retention (EPR) effect is a unique phenomenon of solid tumors related to their anatomical and pathophysiological differences from normal tissues. For example, angiogenesis leads to high vascular density in solid tumors, large gaps exist between endothelial cells in tumor blood vessels, and tumor tissues show selective extravasation and retention of macromolecular drugs. This EPR effect served as a basis for development of macromolecular anticancer therapy. We demonstrated methods to enhance this effect artificially in clinical settings. Of great importance was increasing systolic blood pressure via slow angiotensin II infusion. Another strategy involved utilization of NO-releasing agents such as topical nitroglycerin, which releases nitrite. Nitrite is converted to NO more selectively in the tumor tissues, which leads to a significantly increased EPR effect and enhanced antitumor drug effects as well. This review discusses molecular mechanisms of factors related to the EPR effect, the unique anatomy of tumor vessels, limitations and techniques to avoid such limitations, augmenting tumor drug delivery, and experimental and clinical findings.

Contents

Abbreviations: NO, nitric oxide; SMA, styrene maleic acid copolymer; NCS, neocarzinostatin; CT, computed tomography; AUC, area under the concentration–time curve; IgG, immunoglobulin G; HPMA, *N*-(2-hydroxypropyl)methacrylamide; α_2-M, α_2-macroglobulin; XO, xanthine oxidase; RES, reticuloendothelial system; VEGF, vascular endothelial growth factor; SEM, scanning electron microscope; AT-II, angiotensin II; iNOS, inducible nitric oxide synthase; $ONOO^-$, peroxynitrite; VPF, vascular permeability factor; PG, prostaglandin; SBTI, soybean trypsin inhibitor; cPTIO, carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-oxide; $O_2^{-\bullet}$, superoxide anion radical; MMP, matrix metalloproteinase; NG, nitroglycerin; COX, cyclooxygenase; ACE, angiotensin-converting enzyme; HCC, hepatocellular carcinoma; ISDN, isosorbide dinitrate; pO_2, partial oxygen pressure.

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1. Introduction

1.1. General problems in development of cancer treatment even after 50 years of endeavor

Cancer remains the major cause of death in most advanced countries in the world, and the incidence of cancer increases as populations age. The best treatment of malignancies such as gastric, colonic, and cervical cancers is surgical removal of early-stage tumors that are small and confined to a limited area, without metastasis. Chemotherapy, and to a limited extent radiotherapy, have been the last resort to control cancer. However, conventional chemotherapy, which utilizes small molecular drugs, is far from successful, similar to the situation that we have experienced with antibiotics given for microbial infections. This problem derives mostly from the lack of tumor selectivity, or so-called selective toxicity, of these agents, so that severe adverse effects limit usage. Thus, an urgent need exists to develop drugs with high selectivity to target tumors, which may greatly reduce drug toxicity and enhance the therapeutic efficacy of chemotherapeutics.

Another so far unsuccessful direction of recent cancer treatment is so-called molecular target therapy, which usually focuses on specific kinases or receptors that are overexpressed in cancer cells or tissues. Recent clinical results for those molecular target drugs have been disappointing [1–3]. The benefit for patients undergoing these treatments is a 1–2 month extension of the usual 3- to 5-year overall survival [1,3]. In another study, a combination of two different types of molecular target drugs resulted in shorter overall survival [2]. Adverse side effects were not easily overcome, and the frequency of medical emergencies was not reduced. Among these drugs, one exception was imatinib (Gleevec), which is used for chronic myelogenous leukemia. However, most cases demonstrated drug resistance after several months of its use. The problems associated with molecular target drugs probably relate to the intrinsic genetic diversity of human solid tumors, which these drugs do not account for [4,5]. Usually, multiple genes or their product proteins, which make up sophisticated networks, support or promote the growth, cell regulation, invasion and metastasis of tumor cells. These genes are now known to undergo extensive mutations. Findings for 11 patients with breast cancer and 11 patients with colorectal cancer showed that individual tumors demonstrated an average of approximately 90 mutated genes, and 189 genes mutated at a significantly high frequency [5]. These data mean that the patients had only a small (few percent) chance of the likelihood of a positive response to the molecular target drugs. In addition to the high frequency of occurrence of mutant genes, redundant genetic and molecular or metabolic pathways, which constitute the backup system of vital molecular pathways, may invalidate the single gene or receptor concept and single pathway assumption. Thus, such a highly specific molecular approach, targeted to even a single epitopic antigen, receptor, or kinase, seems to be an imperfect if not an unwise approach.

Another problem may reside in the *in vitro* screening method for cancer chemotherapeutics. This method utilizes the individual cancer cell type panel model, and a drug is thus screened on the basis of tumor cell type. However, even after more than 30 years of screening at least 50 cell types, such as glioblastoma, malignant melanoma, hepatoma, pancreatic cancer and cervical cancer, no revolutionary discovery of new useful drugs has been reported. One problem with this screening system is probably related to a lack of considering pharmacokinetics and the vascular phenomenon named the *enhanced permeability and retention* (EPR) effect, so that only cytotoxic compounds were identified.

1.2. The EPR effect: the cutting edge

The greatest breakthrough leading to more general targeted antitumor therapy was the discovery of the EPR effect, as commented by Torchilin [6], (in this issue of ADDR).

The EPR effect was first reported by Matsumura and Maeda in 1986 [7] and was described in greater detail and validated by Maeda et al. [8–14]. Their investigations showed that most solid tumors have blood vessels with defective architecture and usually produce extensive amounts of various vascular permeability factors. Most solid tumors therefore exhibit enhanced vascular permeability, which will ensure a sufficient supply of nutrients and oxygen to tumor tissues for rapid growth. The EPR effect considers this unique anatomical–pathophysiological nature of tumor blood vessels that facilitates transport of macromolecules into tumor tissues. Macromolecules larger than 40 kDa selectively leak out from tumor vessels and accumulate in tumor tissues. In contrast, this EPR effect-driven drug delivery does not occur in normal tissues [7–14]. This unique phenomenon in solid tumors—the EPR effect—is thus considered to be a landmark principle in tumor-targeting chemotherapy and is becoming an increasingly promising paradigm for anticancer drug development. For example, Doxil, which is a PEGylated (polyethylene glycol-coated) liposome-encapsulated formulation of doxorubicin, was approved for treatment of Kaposi sarcoma and other cancers. Many other polymeric or micellar drugs are in clinical stage development (phases I and II) [15,16], of which only a few are reviewed in this special issue. Compared with conventional anticancer drugs, most of which are small molecular drugs, these macromolecular drugs have superior *in vivo* pharmacokinetics (e.g., a prolonged plasma half-life) and, more important, greater tumor selectivity, so that they produce improved antitumor effects with no or less adverse reactions [15–17].

The EPR effect has thus now become the "gold standard" in anticancer drug design and anticancer strategies using macromolecular agents, including gene delivery, molecular imaging, antibody therapy, micelles, liposomes, and protein–polymer conjugates (see Torchilin [6] in this issue of ADDR). As evidence of this status, the numbers of citations related to the EPR effect have been progressively increasing in recent years (Fig. 1).

1.3. Problems related to the EPR effect and their solutions

Regardless of the popularity of EPR effect-based drug delivery, many problems with that strategy still exist. We know that large tumors show great pathophysiological heterogeneity. Although we have identified many factors that affect vascular permeability in tumors, as described in the later sections of this article, some parts of

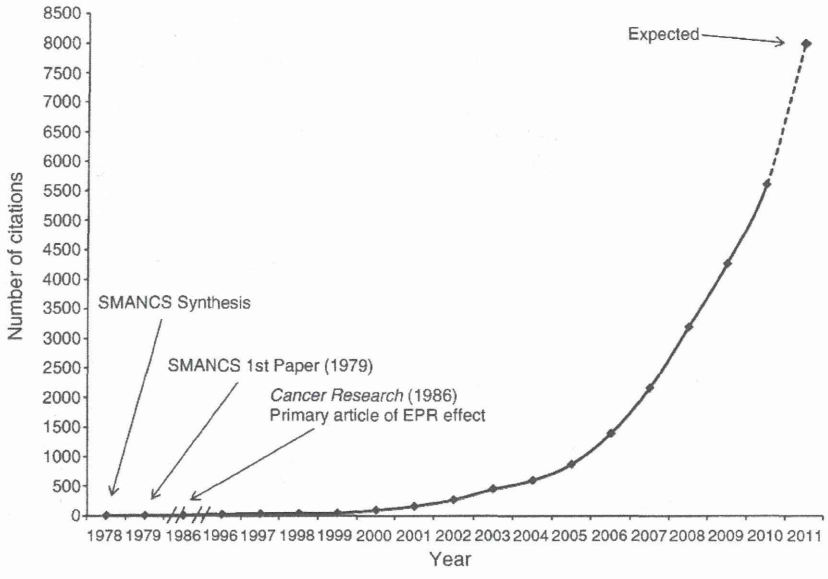

Fig. 1. Number of citations on the EPR effect.
Data are collected from the database of Science Direct, SciFinder and Web of Science.

tumors, particularly the central area of metastatic cancers, do not exhibit the EPR effect and show less accumulation of macromolecules than other parts [18,19]. Most of the *in vivo* experimental studies used mouse tumors that were usually 0.5–1 cm in diameter at least; larger tumors (e.g., 1–2 cm in diameter) tend to contain more necrotic tissues or highly hypovascular areas (with thus less chance of vascular leakage and less tumor growth because of the lack of blood vessels).

In an effort to solve some of these problems, we recently developed methods to intensify the EPR effect and to achieve a more homogeneous drug delivery to tumors, either by elevating blood pressure or by applying nitric oxide (NO)-releasing agents, as described later. The former method utilizes hydrodynamic enhancement of drug delivery; the latter is via generation of NO in tumor tissues. These developments allow one to envision improved cancer chemotherapy via macromolecular drugs in clinical situations.

This review summarizes and discusses the principle of the EPR effect, especially factors influencing this effect, its limitations and methods of avoiding such limitations. In addition, augmenting the EPR effect (tumor drug delivery) and reducing the heterogeneous consequences of the EPR effect to improve clinical outcome are also discussed.

2. The EPR effect: history and principle

In 1979, Maeda et al. [20] reported the first synthesis of the anticancer protein neocarzinostatin (NCS) conjugated with a polymer (styrene maleic acid copolymer, SMA), which named SMANCS. Later studies found SMANCS to accumulate to a greater degree than NCS in tumor tissues [7–14,21,22]. In addition, this biocompatible polymer conjugation of the protein prolonged the plasma half-life, often up to 200 times longer compared with unmodified free NCS or low-molecular-weight drugs [7,20–22]. Furthermore, this unique and important phenomenon was demonstrated with other plasma proteins of different molecular sizes. Proteins larger than 40 kDa showed selective accumulation in tumor tissues, to a degree far more than that observed in normal tissues, and these proteins were retained in tumor tissues for long periods: at 24 h after intravenous (i.v.) injection, the accumulation of most polymeric macromolecular drugs in tumor tissues was more than 10–200 times higher than that in normal tissues and organs, such as skin, muscle, heart, and kidney [7,9,10,15–17,23–28]. These findings led to generalization of the concept of the EPR effect. SMANCS, which took the advantage of the EPR effect, became the first macromolecular anticancer drug approved for use in clinical settings in 1993.

A typical experiment that illustrates the EPR effect involves using an i.v. injection of the dye Evans blue, which binds to plasma albumin and behaves *in vivo* like a true macromolecule, i.e., a putative macromolecular drug. Fig. 2A shows that 24 h after i.v. injection of Evans blue/albumin complex, the blue dye was seen in certain tumor sites, but normal tissues (e.g., skin) showed no blue staining. Moreover, in experimentally induced tumors larger than 3 cm in diameter, the blue dye was found primarily in the tumor periphery, where tumor growth and angiogenesis predominate (Fig. 2B). Frequently, as mentioned earlier, central regions of tumors are partly necrotic or hypovascular, so that area demonstrated no significant accumulation of blue dye (Fig. 2B). These findings suggested that the EPR effect (accumulation of macromolecular drugs) is a blood vessel-dependent phenomenon, which is discussed below in detail. Also, for example, in cancer patients, when hepatoma patients received intraarterial (i.a.) SMANCS in Lipiodol (i.e., a formulation of SMANCS with the lipid contrast agent), imaging of tumor-selective drug delivery became possible by use of computed tomography (CT). CT showed Lipiodol retention as high-density staining (Fig. 2C). The tumor/blood ratio of drug distribution had increased more than 2000 times, and that retention of Lipiodol could last for more than 2–3 months [27–31]. This tumor-selective delivery of SMANCS consequently led to a markedly regressed tumor (Fig. 2D), as well as prolonged survival of patients, without serious adverse side effects [18,19,29–31].

2.1. The EPR effect: a molecular size-based phenomenon

As mentioned above, the EPR effect is a molecular weight-dependent phenomenon: molecules or particles larger than 40 kDa, which is the threshold of renal clearance, showed a prolonged circulation time (thus, a much increased $t_{1/2}$) and hence very slow clearance from the body, with a higher AUC (area under the concentration–time curve) (Fig. 3). These molecules thus gradually permeated tumors in a selective fashion. In addition, these accumulated macromolecular drugs remained in tumors for a relatively long time (e.g., several days) [7–14]. The EPR effect was observed not only with proteins including immunoglobulin G (IgG), but also with drug–polymer conjugates, micelles, liposomes,

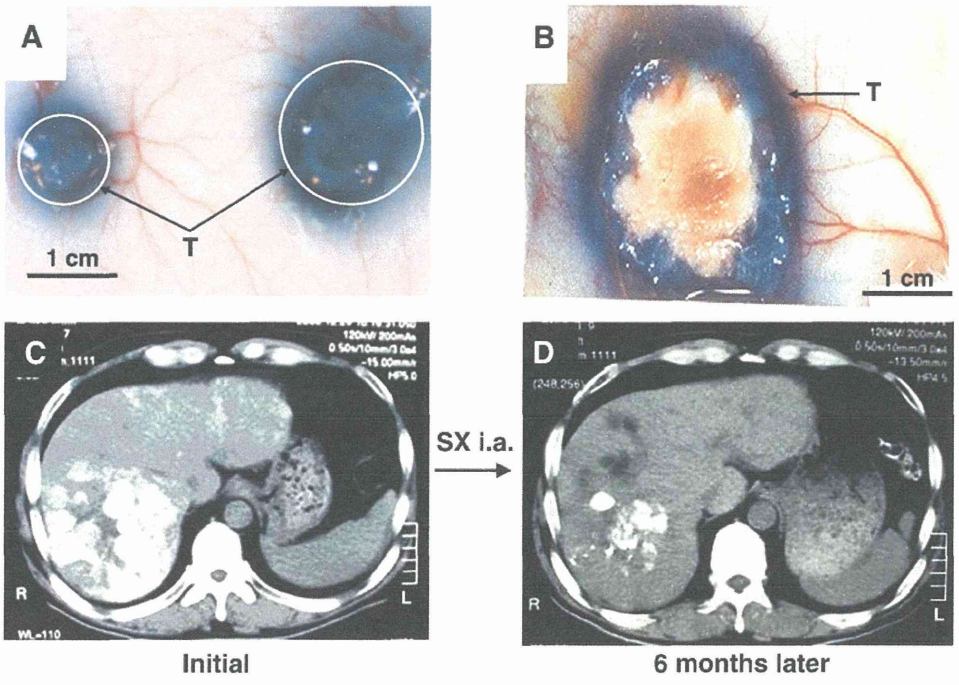

Fig. 2. Experiments illustrating the EPR effect. The EPR effect is shown by the putative macromolecular drug Evans blue/albumin complex (A, B), and by tumor-selective accumulation of the macromolecular anticancer drug SMANCS dissolved in Lipiodol (C, D). (A and B) Macroscopic images of tumor implanted in the skin of mice, at 24 h after i.v. injection of Evans blue (10 mg/kg). The tumor, *T* (circles and arrows), became progressively blue after injection. Normal tissue had no blue color. However, the tumor periphery (outside the circles in A) showed slight extravasation, which is thought to be the result of vascular mediators that diffused out of the tumor tissues and demonstrates the vascular permeability in the vicinity of the tumor, as discussed in elsewhere. (C and D) Computed tomography (CT) of a massive hepatocellular carcinoma after administration of SMANCS (SX)/Lipiodol (LP) given via the hepatic artery. Image was taken 2 days after the initial SX/LP injection, with the white areas in the right lobe (R) indicating tumor-selective uptake of the drug (C). After 3 injections of SX/LP in 6 months, tumor size was markedly reduced (D). The drug selectively retained in the tumor for a long time. R and L indicate right and left sides of the patient, respectively.
C and D are modified from Ref. [29] with permission.

nanoparticles of poly(lactic-co-glycolic acid) (PLGA), DNA polyplexes and lipid particles [7–14,32]. We initially examined *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer, with a molecular size up to 778 kDa [33] and α_2-macroglobulin (α_2-M) (720 kDa) [20], both of which exhibited the EPR effect. Moreover, earlier work using *Lactobacillus* sp. and a more recent study using *Salmonella typhimurium* suggested that the EPR effect functions even for bacteria larger than 1000 nm [34–36].

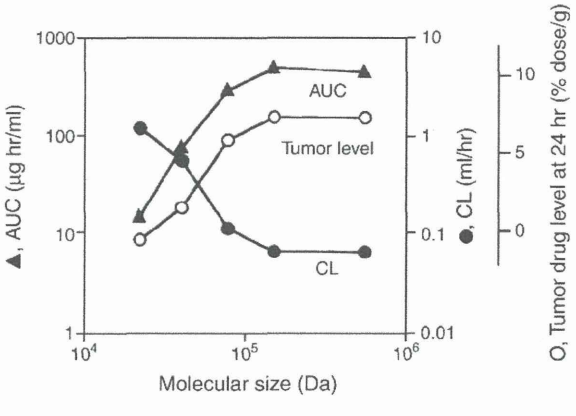

Fig. 3. Relationship of the molecular size of drugs to plasma drug concentration (AUC), renal drug clearance (CL) and intratumor drug uptake (as expressed by a percentage of injected dose). Mice received i.v. injections of putative polymeric drugs, which are ^{125}I-labeled Tyr-HPMA-copolymers of various molecular sizes, at 1.8×10^6 cpm per mouse. The tumor model was S-180. At various times (up to 24 h) after drug administration, mice were killed, and organs, tissues including blood and tumors were collected for counting of radioactivity, to estimate the drug distribution.
From Ref. [33] with permission.

However, the *in vivo* behavior of macromolecules, especially proteins, is one concern. Most proteins, when they are denatured, are of non-self origin, or are less biocompatible, can be cleared rapidly from the circulation via scavenger receptors or other mechanisms. For example, an asialoglycoprotein receptor of liver cells (parenchymal hepatocytes) rapidly cleared desialylated serum glycoproteins from the circulation [22]. Xanthine oxidase (XO) that is a 298-kDa molecule, is rapidly taken up by vascular endothelial cells after i.v. injection because of its high binding affinity to sulfated glycosaminoglycans on the endothelial cell surface; thus, no significant tumor uptake occurred in a mouse tumor model [37]. However, after PEGylation of XO, which involved masking the ε-amino groups of the lysine residues of XO that play a critical role in binding XO to vascular endothelial cells, tumors showed markedly increased drug (PEG-XO) accumulation, and thereby a marked antitumor effect was achieved [37]. Also, modifications or conformational changes of proteins may greatly affect their *in vivo* half-lives. One example involves α_2-M, a multifunctional protease inhibitor in plasma. In the normal state, native α_2-M has a plasma half-life of 140 h in mice. However, the plasma half-life of a α_2-M-plasmin complex decreased dramatically to only 5 min [22]. Thus, molecular weight is not necessarily the key determinant for attaining a long plasma half-life and a functional EPR effect. Biocompatibility in the broad sense is the key.

2.2. Problems and warnings during macromolecular drug development based on the EPR effect

As just stated, molecular weight is not the sole determinant of the EPR effect; other factors such as the surface charge and an *in vivo*

surveillance system for macromolecules (i.e., scavenger receptors of the reticuloendothelial system, RES) are quite important. The vascular endothelial luminal surface is known to carry a negative charge, so basic proteins with positive charges or cationic polymers rapidly bind to vascular endothelial cells, which results in a lower AUC, shorter plasma $t_{1/2}$, and a consequently reduced tumor drug accumulation by means of the EPR effect [38,39].

Acidic or neutral particles are thus expected to have a longer plasma half-life. However, the RES in the liver and spleen reportedly showed faster uptake of negatively charged nanoparticles and liposomes than that of neutral particles [40]. Nevertheless, α_1-acid glycoprotein with a pI of about 3.5 exhibited a long plasma half-life (e.g., 19 h for human α_1-acid glycoprotein in the rat; Ref. [41]). Also, in our previous work, we found that the acidic polymer-conjugated proteins SMANCS (pI of ~3.0) and serum albumin (pI of 4.8) showed long plasma half-lives and high tumor concentrations, although accumulation of SMANCS in the liver was also high [7,22]. These findings suggest that a surface charge may not cause all polymeric drugs and proteins to behave in the same way. Generalization on the basis of these data may therefore be difficult.

The RES, which demonstrates a rich presence in the liver and spleen, can be a major obstacle to tumor delivery of macromolecular drugs, as observed with liposomes in the 1960s to early 1970s [42]. To prevent phagocytic clearance by the RES, the most commonly used strategy is to conjugate PEG onto the surface of proteins or nanoparticles. The result is known as a stealth particle or liposome [42–44], which contains a hydrated water barrier that provides good steric hindrance to the attachment of phagocytes. PEGylation therefore reduces the rate of RES uptake and increases the circulation half-life of various types of nanoparticles, including liposomes [43,44], polymer-based nanoparticles [45] and hybrid nanoparticles [46]. PEGylation thus benefits EPR-based targeting of drugs to tumors.

However, it has been reported that injection of PEGylated liposomes will elicit PEG-specific IgM, thus inducing rapid elimination and enhanced hepatic uptake of a second dose of PEGylated liposomes, which is known as accelerated blood clearance phenomenon and is becoming a barrier to the pharmacokinetics and pharmacodynamics (PK/PD) of PEGylated liposomes and particles [47,48]. Investigations regarding this point are therefore necessary and will greatly improve the therapeutic efficacy of PEGylated drugs. In addition, certain PEG-modified macromolecules and particles are also now understood to have a slower uptake into tumor cells than non-PEGylated molecules [49]. This finding is believed to be one barrier against efficient drug delivery to tumor cells; this situation has been named the PEG dilemma [49]. Nevertheless, suppressed cellular uptake, perhaps a common characteristic of most PEGylated drugs, constitutes an important effect for achieving a longer plasma half-life. Therefore, avoidance of the PEG dilemma in targeted tissues (e.g., tumor) is becoming a critical issue. Developing suitable PEGylation strategies to achieve a longer plasma half-life, as well as better intracellular trafficking, is important. For example, one option may be using bonds that could be eventually cleaved by proteases or similar agents near tumor cells [49,50]. A different approach to avoiding the PEG dilemma may involve, as another example, using SMA conjugation or SMA micelles, in which the hydrophobic component of SMA would confer a higher affinity for cell membranes and improve endocytotic uptake in significant extent [51].

2.3. Problems unrelated to EPR effect: various barriers to drug delivery to target tumor tissues

Walls of blood vessels are the first barrier to drug delivery to target tumor tissues. However, according to the mechanism of the EPR effect, macromolecular drugs could easily reach the interstitium of tumor tissues from leaky tumor blood vessels. Vascular walls in tumors thus do not serve as a barrier for macromolecular drugs or nanomedicines; instead, they facilitate selective delivery of macromolecular drugs to tumor tissues.

The second barrier to successful delivery of macromolecular drugs to tumor cells is the requirement to cross matrix tissue surrounding target cells and target molecules in cells. Many tumor tissues are surrounded by coagulation-derived matrix gel such as fibrin gel or stromal tissues or are nodules encapsulated by fibroblasts. For these tumor tissues, release of low-molecular-weight drugs or cleavage from chains of polymer (e.g., PEG, as discussed above) and disintegration of micelles and liposomes may be an essential point. Moreover, macromolecules themselves can diffuse across matrix tissue for a considerable distance: for instance, IgG (160 kDa) can freely diffuse across 5 mm overnight in 1% agar gel. This second barrier may therefore not be such a serious problem for delivery of macromolecular drugs to tumors.

The third barrier to reaching target molecules is the cell membrane. For example, as described above for the PEG dilemma, PEG-coated macromolecules have a hydrated barrier on the surface that would resist endocytic uptake [49]. The use of short PEG (e.g., Mw<1000) or insertion of protease sensitive peptide between PEG and macromolecules (as described above) may be effective strategies to overcome the PEG dilemma [49,50]. Under these circumstances, an efficient internalization mechanism or transporter system is often better than simple free diffusion. Receptor-mediated endocytosis is thus expected to be an effective means to deliver macromolecular therapeutic agents into tumor cells. One such method uses conjugation of transferrin as a ligand because the transferrin receptor is significantly upregulated in many, if not all, malignant cells [52]. Nanoparticles with their surface modified with transferrin showed a markedly increased tumor-targeting property and intracellular uptake [53,54]. These points are also discussed in this special issue by Harashima and Maruyama.

Utilization of a transport system of cells such as the ATP-binding cassette (ABC) transporter would also be better than free diffusion of drugs. For instance, pirarubicin, which is a pyranyl derivative of doxorubicin, has a 3- to 4-fold faster cellular uptake than doxorubicin (another anthracycline), perhaps because it can utilize the glucose (pyranose) transport system. Pirarubicin micelles are thus advantageous because once the drug is released into the area around tumor cells, free pirarubicin is rapidly—more rapidly than doxorubicin—taken up by the cells [26,27].

The fourth barrier to effective macromolecular anticancer therapy is the release rate of the active principle of the drug from liposomes, micelles, or drug–polymer conjugates. The EPR effect will drive the macromolecules into tumor tissue. Nevertheless, the ultimate goal is access of active drugs to target sites. Therefore, the release rate at the target must be optimal (e.g., 3–10% per day), because too slow a release results in insufficient concentrations of active drugs at sites of action. Release that is too rapid would lead to a high concentration of free drug in circulation but no drug accumulation in the tumor, the results thus being a considerably lower therapeutic effect and undesired systemic toxicity. Micellar drugs that have a very rapid drug release (a micelle burst) after injection, e.g., 50% release within about ~30 min for several drugs, often demonstrate no EPR effect and an unsuccessful therapeutic effect. Liposomes are prepared to have adequate stability in solution (or an adequate shelf life) due to their cholesterol-enriched harder lamellar structure, so their rate of drug release could become too slow. Thus, micelles or liposomes with too stable an encapsulation construct, or too labile a composition, have *in vivo* pharmacological properties that make them unfit for clinical use, even though their *in vitro* data are excellent. Therefore, the optimal kinetics of drug release should be studied *in vivo* because cell-free *in vitro* systems may not accurately reflect *in vivo* conditions.

Similarly important is release of drugs from prodrugs or drug–polymer conjugates via ester or amide bonds; such a release may have species differences. For example, mice and humans are much different in

terms of carboxylesterase activity [55]. Drug release data for mice may thus need reevaluation for humans. All these issues are important for understanding and utilizing the PK/PD of drug–polymer conjugates, drug-encapsulated macromolecular drugs, and nanomedicines.

3. Unique features of blood vessels in tumors: angiogenesis, hypervascularity, irregularity of blood flow, extensive vascular permeability, and abnormal lymphatic drainage

3.1. Abnormality of tumor blood vasculature: morphology

In contrast to normal tissues and organs, most solid tumors show a higher vascular density (hypervasculature), especially when tumors are small, some exceptions being pancreatic and prostate cancers and large metastatic liver cancers. This finding may relate to the heterogeneity of the EPR effect, as discussed above. Tumor angiogenesis is now well known to be one of the most important features that sustains rapid tumor growth. Folkman [56–58] first demonstrated that tumors generate an angiogenesis-stimulating factor (now called vascular endothelial growth factor, or VEGF). Tumor angiogenesis was said to begin as the tumor diameter becomes larger than 0.8–1.0 mm [56–58], and the neovasculature being formed maintains the tumor blood supply. Our scanning electron microscopy (SEM) observations showed the presence of tumor vascular angiogenesis (vascular bed) even when tumor nodules were smaller than 0.2 mm [12,59].

The newly formed tumor blood vessels usually had an abnormal architecture, including defective endothelial cells with wide fenestrations, irregular vascular alignment, lack of a smooth muscle layer or innervation, wide lumen and impaired functional receptors for angiotensin II (AT-II) [10,12,29,59–65]. Blood flow behavior, such as direction of blood flow, was also irregular or inconsistent in these vessels [61]. Suzuki et al. [62] described unresponsiveness of tumor blood vessels to AT-II, and Hori et al. [61,66] observed tumor blood flow only once in 15–20 min, after which it stopped for a while; in addition, blood frequently flowed in the opposite direction.

3.2. Lymphatic clearance of tumor tissue and lymphatic metastasis

Tumor tissues usually lack effective lymphatic drainage [7,9,28–30,67]. In normal tissues, the lymphatic system can effectively recover macromolecules and lipid particles from the interstitial space. For instance, Lipiodol (iodinated poppyseed oil) is used for lymphangiography, because it is primarily recovered via the lymphatic system, and its presence in lymphatic vessels can be visualized with an X-ray system. The same recovery route was observed for macromolecular drugs. Maeda's group was the first to use Lipiodol as a carrier of the drug SMANCS. They found that when SMANCS solubilized in Lipiodol was injected into a tumor-feeding artery (e.g., via the hepatic artery for hepatomas and the renal artery for renal cell carcinomas), it selectively remained in tumor tissues, not in normal tissues [18,30,67,68]. Similarly, when the Evans blue/albumin complex was injected i.v. into tumor-bearing mice it accumulated and remained in the tumor for more than a week, but it was gradually cleared from nontumor tissue by normal lymphatic function [7]. As shown in Fig. 2A, Evans blue/albumin extravasated out of tumor tissue (i.e., into normal skin seen outside of the encircled tumor tissue), was gradually carried away via the lymphatic system, and disappeared in 1–2 weeks. No such clearance of Evans blue/albumin was found for tumor tissues.

In addition, although the lymphatic system does not function properly in tumor tissues, it is the major route for metastasis of tumor cells into normal tissues. Lymphatic metastasis is one of the most formidable consequences of cancer progression, and its control is critically important [20,28,69,70]. SMANCS, as originally developed [20], exhibited lymphotropic accumulation similar to that noted for NCS [69]. We also reported significant antimetastatic activity of SMANCS in rat and rabbit tumor models [20,28,70,71]. In addition, in metastatic liver cancer originating from colon and gastric cancer in humans, arterial administration of SMANCS with Lipiodol resulted in accumulation of the drug in metastatic lymph nodes, with a marked therapeutic effect [72]. This accumulation of SMANCS in lymph node metastases did not occur via the lymphatic route but by the arterial blood supply, which thus demonstrated the EPR effect [72]. Macromolecular drugs should thus be effective for diagnosis and treatment of lymphatic metastasis.

These architectural and anatomical features of a tumor's vascular system constitute the foundation of the EPR effect, which leads to extravasation of macromolecular and lipid drugs. In our recent collaboration with Professor Christophi's group at the University of Melbourne, we used a metastatic liver tumor model in mice to validate the anatomical characteristics of tumor blood vessels. In contrast to blood vessels in normal tissues, which possessed a uniform network and orientation (Fig. 4A–C), tumor blood vessels clearly showed abnormal vascular networks and exhibited high permeability, as evidenced by leakage of acrylic polymer resin as seen with SEM (Fig. 4D,E, indicated by arrows in E). Moreover, EPR effect-based treatment of tumor micronodules by use of the micellar form of pirarubicin completely destroyed the vascular bed of the tumor micronodules, so that the micronodules were no longer visible (Fig. 4F).

4. Factors involved in the EPR effect

Vascular mediators involved in the EPR effect include the following: (a) bradykinin (kinin), which is produced via activation of the kallikrein–kinin system involving a proteolytic cascade [9,28,73–76]; (b) NO generated from L-arginine by the inducible form of NO synthase (iNOS) in leukocytes and tumor cells [76–78], as well as peroxynitrite ($ONOO^-$), an oxidative derivative of NO [79]; (c) prostaglandins (PGs) [76,80]; (d) angiogensin-converting enzyme (ACE) inhibitors [66] and (e) vascular permeability factor (VPF)/VEGF and other cytokines [81–85].

Reducing pericyte coverage of tumor blood vessels by using transforming growth factor-β inhibitor was recently reported to greatly increase the intratumor uptake of nanoparticles [86], which suggested that this inhibitor, in combination with macromolecular anticancer drugs, has the potential to obtain better accumulation of drugs in tumors and thus an improved therapeutic effect. All the factors mentioned above are known as inflammatory mediators. That is, tumor tissues are like inflammatory tissues and show increased extravasation of macromolecules in plasma. The EPR effect can also be observed in inflammatory tissues. As described above, normal tissues surrounding tumors were affected by these vascular mediators and, similar to inflammatory tissues, showed extravasation of Evans blue (Fig. 2A).

4.1. Bradykinin (kinin)

Kinin is a major mediator of inflammation that induces extravasation and accumulation of body fluids in inflammatory tissues (edema); it is the major cause of pain in inflammation [87,88]. Maeda et al. [73] found that tumors have, in addition to bradykinin (also referred to as kinin), [hydroxyprolyl^3]bradykinin, which is a derivative of kinin that has the third amino acid replaced by hydroxyproline. High levels of the derivative were found in blood plasma and in peritoneal and pleural fluids in carcinomatosis in patients with advanced cancer [73–75]. This increased kinin is generated by tumor cells through activation of the Hageman factor (factor XII) and then the pathway of prekallikrein to kallikrein to kinin [74,76]. Administration of kinin at the nanomolar level into the skin of guinea pigs significantly increased the permeability of blood vessels and thus the accumulation of Evans blue at the injection site [60]. Inhibition of kinin generation by means of kallikrein inhibitors (e.g., soybean

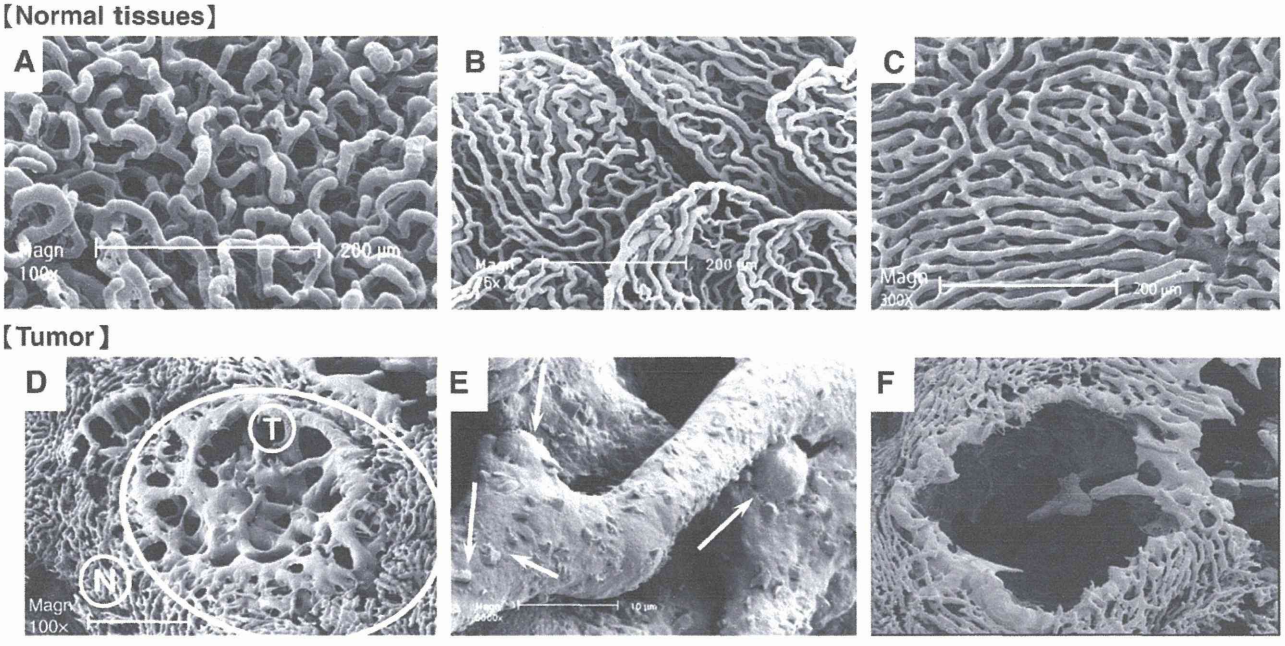

Fig. 4. SEM images of blood vessels in various normal tissues (A–C) and metastatic liver tumors (D–F). Normal capillaries of the pancreas (A), colon (intestinal villi) (B), and liver (sinusoid) (C) are shown. (D) Metastatic tumor nodule (circled area identified with T) in the liver, the normal liver tissue is indicated with "N." (E) Tumor vessels at the capillary level (larger magnification), with a rough surface and an early phase of polymer-extravasating vessels (arrows). Normal tissues show no leakage of polymeric resin (A–C), whereas the tumor nodules clearly demonstrate tumor-selective extravasation of polymer (via the EPR effect) (D, E). After i.v. injection of the macromolecular anticancer drug (SMA-pirarubicin micelles), the tumor vascular bed (visible in D) was completely disintegrated, as shown by an empty void (F).
Modified from Ref. [11] with kind permission of J. Daruwalla and C. Christophi.

trypsin inhibitors, SBTIs) or the kinin antagonist HOE-140, however, significantly suppressed fluid accumulation in mice bearing ascites tumor [73–76].

Moreover, we and other research groups previously reported that the kinin type 2 (B2) receptor was highly expressed in human and animal tumor tissues [89–91]. Kinin is also known to activate endothelial cell-derived NO synthase [92], which ultimately leads to an increase in NO, which is an important mediator of tumor vascular permeability, as described below. Therefore, vascular permeability in tumors is commonly associated with kinin, both directly and indirectly.

4.2. NO and its derivatives, and collagenase (matrix metalloproteinase)

NO is a vital molecule in living creatures, since it has multiple direct and indirect roles as a signaling messenger. NO is produced from L-arginine by NOS in the presence of oxygen. In inflammation and cancer, NO is extensively produced from a greatly increased number of infiltrated leukocytes, in which iNOS plays a major role. To study the role of NO in cancer, we prepared an oily formulation of NO (a solution of NO in medium-chain triglycerides). Intradermal injection of this formulation into guinea pigs caused marked extravasation of Evans blue-/albumin complex at the injection site [78]. The extravasation was significantly inhibited by the NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-oxide (cPTIO) [77]. In *in vivo* experiments with a tumor-bearing mouse model, we showed that both cPTIO and the NOS inhibitor N^{ω}-monomethyl-L-arginine (L-NMMA) inhibited extravasation of Evans blue in tumors [76]. Tumors also showed extensive iNOS expression, which indicated that tumors produce significantly more NO compared with normal tissues [77,93] (Fig. 5A). The amount of NO produced in tumors has a positive association with tumor weight—up to 1.75 g in AH136B tumor-bearing rats and 250 mg in mice bearing sarcoma 180 (S-180) tumors; this result corresponds to the extravasation of the Evans blue/albumin complex (the EPR effect) [77,78] (Fig. 5B,C). iNOS knockout mice evidenced clearly delayed tumor growth after tumor cells were injected into the mice [94]. Thus, NO generation is important for tumor growth, and to maintain the supply of nutrients and oxygen.

Like NO, oxidized products of NO including $ONOO^-$ and nitrogen dioxide potentiated the EPR effect. Among the NO derivatives, $ONOO^-$ is a strong oxidizing and nitrating agent, which forms via the reaction of NO with superoxide anion ($O_2^{\cdot-}$) at a diffusion-limited rate [95,96]. Also like NO, $O_2^{\cdot-}$ is generated extensively in tumor and inflammatory tissues, primarily via NADPH oxidase and cytochrome b_5 reductase in infiltrated macrophages and neutrophils, plus XO [97–100]. In addition, Maeda's group showed that NOS can catalyze the generation $O_2^{\cdot-}$. The reductase domain of iNOS uses oxygen and nitroguanosine (a nitrosated product of guanosine by $ONOO^-$) as substrates to yield $O_2^{\cdot-}$ [101].

Intradermal injection of $ONOO^-$ increased extravasation of Evans blue/albumin in a dose-dependent manner at the site of injection (Fig. 6A). This extravasation lasted for a relatively long time after $ONOO^-$ administration (Fig. 6B) [79], even though the half-life of $ONOO^-$ at physiological pH is only a few seconds [102]. This finding suggests a secondary or indirect mechanism in $ONOO^-$-induced enhancement of the EPR effect. One major mechanism that we demonstrated involves activation of matrix metalloproteinases (MMPs) [79]. MMPs are classified as zinc-dependent neutral endopeptidases that are expressed at high levels in tumor cells and play important roles in tumor invasion, metastasis, and angiogenesis [103–107]. Activation of MMPs causes disintegration and remodeling of the extracellular matrix, in addition to facilitating vascular permeability via degradation of matrix proteins as a result of collagenolytic action; MMPs probably affect blood vessels as well. $ONOO^-$ can be decomposed to generate NO, which leads to functioning of the EPR effect. It may also enhance the EPR effect through a kinin cascade via activation of MMPs as follows: $ONOO^- \rightarrow$ proMMP → MMP → plasminogen/miniplasminogen → prekallikrein → kallikrein → kinin [79].