Proteases in cancer drug delivery

Jennifer Vandooren[1], Ghislain Opdenakker[1], Paul M. Loadman[2], and Dylan R. Edwards[3,4]

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[1] KU Leuven – University of Leuven, Department of Microbiology and Immunology, Rega Institute for Medical Research, Laboratory of Immunobiology, B-3000 Leuven, Belgium

[2]Institute of Cancer Therapeutics, School of Life Sciences, University of Bradford, Bradford, Yorkshire BD7 1DP, United Kingdom.

[3] School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK and

[4] The Genome Analysis Centre, Norwich Research Park, Norwich, NR4 7UH, UK.

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Address for correspondence: School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK;

email: dylan.edwards@uea.ac.uk

Abbreviations: ABP: Activity-Based Probe; ACPP: Activatable Cell-Penetrating Peptide; BD: Blocking Domain; ECM: Extracellular Matrix; MMP: Matrix Metalloproteinase; MMPI: MMP Inhibitor; NP: Nanoparticle; PAP: Protease-Activated Prodrug; PEG: Polyethylene glycol;

Abstract

Whereas protease inhibitors have been developed successfully against hypertension and viral infections, they have failed thus far as cancer drugs. With advances in cancer profiling we now better understand that the tumor degradome forms a complex network in which specific nodes determine the global outcome of manipulation of the protease web. However, knowing which proteases are active in the tumor micro-environment, we may tackle cancers with the use of protease-activated prodrugs (PAPs). Here we exemplify this concept for metallo-, cysteine and serine proteases. PAPs not only exist as small molecular adducts, containing a cleavable substrate sequence and a latent prodrug, they are presently also manufactured as various types of nanoparticles. Although the emphasis of this review is on PAPs for treatment, it is clear that protease activatable probes and nanoparticles are also powerful tools for imaging purposes, including tumor diagnosis and staging, as well as visualization of tumor imaging during microsurgical resections.

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1. Introduction

The human genome encodes 588 proteases that assemble into five distinct catalytic classes: metallo-, serine, threonine, cysteine and aspartic proteases [1]. Comprising approximately 2% of protein coding genes, the components of the "degradome" are important for a host of normal physiological and developmental processes, and their dysregulation is associated with pathogenic mechanisms underlying cancer and many other human diseases. This has made proteases attractive targets for drug development - however, with a few important exceptions, success has been elusive. Among the spectacular victories are the stories of the metalloproteinase inhibitors that block angiotensin converting enzyme (ACE) for lowering blood pressure [2] and the inhibitors of the aspartyl protease encoded by the human immunodeficiency virus which is required for human immunodeficiency virus (HIV) virion assembly [3]. But in contrast, the potent broad-spectrum matrix metalloproteinase (MMP)

inhibitors that were developed in the 1990's as anti-cancer agents met with disappointment in the clinic [4, 5].

There are several reasons for the failure of the MMP inhibitors (MMPIs) as cancer therapies. The central issue is that MMPs were initially perceived to act primarily as destroyers of the extracellular matrix (ECM), thus endowing cancer cells with the ability to invade and metastasize. We now know that this is a woefully simplistic view of the pathophysiological roles of these enzymes: subsequent work in the first decade of this century has highlighted their importance as regulators of the entire extracellular signaling milieu, thereby influencing pathways that control cell proliferation, survival, adhesion, migration, differentiation and their interactions with other cells [6, 7]. Indeed, although some MMPs such as membrane type-1 MT1-MMP (MMP14) are essential for cancer cell invasion through collagenous matrices [8], many MMPs can act either to promote or enhance tumor cell aggressiveness depending on the tumor type and its stage of progression [9-11], and some, such as MMP8, appear to be fundamentally tumor- and metastasis-suppressive [12, 13]. The first generations of MMPIs lacked selectivity, not only inhibiting many of the 24 human MMPs but also some of the other large families of related metalloenzymes such as the ADAMs (a disintegrin and metalloproteinase) and ADAMTS (ADAMs with thrombospondin-like motifs), whose existence was unknown when these drugs were developed. Together with the MMPs, these enzymes have important functional roles in organ homeostasis throughout the body, including the status of the immune system, and their inhibition no doubt contributed to the unacceptable side-effects of the MMPIs.

The multiple roles of proteases and the complex repertoire of protease families introduce another important consideration in relation to the problems associated with the MMPIs, which relates to the interconnectedness of what has been called the “protease web” [14, 15]. Increasingly we have become aware that in general proteases do not act in simple linear pathways like the clotting cascade, but instead are found to be working in concert via networks of enzymes of multiple catalytic classes, which regulate and coordinate each other’s activities and expression to determine tissue homeostasis. Functional inhibition of one enzyme or set of enzymes – for instance by MMPIs - can disturb this homeostatic balance, leading to alterations in the protease web whereby different protease activities are manifest, taking over roles originally performed by the inhibited MMPs, or unleashing activities that have deleterious consequences for the tissue or the body as a whole. These “systemic protease web-associated modulations” (spam) as a result of MMP inhibition can lead to the paradoxical promotion of metastasis *in vivo*, as we discussed in an earlier review [16].

Taking these considerations together, it is clear that although several protease systems are upregulated or functionally activated in the extracellular tumor environment - particularly the serine proteases of the plasminogen activation pathway, the MMPs and cysteine cathepsins - their blockade may not have the hoped-for anti-cancer therapeutic effects, even with highly specific inhibitors. (Parenthetically though, we should note that there is renewed enthusiasm for the clinical potential of selective MMP inhibitors [5]). But instead of trying to inhibit the actions of proteases to prevent cancer growth and spread, we can turn the problem on its head: an exciting and logically appealing alternative strategy takes advantage of the elevated activities of particular proteases in cancer tissues to serve as "triggers" for the localized release

of toxic agents from otherwise inactive prodrugs. This is an attractive concept because many conventional chemotherapeutic agents are non-selective, and therefore toxic to both cancer and normal host cells, resulting in dose-limiting systemic toxicities. Incorporation of a short peptide as a pro-moiety can render the agents inactive and therefore reduce their toxicity, until they enter the tumor environment and encounter the high-levels of a specific cleaving enzyme (Figure 1A).

As we will discuss further, the Protease-Activated Prodrug (PAP) concept can be refined by adding on targeting moieties to direct the agent to specific tissue locations. Such targeting moieties could be antibodies that recognize tissue-restricted markers, or ligands for cellular receptors. Linking the cytotoxic agents to high molecular weight carriers or nanoparticles can offer enhanced pharmacokinetic properties by reducing urinary excretion through the kidneys, thereby increasing retention in the circulation. Within tumors, the leakiness of the vasculature can passively contribute to increased accumulation of such high-molecular weight prodrugs via a mechanism known as “enhanced permeability and retention” [17]. The key factor in these scenarios is that the protease-cleavable pro-moiety must be stable while the agent is in transit in the circulation, but be rapidly cleaved when it encounters the tumor-restricted protease. Secreted and transmembrane proteases that are highly expressed and activated in the tumor environment are thus ideal candidates as prodrug activators, and we will discuss examples from several catalytic classes. From the standpoint of cancer therapy, an additional attraction is that because the proteases responsible for prodrug cleavage may come not just from cancer cells but also from the stromal components of tumors, release of the active drug directly into the tumor microenvironment does not depend on a target expressed only by the cancer cells.

Instead it is the entire tumor ecosystem that represents the target. Thus compounds such as colchicine that act primarily as vascular disrupting agents (VDAs) are associated with significant systemic toxicity in their native forms, but can be delivered as prodrugs to cause localized disruption of the tumor vasculature, with a substantial improvement in the therapeutic window, as exemplified by the ICT2588 PAP shown in Figure 1B [18-20].

The PAP concept extends into other therapeutic and "theranostic" applications. For example, protease-activatable therapeutics have also been tested for their use in gene therapy. Protease-activatable retroviral vectors allow the transfer of a gene into cancer cells expressing a unique set of proteases [21]. In this system a protease-cleavable sequence and a blocking domain (BD) are incorporated into a plasmid. The blocking domain consists of a sequence coding for a growth factor (e.g. epidermal growth factor, insulin growth factor and stem cell factor). The presence of the growth factor on the virus envelope results in the binding of the virus to growth factor receptors and the subsequent sequestration of the virus to the receptor results in a low infectivity. However, this infectivity is restored when the protease-activatable sequence is cleaved, resulting in the release of the BD [21-23]. Also, the PAP principle is being applied for development of cancer imaging agents for diagnosis and monitoring responses to therapy. For instance, activity-based probes (ABPs) that carry quenched fluorophores linked via protease-cleavable peptides can act as proteolytic beacons, whose cleavage generates optical sensors that can be useful for assessing protease activity *in vivo*, and also potentially as diagnostic tools for imaging the tumor microenvironment [24, 25]. Likewise protease-modulated contrast agents for magnetic resonance imaging (MRI) are being developed [26].

Another PAP strategy involves activatable cell penetrating peptides (ACPPs) which are peptides composed out of a protease-cleavable sequence separating a polycationic cell penetrating peptide (CPP) from a quenching polyanionic inhibitory domain. With CPPs attached to a cytotoxic warhead (such as a drug or an oncolytic virus) or a contrast agent for imaging, these entities can all be taken up by cells. With the ACPP attached, the polyanionic inhibitory peptide reduces cellular uptake by neutralizing the CPP. Upon cleavage of the protease-cleavable sequence, the CPP becomes active and can carry its payload inside the cells [27-29]

2. Extracellular protease systems for PAP development

From a historical perspective, the term "prodrug" originated in the 1950's [30], and encompasses around 10% of drugs currently approved for use worldwide [31, 32]. It applies broadly to drugs that are inactive in their native state but require some form of modification to generate the active entity, for instance by enzymatic conversion or chemical transformation. The objectives in prodrug design are to enhance the pharmacologic properties of a parent drug, for instance by improving its solubility, stability or clearance from the body, and to reduce side-effects or toxicities [31, 33]. A range of enzyme types are enlisted for prodrug activation, including hydolases, transferases, oxidoreductases, lyases and proteases. Protease-activated prodrugs (PAPs) therefore represent a particular type of prodrug, but one that is growing in numbers and varieties of design as our knowledge of the cancer degradome has burgeoned.

The ideal characteristics for a protease target for the development of PAPs are:

1) The enzyme or enzyme system is well-characterized biochemically and its cleavage preferences are known.

2) The protease is present and active at elevated levels in the tumor environment and has low expression or lack of activity in normal tissues.

3) It is absent or inactive in the circulation to minimize systemic toxicity.

4) It shows high affinity and selectivity for the designed prodrug, leading to rapid release of the active form of the drug *in vivo*.

With our improved knowledge of cancer proteases gained from experience with the clinical use of protease inhibitors discussed earlier, these four requirements can be translated in various ways. The first requirement means that the protease needs to be situated at an important central node in the tumor protease web and that it has high selectivity to a unique substrate. The second rule implies that the protease originates either from the tumor cells themselves or from tumor-activated resident stromal cells or leukocytes recruited into the micro-environment, thus providing a type of “bystander effect”. The third characteristic of circulatory latency is often guaranteed by α2-macroglobulin that inactivates all classes of proteases in the circulation. Finally, the fourth requirement is the most challenging one and is presently often solved with the use of highly specific monoclonal antibodies or with protease-specific exosite designs [34].

Key to the development of all PAPs is the selection and optimization of the protease-cleavable pro-moiety, which has been possible through analysis of phage display peptide libraries and more recently through “reverse degradomics” [35-37], which is the definition of all (major)

proteases acting on a single peptide substrate. The next critical consideration is the nature of the therapeutic agent or “warhead” to be delivered. Two of the most widely employed categories of warhead are: a) small molecule conventional cancer chemotherapeutic drugs (eg doxorubicin and paclitaxel) and other generally cytotoxic agents (eg thapsigargin) and b) natural bacterial toxin proteins such as anthrax and diphtheria toxins. However, many other types of agents could be envisaged.

Many protease systems are ideally positioned to be useful in the context of cancer, and in the sections that follow we will explore progress with PAPs developed for activation by extracellular proteases of the serine, cysteine and metalloproteinase classes. Much of the detailed information will be found in Tables 1-3, with the text here describing some of the main conceptual developments.

2.1 Serine protease-activated therapeutics

Serine proteases constitute one of the largest catalytic classes in the human degradome and they play a major role in cancer both as biomarkers of disease progression and as key effectors of pathology. Principal players are the components of the plasminogen activation pathway, whereby urokinase plasminogen activator (uPA), bound to its cell surface receptor (uPAR) is responsible for proteolytic activation of the broad-spectrum serine protease plasmin from plasminogen [38]. Plasmin in turn is responsible for proteolytic activation of several pro-MMPs, including MMP-1, 3 and 9, thereby effecting degradation of the ECM and other key extracellular regulators [7, 39, 40].

The increased levels of active uPA and plasmin in the tumor microenvironment make them excellent candidates for the development of selective PAPs [41]. Also another serine protease, prostate specific antigen (PSA) which is a member of the kallikrein family, has been exploited for development of PAPs for prostate cancer therapy [42-47].

2.1.1: Prodrugs based on the plasminogen activation pathway

Although plasminogen is an abundant (1-2μM) zymogen in plasma, its activation is temporally and spatially restricted, either to fibrin clots where it is activated by tissue plasminogen activator (tPA) or to the cell surface where it interacts with the uPA/uPAR complex [48]. Pro-uPA is usually produced by tumor-associated stromal cells in many cancer types, with uPAR being expressed by cancer cells. In the systemic circulation, active plasmin is rapidly neutralized by binding to α2-antiplasmin or α2-macroglobulin. As a result both plasmin and uPA fulfil the criteria described in section 2.1 and have been able to be exploited for the development of PAPs for cancer therapy, though most progress has been made with uPA-activated agents. Several reviews have described the design and properties of these PAPs [31, 41, 49, 50], so we have summarized the approaches and key findings in Table 1.

Small molecule chemotherapy-based PAPs: The first plasmin-activated prodrug was generated by attaching the plasmin-cleavable sequence Val-Leu-Lys to the anthracycline anti-tumor drug doxorubicin (Adriamycin) [51]. However this design was not optimal for cleavage, and was improved substantially with the inclusion of a spacer between the cleavable pro-moiety and the doxorubicin [52]. An excellent example of how this design strategy can subsequently evolve is provided by the development of a PAP based on doxazolidine, a more cytotoxic formaldehyde-

conjugated version of doxorubicin, but which has a very short half-life in physiological conditions. The resulting prodrug, GaFK-Doxaz, showed excellent stability in plasma and had low cell permeability, but was readily activated by plasmin and cathepsin B, resulting in low nM inhibition of growth of a wide variety of cancer cells [53].

uPA activated toxin prodrugs (anthrax and diphtheria)

Bacterial toxins are potent cell killing agents that have been adapted for use as cancer therapeutics by adapting them as PAPs (reviewed in [49]). Anthrax toxin has three components, namely protective antigen (PrAg), lethal factor (LF) and edema factor. PrAg is an 83kDa protein responsible for delivery of the effector proteins inside the susceptible cell by binding to cellular receptors, tumor endothelial marker 8 (TEM8) or ciliary morphogenesis gene 2 (CMG2), whereupon it is cleaved by the cell surface pro-protein convertase furin to a 63kDa form that oligomerises, creating a docking site for LF and edema factor. This complex is then internalized via endocytosis, and in the acidic endosomal environment the PrAg oligomer forms a pore that allows release of LF and edema factor into the cytosol, which bring about cell death, since LF is a metalloproteinase that cleaves mitogen-activated protein kinase kinases, disrupting essential cell signaling pathways [54]. Tumor selective anthrax biotoxins have been generated by substituting the furin cleavage site within PrAg (RKKR) with a uPA cleavable motif, SGRSA, generating the modified toxin PrAg-U2 [55]. The cytotoxicity of LF was also enhanced by creating a composite with Pseudomonas exotoxin A, resulting in a fusion protein called FP59. This combination of PrAg-U2 and FP59 generated a potent PAP, which was strictly dependent for its action on the presence of an active cell surface uPA/uPAR system [55]. A similar strategy generated a version of PrAg activated by MMPs (PrAg-L1), and a combined system involving

intermolecular complementation that requires both cell surface MMP and uPA activities, IC-PrAg [56]. These systems have each shown excellent tumor killing potential in human non-small cell lung xenografts [57] and murine B16-BL6 melanoma syngrafts [58], though with dose-limiting gastrointestinal toxicities. However the dual MMP/uPA-activated IC-PrAg when coadministered with LF has proved the most effective, working effectively against established tumors at doses well below those where toxicity is first encountered [58]. In a further refinement, PrAg has been engineered by creation of a form that is incapable of forming oligomers (D512K), but which can be rescued via a complementary mutation in a separate PrAg molecule. These PrAg variants are individually non-toxic, only becoming toxic when used in combination, where they were shown to have a potent effect against A549 xenografts [59].

Parallel strategies have been used for the development of diphtheria-based pro-toxins [49]. Diphtheria toxin (DT) has cell-binding, translocation and catalytic domains, separated by a furin-sensitive loop. Replacement of the furin site with one cleaved by the uPA/uPAR system, and the cell-binding domain with granulocyte-macrophage colony stimulating factor (GM-CSF) generated a recombinant DTU2GMCSF toxin that is potently cytotoxic for leukemic cells [60]. However further development of this agent has not yet been reported.

2.1.2: PSA-activated prodrugs

Prostate-specific antigen (PSA), or kallikrein-3, is a serine protease that is in routine use as a serological diagnostic marker for prostate cancer. Expressed by the prostatic epithelium, its normal function is to cleave semenogelin in the ejaculate to allow sperm to swim freely: in the circulation, PSA is inactive due to complex formation with the plasma protease inhibitors α-

chymotrypsin and α2-macroglobulin, so active PSA is only found in the vicinity of prostate cells [61]. The identification of a heptapeptide sequence based on semenogelin that is rapidly cleaved by PSA led to the development of a doxorubicin-based prodrug (L-377202) that had an improved therapeutic index compared to doxorubicin alone in preclinical studies [42, 43]. Subsequently this strategy was applied to generate PSA-activated prodrugs based on vinblastine [44], 5-fluorodeoxyuridine [62], and paclitaxel [63]. But despite initial promise, there have been no reports of clinical trials since 2008 when this topic was reviewed previously [49]. However, another type of PAP based on thapsigargin is showing promise [47]. Thapsigargin is particularly attractive as a warhead since it is a potent inhibitor of SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) which is toxic to non-growing as well as proliferating cells, and thus could potentially target slow-growing cancer stem cell populations. The PAP strategy led to development of a PSA-cleavable form of thapsigargin (G115) which showed complete inhibition of growth of PSA-expressing xenografts in vivo [47]. Subsequently a thapsigargin analogue with a PSA-cleavable linker covalently coupled to a N-(2hydroxypropyl) methacrylamide copolymer as macromolecular carrier was generated which also showed good efficacy and low toxicity [64]. However, further clinical development of thapsigargin-based PAPs has switched attention to focus on prostate-specific membrane antigen (PSMA)-activated prodrugs [65, 66], which will be considered in section 2,3 as this enzyme is a metalloproteinase.

2.2 Cysteine and Aspartyl Cathepsin-Activated Anticancer Therapeutics

Cysteine and aspartyl cathepsins are a popular target for prodrug design due to their roles in tumor progression [67]. The natural milieu of these enzymes is the lysosomal compartment, where a low pH ensures optimal catalytic activity of these acid proteases. A novel approach has

been designed by Ueki *et al.* taking advantage of the overexpression of histone deacetylase (HDAC) and the cathepsins within the tumor environment [68]. Puromycin is an aminonucleoside antibiotic capable of inhibiting protein synthesis causing ribosomal disruption during translation and can show significant cytotoxicity *in vitro*. Ueki *et al*. have attached an acetylated lysine group to the puromycin to deactivate it and create a non-toxic prodrug. The acetyl group is removed by HDACs leaving lysine which in turn is removed by Cathepsin L to leave the activated puromycin. *In vivo* studies have confirmed activity against human tumor xenografts [68].

In an attempt to combat some of the drawbacks of gemcitabine administration, such as a short half-life and a range of side-effects, gemcitabine was de-activated by covalently coupling to cholesteryl hemisuccinate. The non-toxic prodrug is reported to spontaneously form nanoparticles. These nanoparticles released gemcitabine in a lysosomal mimicking environment (pH 5.0) but not at a physiological pH (pH 7.4) with the gemcitabine release being greatly enhanced by cathepsin B. These nanoparticles enhanced the cellular uptake of gemcitabine by 15-fold when incubated with cells [69].

Floxuridine is an analogue of 5-fluorouracil with similar problematic side-effects. Two successful prodrugs of Floxuridine were developed by attaching phenylalanine and either tyrosine or glycine creating 5'-*O*-L-phenylalanyl-L-tyrosylfloxuridine and 5'-*O*-L-phenylalanyl-L-glycylfloxuridine. Both molecules were activated by cathepsins B and D suggesting these may be good candidate prodrugs for further development [70].

Pancreatic ductal adenocarcinoma (PDAC) is a difficult disease to treat. 5-aminolevulinic acid (5-ALA) can be used clinically as a photosensitiser in tumor treatment and imaging but does have

off target effects due to its inability to concentrate solely in the tumor tissue [71]. 5-ALA has been developed as a Cathepsin E-activatable prodrug, (H-Arg-Gln-Ala-Gly-Phe-Ser-Leu-5-ALA-OH) to take advantage of the overexpression of cathepsin E in PDAC [71]. The 5-ALA prodrug was shown to activate within the Cathepsin E-positive tumor but not in the normal pancreatic tissue. It is concluded that cathepsin E is a specific and effective enzyme activation system for the development of prodrugs in PDAC treatment.

Many other groups have developed doxorubicin prodrugs over the years which are capable of being activated by the cathepsins. Some time ago a doxorubicin prodrug Ac-Phe-Lys-PABC-DOX (PDOX) was developed by Dubowchik *et al.* [72] but has recently been shown to have activity against hepatocellular carcinoma in model systems [73]. A review by Zhong *et al*. [74] covers many of the cathepsin-activated doxorubicin based prodrugs developed. Other recent reviews which cover cathepsin activated probes are very useful for future prodrug design [75] as are the reviews on cathepsin L as target in cancer treatment [76] and the role of cysteine cathepsins in the degradation of the extracellular matrix [77].

2.2.1 Legumain-activated anticancer therapeutics

Legumain is a cysteine protease and an attractive target for prodrug design as it is overexpressed in the majority of human solid tumors including breast [78], ovarian [79], colon [80] and prostate [81]. It has restricted substrate specificity cleaving peptides on the C-terminal side of asparagines [82]. Furthermore, legumain is also expressed by intratumoral blood vessels and Tumor-Associated Macrophages (TAMs) and is involved in promoting cell migration in

invasion [83], which support its attractiveness as a target for tumor-selective drug delivery [83, 84].

Several groups have reported prodrugs of dolastatins or related molecules. The dolastatins are exceptionally potent pentapetide antineoplastic agents (in the pM range) which bind to tubulin close to the vinca binding site and disrupt microtubule function, however, their side-effects are considerable. Their potency makes them ideal candidates for prodrug design to enable selective delivery to tumors. Bajjuri *et al.* [85], have developed both didesmethylauristatin E (DDAE) and monomethylauristatin E (MMAE) legumain-activatable prodrugs by tethering an asparagine containing tripeptide linker (Alanine-Alanine-Asparagine) to either DDAE or MMAE. Several analogues were developed which showed considerable activity against an MDA-MB-435 (transfected with legumain) cell line but not in the wild type. This improved activity was demonstrated to be legumain-catalysed. Liu *et al.,* [83] have developed similar MMAE based prodrug molecules with a tripeptide (Alanine-Alanine-Asparagine) linker but capped with an inhibitor of integrin αvβ3. The inclusion of an integrin inhibitor enables the prodrug to bind to the cell surface integrin αvβ3 prior to activation by legumain. *In vivo* studies suggested that the MMAE integrin-binding prodrug was more effective than the cytotoxic MMAE alone.

As we have seen, doxorubicin is a common candidate for prodrug design. Several legumain-cleavable peptide-conjugates of doxorubicin were synthesised by Wu *et al.,* [84]. However, these intact prodrugs incorporated a succinyl group which prevented cell-permeability. Doxorubicin therefore was prevented from entering the cell until it had been activated in the tumor microenvironment by legumain. Within the tumor microenvironment, however, legumain is most highly expressed by tumor-associated macrophages (TAMs) and not by the

tumor cells themselves [84-87]. In this way the activated drug was suggested to have much greater antitumor efficacy by functioning through a "bystander effect" upon both tumor and stromal cells, rather than just selectively deleting the target-producing cells from the tumor. The most effective of the legumain-activated doxorubicin prodrugs, LEG-3, comprised doxorubicin bound to a tetrapeptide (Leu-Asn-Ala-Ala), endcapped with the succinyl group [86]. From *in vivo* studies, LEG-3 possessed enhanced efficacy compared with doxorubicin alone, in murine syngeneic tumor models and human tumor xenografts, including a doxorubicin-resistant prostate cancer model [84].

The same legumain specific tripeptide has been used by Smith *et al.,* [88] to develop a legumain activated colchicine prodrug. Colchicine was linked to a peptide sequence (Suc-Ala-Ala-Asn-Val-colchicine) to develop a prodrug which was more toxic to cells expressing active legumain than cells only expressing the 56 kDa prolegumain and this activity could be inhibited by a legumain inhibitor cystatin E/M.

A further legumain activated prodrug with etoposide as the active component was synthesized by Stern *et al.* [89]. The prodrug (carbobenzyloxy-alanine-alanine-asparagine-ethylenediamine-etoposide), released the etoposide upon cleavage by recombinant human legumain, and showed an inhibitory effect on the proliferation of legumain expressing 293 HEK-Leg cells. The authors suggest a novel platform for prodrug therapy activated by legumain as a promising approach for cancer therapy.

2.3 Metalloproteinase-activated therapeutics

The metalloproteinases encompass several families of proteases of which their catalytic activity depends on the presence of a metal ion [90]. Three often studied subfamilies are the matrix metalloproteinases (MMPs), ADAMTS (a disintegrin-like and metalloproteinase domain with thrombospondin type 1 repeat) proteinases and astacins due to their functions in extracellular matrix metabolism [91]. The MMPs are a family of 24 Zn^{2+}-dependent endopeptidases, including gelatinases, collagenases, stromelysins, matrilysins and membrane-type MMPs [90]. This class of proteases has a broad range of extracellular [39, 92], intracellular [93] and membrane-bound substrates [94]. In addition, MMPs also have non-catalytic functions such as the stimulation of cell signaling pathways [95-97] and they can act as transcription factors [98, 99]. Consequently, MMPs are implicated in a vast range of physiological and pathological processes ranging from bone growth, neurological development and the migration of immune cells to pathological bone resorption, cancer development and autoimmune disease [7, 25, 92].

In cancer, increased levels of several members of the MMP family are correlated with tumor progression, including MMP1 (collagenase-1), MMP2 (gelatinase A), MMP9 (gelatinase B), matrilysin (MMP7) and MMP14 (membrane-type-1-MMP) [100, 101]. As discussed above for other protease classes, MMPs act in a network, rather than in a cascade [14, 16, 39]. Therefore, for specific cancer types, grades and stages, it is critical to know which MMP determines the most important node of the network. Whereas until now this search has been difficult, with the use of novel broad profiling systems and better tumor markers, it is envisaged that it will be possible to target individual cancers with more precision and less side-effects. The development of tumor protease-based probes for MMPs has diagnostic and therapeutic implications. For

instance, fluorescent MMP-activity-based probes may be used by surgeons to delineate the tumor margin during resection. These applications will not be discussed here in-depth. We will rather focus on therapeutic applications of conjugates between MMP substrates and cancer drugs. Several aspects have been covered recently in an excellent review by Tauro *et al*. [24].

Many types of metalloproteinase-activated drugs have been developed, and an overview of these drugs is given in Table 3. One such drug relates to MMP-activated anthrax toxin, which parallels the approach described in section 2.1 for the engineered uPA-activated toxins. This concept was exploited by replacing the furin-cleavage site by substrate sequences for MMP2 and MMP9. *In vitro*, selective killing of MMP-overexpressing cell lines was witnessed [102]. As we have seen combined use of the MMP- and uPA activatable anthrax toxins has shown optimal effects in preclinical mouse models [56, 58].

As mentioned in section 2.1.1, an interesting recent PAP development involves the generation of a prostate-specific membrane antigen (PSMA)-activated thapsigargin prodrug [65, 66]. Despite its name, PSMA is not restricted to prostate cells, but is highly expressed by the neovasculature in most solid tumors. It is a type II transmembrane metalloproteinase with glutamate carboxypeptidase activity which cleaves poly-γ-glutamyl peptides [65], which has enabled the design of a thapsigargin derivative, G202, now termed “mipsagargin” [103]. Mipsagargin has shown substantial tumor regression against a variety of human xenografts in vivo and shows low toxicity, with no evidence of myelosuppression in phase I clinical trials [104]. Two patients suffering from hepatocellular carcinoma showed prolonged benefit so the drug has now progressed to phase II trial for HCC [65]. From the same group, a PSMA-activated cell-penetrating peptide conjugate has also been shown to have a strong cytostatic effects on

xenograft growth in vivo and also to be useful for optical tumour imaging when labeled with a near-infrared fluorophore [105].

3. Protease-activated nanoparticles

Nanoparticles are hailed as the new generation of diagnostic and therapeutic tools, according to a recent review [106]. The physico-chemical properties of nanoparticles are influenced by their material composition and size, making them highly tunable for diverse purposes, including carrying cytotoxic cargoes or contrast agents for imaging. Here we will place emphasis on peptide-decorated nanoparticles in which the peptides contribute as functionalizing systems to influence nanoparticle size, targeting or ability to be endocytosed. An attractive potential scheme for bringing these various aspects into play for the development of nanoparticles with optimized pharmacokinetics and delivery to tumors is shown in Figure 2. Here we envisage a local tumor protease (eg MMP9) acting on the decorated nanoparticles to alter their properties and direct them to display surface targeting factors that enhance tumor cell-specific interactions. However, the effects of nanoparticle cleavage could be many-fold: it might release active drugs (including the surface peptides), modify the nanoparticle diameter (affecting local uptake in tumor tissues), bind to specific targets (e.g. for directing internalization into cancer cells), change their physical properties (eg aggregation, which may be useful for imaging purposes), act as an (ant)agonist (in receptor interactions) and various combinations thereof. As previously documented, a number of caveats exist in that protein-coated nanoparticles are seen by the immune system as simple viruses and thus provoke both innate and adaptive immune reactions [107]. In the context of tumor therapy this may result in a positive bystander effect. However, it needs to be recognized that with immune recognition of nanoparticles, the

innate immune reaction may also cause collateral tissue damage and an adaptive immune reaction against them may hamper repeated use and may lead to avoidable complement-mediated systemic reactions.

In several cases it has been shown that small changes in the chemistry of nanoparticles can have drastic, and sometimes unpredictable, implications for their successful application [108]. The success of the nanoparticles depends on many factors including safety, stability in the circulation, escape of the macrophage phagocytic system and immune evasion, selective uptake by target cells and effective cytotoxicity or detection properties which each depend on the chemical characteristics of the nanoparticles.

In most therapeutic studies so far, nanoparticles have been designed to deliver cytotoxic substances into the tumor environment. In the simplest design, the drug of choice is encapsulated into the nanoparticle (which may be polymeric structures, lipids, proteins, organometallic compounds, or viruses) that is then endocytosed by the cancer cells. Several studies have evaluated the size threshold for passive diffusion of nanoparticles into tumor tissue and found that the most effective uptake is seen for particles with sizes smaller than 200 nm [109-111]. A logical strategy to retain nanoparticles in and around tumor tissue is to decorate the particles with cell-interactive ligands which can be peptides, proteins (antibodies), small molecules or other receptor ligands [111, 112]. An excellent example here is the somatostatin receptor in pancreatic cancer [113], which is being applied for nanoparticle-mediated therapy. However, such modifications alter the chemical characteristics of the nanoparticles (adding cationic or hydrophobic regions) and they may thus be more easily removed out of the circulation by the reticuloendothelial system (RES). This can be overcome

by adding hydrophilic groups such as polyethylene glycol (PEG) chains - though this would be at the expense of the targeted binding activities of the nanoparticles [114]. One way to address this problem is to develop nanoparticles with a removable hydrophilic layer [115]. Several groups [108, 114, 116-118] have now developed nanoparticles that are shielded for longevity in the circulation by virtue of an outer PEG coating, which can be removed in the cancer environment by proteolysis. A key aspect of the tumor milieu is the presence of inflammatory cells - neutrophils and macrophages – which deliver copious quantities of proteases, in particular neutrophil elastase (a serine protease), MMP8 (neutrophil collagenase) and MMP9 (gelatinase B). In the schematic in Figure 2 we highlight the utility of MMP9 in this nanoparticle de-shielding role. This strategy has been used with PEG-poly-caprolactone nanoparticles with an MMP9 cleavable linker attached to a cell penetrating peptide for proof-of-principle studies with glioblastoma cells in vitro [119]. In a different type of approach in which the pores of silica nanoparticles were tightly capped with avidin-biotin complexes, MMP9 was shown to be able to open the “valves” to allow local release of the chemotherapeutic drug payload [120].

There are also significant opportunities for use of protease-activated nanoparticles for tumor imaging purposes. For example, PEG-conjugated, uPA-activatable gold nanorods have been developed, whereby degradation of the uPA substrate sequence resulted in the aggregation of the gold nanorods which could be monitored as a decrease in light absorption [116]. These gold nanorods are likely to prove useful for imaging and photothermal therapy. In another strategy, nanoparticles were developed which aggregate only in the presence of MMP2 and MMP7. For this, one set of nanoparticles (biotin ligand particles) were shielded with an MMP2 substrate polymer and another set of nanoparticles (streptavidin receptor particles) were shielded with

an MMP7 substrate polymer. These nanoparticles self-assemble when in the combined presence of both MMP2 and MMP7, and since these enzymes are often markers of cellular transformation, this could allow development of a system to detect malignant transformation in vivo [121].

But perhaps it is the combination of possibilities for cargo delivery and surface decoration of nanoparticles that offers the most exciting potential for dual therapeutic and diagnostic ('theranostic') applications. This potential is evident in a recent study with functionalized gold nanoparticles carrying doxorubicin via a MMP2-cleavable linker [122]. This group has also generated graphene oxide nanocarriers with the same MMP2-activatable chemotherapeutic payload, where the intrinsic fluorescence of the doxorubicin is blocked by the graphene oxide, and recovered on release from the carrier [123]. Likewise the use of an iron oxide nanocarrier coupled with an MT1-MMP (MMP14)-cleavable azademethylcolchicine payload gives the potential for real-time monitoring of drug delivery and accumulation in tumors by MR imaging [19].

4. Conclusions and Future Directions

This review has covered the conceptual development of PAPs for cancer therapy and recent progress with new agents and technologies. There are compelling cases for the employment of strategies that take advantage of the elevated levels of specific extracellular and cell surface proteases in the tumor microenvironment, and in particular the serine proteases of the plasminogen activation cascade, PSA, the MMPs, and cysteine and aspartyl cathepsins. There is strong evidence that each of these protease systems can be leveraged using a diverse array of

chemotherapeutic weaponry, including potent natural toxins. This is clearly a fertile area for preclinical discovery, and it is gratifying to see some of these agents progressing into clinical trials, such as ICT2588 (Figure 1) and mipsagargin, to name two examples. With the explosive growth of technologies based around multifunctionalized nanocarriers, there is no shortage of potential avenues for further exploration. Hopefully, in the next five years we will see PAPs entering the frontline as therapies for a variety of cancers, based on the recognition of their enhanced therapeutic indices, low toxicities and favourable pharmacokinetics. Combined with the increasing use of genomic technologies (and also potentially degradomics) for evaluation of patients and their tumors these types of agents will help deliver precision medicine and better options for therapy, particularly against the stubbornly intractable cancers such as lung, brain and pancreas.

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Figure Legends

Figure 1: A) Schematic of the basic Protease-Activated Prodrug (PAP) concept. Addition of a peptide pro-moiety renders the drug “warhead” inactive, until the peptide is cleaved by tumor-associated proteases that are either not found or are inactive in the circulatory system.

B) Structure of the MT-MMP activated prodrug ICT2588 and the activated azademethylcolchicine (ICT2522). The dotted line indicates the MT-MMP-selective scissile bond.

Figure 2: The rationale of protease-activated nanoparticle drugs. (**a**) Functionalized NPs which contain a protease-cleavable linker are prepared and administered. NPs with hydrophilic shells such as PEG in the outer layer have extended bioavailability. (**b**) Within the local tumour environment, the protease-cleavable linker is cleaved by active proteases (derived from the tumour cells, activated stromal cells or recruited immune cells) and the outer shell PEG layer is removed. (**c**) Truncation of the linker region results in altered NP characteristics and leads to improved local diffusion of the particles. ‘De-shielding’ of the particle allows other modules (drugs, ligands, antibodies, …) to interact with the ECM and with cells in the tumour micro-environment. (**d**) NPs can be internalized by tumour cells by receptor-mediated endocytosis. (**e**) The load of the particles is released and allows for tumor imaging, induction of tumor cell death or gene transfer.

Table 1: Serine protease-activated therapeutics

Prodrug activator (protease)	Parent drug or agent	Agent name	Cleavable linker sequence*	Cancer types or models	Main findings	Ref
uPA	anthrax toxin	PrAg-U2	*SGRSA*	broad	used with engineered lethal factor FP59; effective tumor growth inhibition in mouse models but gastrointestinal dose-limiting toxicity, overcome with co-administration of dexamethasone	[102]
uPA	diptheria toxin	DTU2GMCSF	*GSGRSA*	acute myelogenous leukemia	diphteria toxin fused to GM-CSF via a uPA-cleavable linker led to selective killing of AML cells expressing GM-CSF receptors	[60]
uPA	gold nanorods		*LGGSGRSANAILEC*	4T1 cell tumors in mice	upon cleavage of the uPA substrate, the gold nanorods aggregate, resulting in higher accumulation in tumor.	[116]
plasmin	doxorubicin, palitaxel	ST-9802; ST-9905	*VLK*	mammary cancer	ST-9905 (with spacer between cleavable peptide and Dox) was substantially more effective than ST-9802 (without spacer) and induced similar tumor growth inhibition as Dox but without apparent toxicity	[51, 52, 124]
PSA	doxorubicin	L-377202	Hyp-*AS*-Chg-*QSL SSKLQ*	prostate	improved therapeutic index compared to doxorubicin alone in preclinical studies	[42, 43, 45, 46]
PSA	thapsigargin	G115	*HSSKLQ*	prostate	complete inhibition of growth of PSA-expressing xenografts in vivo	[47]
PSA	vinblastine		Hyp-*SS*-Chg-*QSSP*	prostate	PSA-dependent cell killing in vitro and tumor growth inhibition in xenografts, with myelopathy and neuropathy side-effects	[44]
factor Xa	retroviral vector with SCF as BD		*IEGR*	hematopoietic stem cells	protease-mediated tunable transduction of EGFR expressing cancer cells or hematopoietic stem cells.	[125]

*Amino acids are indicated in italic with one-letter code.Rare amino acids and other compounds are abbreviated in non-italic font. **Chg**, cyclohexylglycine; **Hyp**, hydroxyproline

Table 2: Cysteine protease-activated therapeutics

Prodrug activator (protease)	Parent drug or agent	Agent name	Cleavable linker sequence*	Cancer types or models	Main findings	Ref
HDAC/cathepsin L	puromycin	Lys(Ac)-Puro	*K*(Ac)-Puro	HCT116 xenograft	activity against human tumor xenografts	[68]
cathepsin B	gemcitabine	CHSdFdC	cholesteryl hemisuccinate	Bxpc-3 cells	enhanced the cellular uptake of gemcitabine	[69]
cathepsins B and D	floxuridine		*FY*	Capan-2 cells	prodrug activated by cells	[70]
cathepsin E	5-ALA		*RQAGFSL*	pancreatic ductal adenocarcinoma	activated within the Cath E-positive tumor but not normal tissue	[71]
cathepsins B	doxorubicin	PDOX	Ac-*FK*-PABC-Dox	hepatocellular carcinoma model	anti-tumor power at least equal to that of free Dox, better anti-metastatic efficacy and reduced toxicity	[72],[73]
legumain	auristatin	DDAE/ MMAE	*AAN*	MDA-MB-435 transfected with legumain	effective *in vitro* and *in vivo,* and reduced toxicity compared to MMAE without prodrug	[85]
legumain	auristatin/ inhibitor of integrin αvβ3	MMAE	*AAN*	MDA-MB-435	*in vivo* studies suggested that the MMAE integrin binding prodrug was more effective than the cytotoxic MMAE alone	[83]
legumain	doxorubicin	LEG-3	*LNAA*-Suc	various	complete arrest of a variety of neoplasms in vivo, including multidrug resistant lines	[84]
legumain	colchicine		Suc-*AANV*	HEK293	more toxic to cells expressing active legumain	[88]
legumain	etoposide		Cbz-*AAN*-AMC	HEK293	showed an inhibitory effect on the proliferation of legumain expressing 293 HEK-Leg cells	[89]

*Amino acids are indicated in italic with one-letter code. Rare amino acids and other compounds are abbreviated in non-italic font. **Ac,** acetyl; **AMC**, amino-4-methyl coumarin; **Cbz,** carbobenzyloxy; **CHSdFdC**, cholesteryl hemisuccinate–gemcitabine; **DDAE**, desmethylauristatin E; **Dox,** doxorubicin; **HDAC**, histone deacetylase; **LEG-3**, N-succinyl-h-alanyl-L-alanyl-L-asparaginyl-L-leucyl-doxorubicin, **MMAE**, monomethylauristatin E; **PABC**, p-aminobenzylcarbonyl; **Puro**, puromycine; **Suc**, succinyl.

Table 3: Matrix metalloproteinase-activated therapeutics

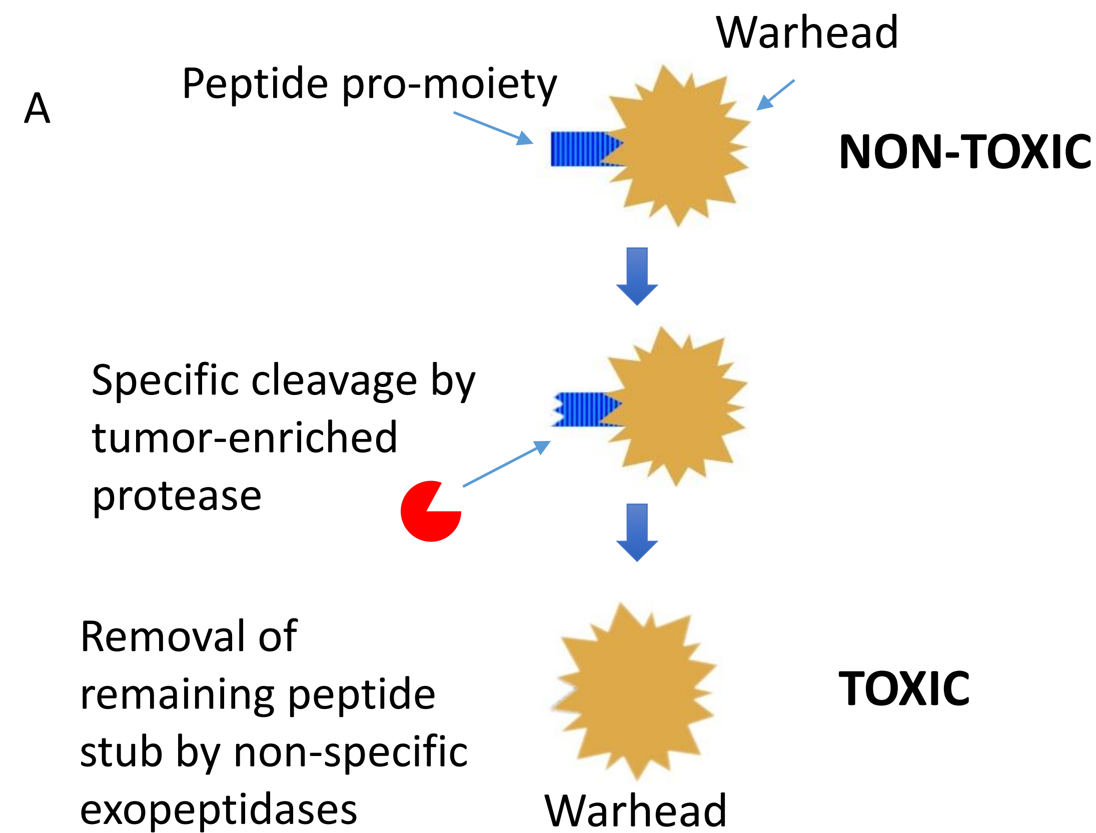
Prodrug activator (protease)	Parent drug or agent	Agent name	Cleavable linker sequence*	Cancer types or models	Main findings	Ref
MMP-2	NP with PTX		*GPLGVRGC*	SKOV-3 ovarian cancer cells	TAT peptide was added to the NPs to potentiate cell surface localization of the nanoparticles	[118]
MMP-2 & MT1-MMP	retroviral vector with EGF as BD and oncolytic MV with MMP-activatable F proteins	MV-MMPA1	*PQGLYA/Q*	liver cancer, human fibrosarcoma cells (HT1080)	virus was restricted to hepatocytes	[21, 27]
all MMPs	retroviral vector with EGF and CD40L as BD		*PLGLWA*	human fibrosarcoma cells (HT1080) and xenografted mice	selective transduction of MMP-rich target cells	[23]
All MMPs	liposomes carrying nucleic acids shielded by a removable PEG-shell		*GGGVPLSLYSGGGG*	human fibrosarcoma cells (HT1080) and subcutaneous tumors in mice	efficient accumulation in tumor and silencing activity	[108]
All MMPs	gold NPs with quenched NIR fluorophore		*PLGVRGC*	mice bearing SCC7 tumors	good performance *in vitro* and *in vivo*	[126]
MMP-2, -8, -9 and -14	CPP with Cy5, CPP with paclitaxel		*PLGLAG*	fibrosarcoma (HT1080), melanoma (B16F10), cervical (Hep2), prostate (PC3), colon (HCT) and mouse breast transgenic (PyMT) xenografts, glioma spheroids and glioma-bearing mice	reduced toxicity	[29, 119, 127]
MMP-2 and MMP-9	Dextran scaffold and methotrexate	MTX-*PVGLIG*-dextran	*PVGLIG*	*in vitro* digestion and cytotoxicity experiments with human fibrosarcoma cells (HT1080) and breast tumor	methotrexate was released in presence of MMP-2 and MMP-9 (*in vitro*). *In vivo*, enhanced efficacy over free MTX and lower toxicity. biodistribution in MMP-overexpressing tumor	[128-130]

				cells (BT-20). Subcutaneous tumor models in mice with human fibrosarcoma cells (HT1080), human glioblastoma (U-87) and human bladder carcinoma cells (RT-112)	xenograft model shows that tumor targeting effect was mainly due to passive targeting and EPR.	
MMP-2 and MMP-9	anthrax toxin PA and LFn domains fused to ADP-ribosylation domain of *Pseudomonas* exotoxin A	PA-L1 PA-L2	*GPLGMLSQ* *GPLGLWAQ*	human fibrosarcoma cells (HT1080), MDA-MB-231 cells and melanoma A2058 cells	selective killing of MMP-overexpressing cells (*in vitro*)	[102]
MMP-9	iron oxide nanoparticles		*GGPRQITAG*-K(FITC)-*GGGGRRRRGRRRRR*	*in vitro* protease incubation assays and molecular modeling	substrate selected for MMP-9 but also detects MMP-2, MMP-7 and MMP-13. Upon degradation the nanoparticle size decreases from ±25 to ±5 nm. No *in vivo* proof of concept.	[131]
MMP-9	activatable low molecular weight protamine		E10-*PLGLAGVSRRRRRRGGRRRR*	*in vitro* protease incubation assays, uptake by C6 glioma cells, tumor spheroid penetration	substrate selected for MMP-9 but also detects MMP-2, MMP-14, MMP-8, and other proteases. Improved glioma-targeting and tumor penetration.	[119]
MMP-2	galactosylated liposomes with NOAC		*GPLGIAGQ*	hepatocellular carcinoma cells (HepG2)	liposomes are not taken up by liver cells due to steric hindrance effect of the PEG groups. Upon encounter of active MMP-2 the PEG shield is removed and internalized through asialoglycoprotein receptor-mediated uptake and increased toxicity in HepG2 cells.	[117]
MMP-2	nanoparticles with NIR fluorophore, cell internalizing peptide and TAMRA labeled protease-		*GK*(TAMRA)*GPLGVRGC*	human fibrosarcoma cells (HT1080) and subcutaneous tumors in mice	accumulation in tumor vasculature and tissue	[114]

	cleavable PEG					
MMP-2	QD with cell penetrating peptide and substrate-PEG		*GGGGPLGVRGGGGK*	*in vitro* cellular uptake experiments with melanoma cells (MDA-MB-435)	treatment with MMP-2 resulted in enhanced cellular uptake of the quantum dots	[132]
MMP-2	aggregating nanoparticles in the presence of MMP-2 and MMP-7		*GPLGVRG*	*in vitro* protease incubation assays	NP switches were successfully developed and aggregated as expected	[121]
MMP-7	Aggregating nanoparticles in the presence of MMP-2 and MMP-7		*VPLSLTM*	*in vitro* protease incubation assays	NP switches were successfully developed and aggregated as expected	[121]

*Amino acids are indicated in italic with one-letter code. Rare amino acids and other compounds are abbreviated in non-italic font. **BD**, blocking domain; **CCP,** cell penetrating peptide; **EGF**, epidermal growth factor; **EPR**, enhanced permeation and retention; **FITC**, Fluorescein isothiocyanate ; **MV**, measles virus; **NP**, nanoparticle; **NOAC**, N4-octadecyl-1-β-D-arabinofuranosylcytosine; **PTX**, Paclitaxel; **PyMT**, polyoma middle T oncogene driven by mouse mammary tumor virus promoter spontaneous model of cancer; **QD**, quantum dots; **SCC**, squamous cell carcinoma; **TAMRA**, tetramethylrhodamine, **TAT**, transactivator of transcription.

Figure 1



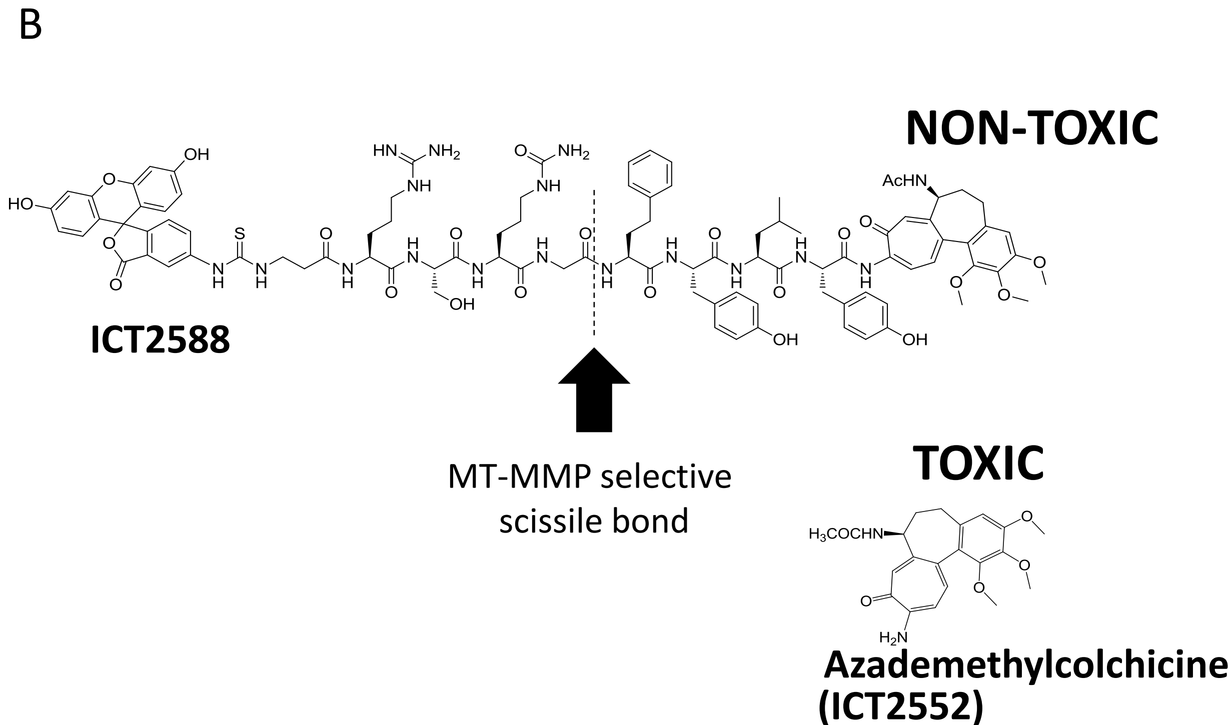
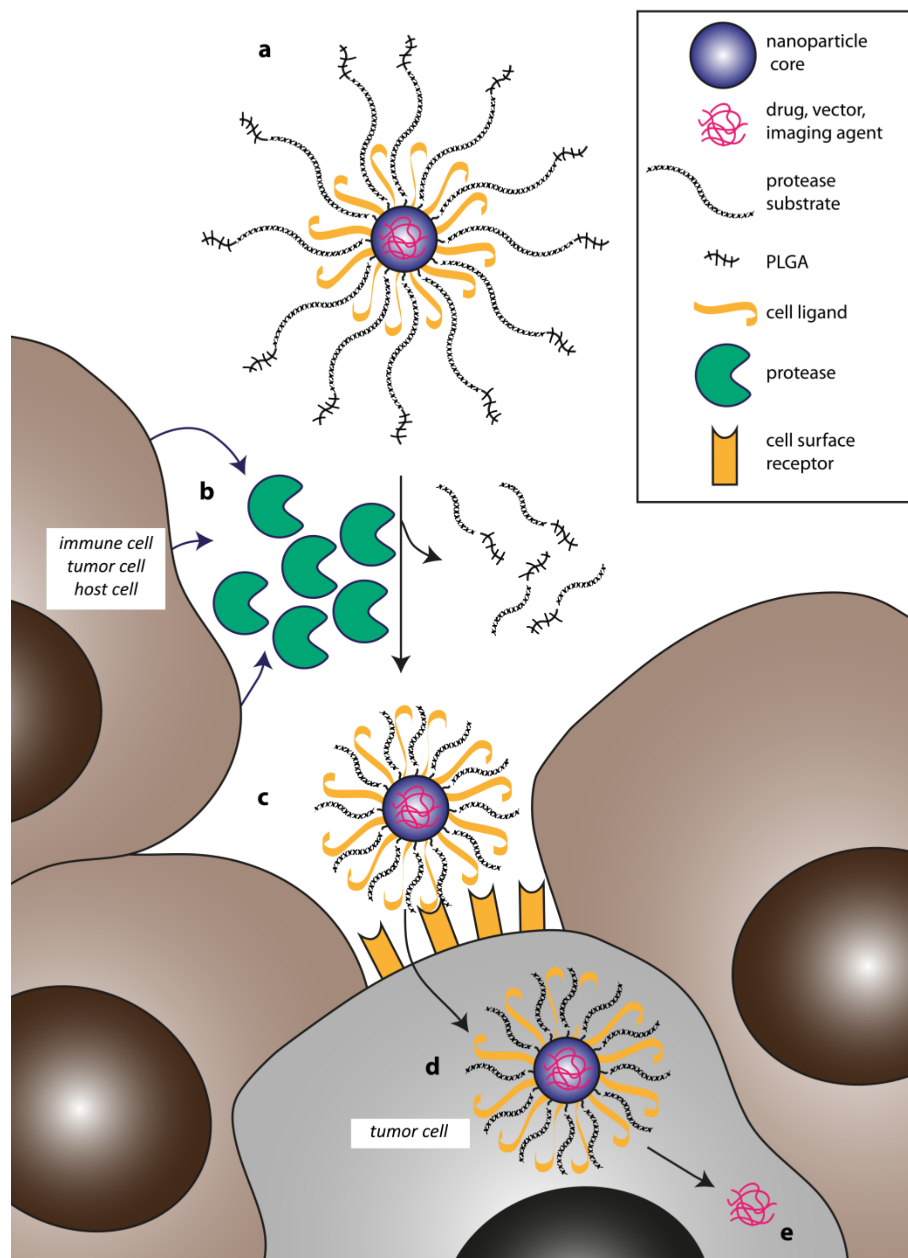


Figure 2



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