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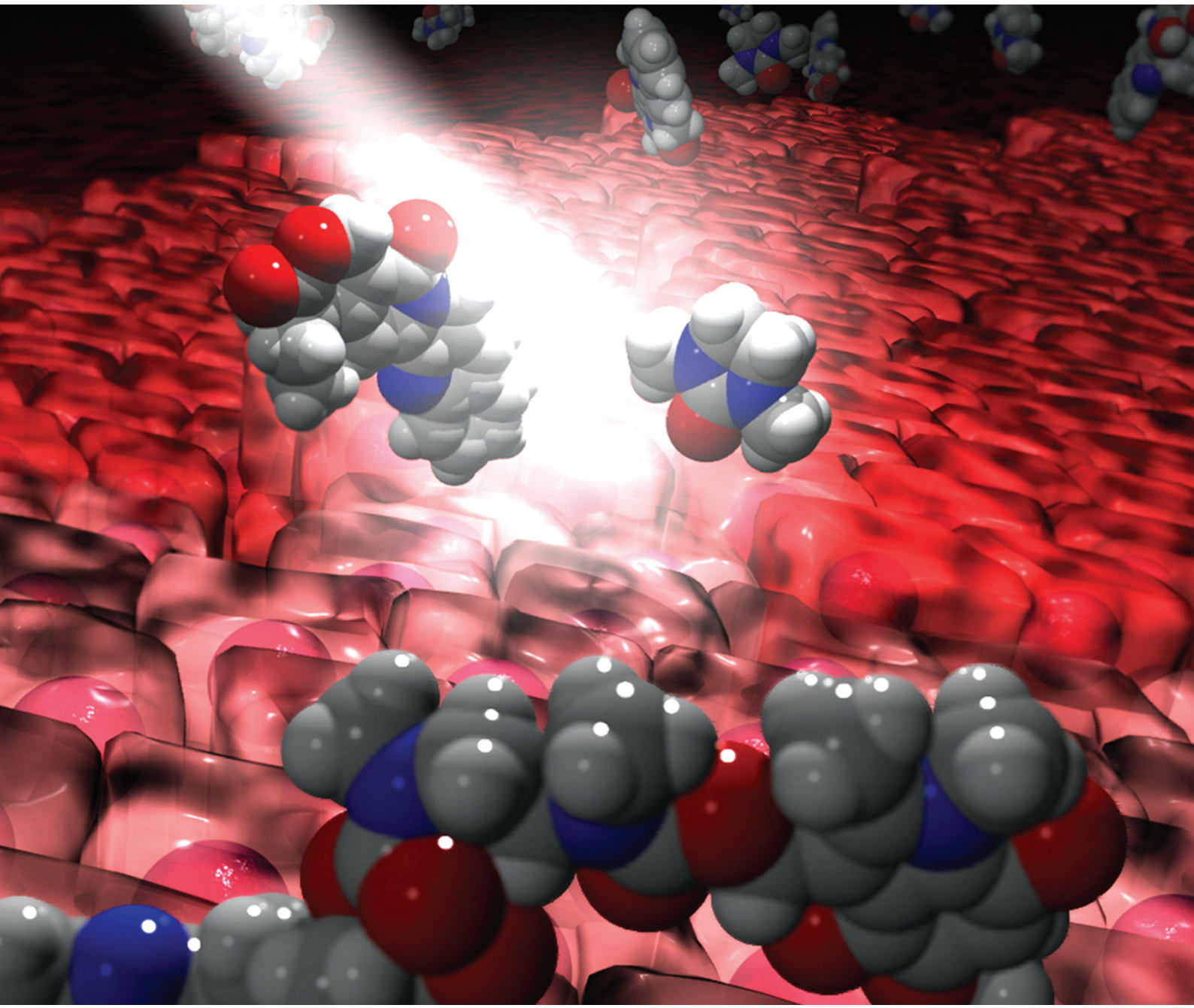

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Current molecular design of intelligent drugs and imaging probes targeting tumor-specific microenvironments

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To address the specific challenges of cancer therapy and diagnosis, a number of approaches have been advocated for the development of tumor-targeting antitumor drugs/prodrugs and non-invasive tumor molecular imaging probes. These intelligent drugs and probes are constructed from multi-functional molecular systems. This review focuses on the molecular design of drugs and imaging probes that target tumor-specific microenvironments such as angiogenesis and hypoxia.

1 Introduction

Ideal chemotherapeutic agents for cancer treatment are intelligent drugs that are selectively toxic to the malignant tumor cells but which lack non-specific toxicity toward normal cells.[1] One often encounters difficulties when designing such drugs because normal cells and cancer cells differ in only a few properties. A number of researchers have attempted to develop anticancer agents that exhibit highly selective cytotoxicity toward cancer cells by reference to cancer-specific characteristics, such as antibody, genes, and elevated levels of certain enzymes and receptors within the cancer cells.[2]

In addition to drug design, it is also important to develop imaging modalities to facilitate cancer diagnosis at the earliest stage and to assess the effectiveness of cancer therapy. The field of noninvasive imaging science has grown exponentially during the past three decades, and a variety of related technologies, such as X-ray computed tomography (CT), ultrasound imaging, magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT) have become indispensable tools in clinical applications. Recent advances in the molecular imaging technologies and imaging probes allow the application of noninvasive imaging to drug discovery, drug target identification, pharmacokinetics, assessment of therapeutic effects, and cancer diagnosis.[3] Thus, the development of molecular imaging probes plays an increasing role in cancer treatment.

In this review, we describe the current state of research on cancer targeting and molecular imaging systems, which are functioning in tumor-specific microenvironments including angiogenesis and hypoxia. This review also refers to our recent research on the development of tumor vascular targeting anticancer prodrugs and imaging probes, hypoxic radiosensitizers, hypoxic cytotoxins, radiation-activated prodrugs, and hypoxia molecular imaging probes.

2 Tumor vascular-targeting anticancer prodrugs and imaging probes

Like all normal cells in the body, cancer cells cannot survive beyond the effective 100 to 200 μm oxygen diffusion distance from

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the vascular system. To grow beyond a small size, a tumor initiates angiogenesis to create new blood vessels for supplying oxygen and nutrients.[4] The tumor angiogenesis and lymphangiogenesis can aggravate a small localized tumor into an advanced malignant tumor with the ability to metastasize to other normal tissues.[4b,5] Thus, the tumor vasculature has been considered an important target for cancer therapy and diagnosis.[6]

Unlike conventional therapies in which attention is mainly paid to cancer cells, tumor vascular therapies concentrate on the tumor blood vessel system to inhibit tumor angiogenesis and thereby block the supply of oxygen and nutrition to cancer cells[7] or to normalize the tumor vasculature and thereby improve the drug delivery efficiency and therapeutic effect.[8] The major challenge for tumor vascular therapies is to develop angiogenesis inhibitors and tumor vascular-targeting drug delivery systems. More than 300 angiogenesis inhibitors have been discovered, about 80 of which are under study in clinical trials. The antiangiogenic therapies usually require a combination with other chemotherapies. Several reviews on antiangiogenic therapies have been published.[9] Here, we focus mainly on the development of tumor vascular-targeting anticancer prodrugs and imaging probes that use tumor-homing chemical antibodies (*e.g.* tumor homing peptides, DNA/RNA aptamers).

The discovery of vascular heterogeneity showed that the vascular endothelium expresses differential molecular markers depending on the tissue localization and functional state.[6a,10] These tissue-specific molecular markers, termed vascular molecular addresses (or vascular zip codes), can be recognized selectively by certain chemical antibodies that can be screened out by phage display technology[11] or systematic evolution of ligands by exponential enrichment (SELEX) technology.[12] In this context, the tumor vascular endothelium carries distinct markers, termed tumor vascular molecular addresses, such as αvβ3, αvβ5, MMPs, NG2, VEGFRs, and other specific isoforms of proteases CD13/APN,[13] which can be selectively targeted by some tumor-homing peptides, DNA/RNA aptamers, special small molecules or some corresponding antibodies. Such findings of specific tumor molecular address systems provide a novel strategy for the development of tumor-targeting anticancer prodrugs and imaging probes.

2.1 Tumor vascular-targeting anticancer prodrugs

Using such a strategy, several novel tumor-targeting prodrugs have been designed, in which anticancer agents are conjugated to tumor-homing peptides. In the first case, Arap and coworkers linked doxorubicin (DOX) to tumor-homing peptides CNGRC or CDCRGDCFC (termed RGD-4C) to generate CNGRC-DOX (**1**) and RGD-4C-DOX (**2**) conjugates (Fig. 1), which target tumor blood vessels and thereby have greater therapeutic efficacy and less toxicity than free doxorubicin in mice models.[14] RGD-4C was shown recently to target tumor vascular marker integrins αvβ3 and αvβ5 selectively, whereas CNGRC targets tumor-specific isoforms of proteases CD13/APN.[13] These findings have encouraged the design of serial tumor vascular-targeting anticancer prodrugs. Scheeren and coworkers developed an integrin αvβ3 targeting plasmin-cleavable doxorubicin prodrug (**3**) (Fig. 1), which shows plasmin-dependent cytotoxicity for endothelial cells and HT-1080 fibrosarcoma cells *in vitro*,[15] and inhibited tumor growth and angiogenesis without systemic toxicity in a tumor-bearing mouse model.[16] Koch and coworkers designed αvβ3 targeting doxorubicin–formaldehyde conjugates acylic-RGD-4C-DOXSF (**4**) and cyclic-(*N*-Me-VRGDf-NH)-DOXSF (**5**) (Fig. 1), as a novel *N*-Mannich base triggered prodrug of doxorubicin.[17] We have recently developed APN/CD13-targeting 5-fluoro-2′-deoxyuridine prodrugs **CNF1** (**6**) and **CNF2** (**7**), which have selective cytotoxicity towards APN/CD13 positive HT-1080 cells.[18]

Most prodrugs that release cytotoxin by hydrolysis usually exhibit poor stability and therefore produce side effects on normal cells *in vivo*. For enzyme-activated prodrugs undergoing enzymatic hydrolysis, tumor-specific enzymes and methods for selective delivery of enzymes or enzymatic genes to tumor tissues are under investigation.[19] To overcome the problem of control release of these drugs, we proposed a novel strategy to produce a tumor vascular-targeting photoactivated prodrug. For the first prototype, we designed the CD13/APN-targeting photoactivated prodrug (**8**) of 5-fluorouracil (5FU) (Fig. 2), by using a tumor homing peptide CNGRC and a photolabile linker. Upon controlled photolysis, such a tumor vascular-targeting photoactivated prodrug is expected to accumulate and to be activated selectively to release anticancer agent 5FU within tumor tissues with outstanding spatial and temporal precision.[20]

Among the other interesting cases, the pro-apoptotic peptide $(KLAKLAK)_2$ conjugated with tumor-homing peptides CNGRC or SMSIARL, shows selective toxicity to angiogenic endothelial cells and effective anticancer activity *in vivo*.[21] Tumor necrosis factor (TNF) coupled to tumor-homing peptides CNGRC or RGD-4C induces selective penetration of TNF into tumor tissues and greater immunotherapeutic properties.[22]

2.2 Tumor vascular-targeting imaging probes

Like the tumor vascular-targeting anticancer prodrugs, many tumor-targeting probes that take advantage of the tumor molecular address systems have been developed recently for tumor imaging techniques, including tumor angiogenesis imaging and tumor lymph imaging.

The first class of probes comprises the tumor vascular receptor-targeting nuclear trace probes, which are applicable to PET and SPECT. As reviewed by Haubner, a series of αvβ3 integrin-targeting radiolabelled RGD peptides, including [^{18}F]galacto-RGD, [^{125}I]gluco-RGD, [^{64}Cu]DOTA-c(RGDyK), and [^{111}In]-DOTA-E-$[c(RGDfK])_2$, have been developed and determined to have potential for αvβ3 integrin-expression monitoring and angiogenesis imaging.[23] Medina and coworkers reported that ^{99m}Tc or ^{125}I labeled peptide CTT (CTTHWGFTLC), inhibitors of MMP-2 and MMP-9 gelatinases, are useful in the early detection and imaging of primary tumors and metastases.[24]

The second class of probes comprises tumor vascular-targeting optical imaging probes, which are derived from the conjugation of tumor-homing peptides with fluorescent dyes. Two groups designed αvβ3 integrin-targeting cyclo(RGDyK)-Cy5.5 (RGD-Cy5.5, **9**)[25] and Cypate-Gly-Arg-Asp-Ser-Pro-Lys-OH (**Cyp-GRD**, **10**),[26] respectively. These two probes were shown to be useful for monitoring αvβ3 integrin expression in a tumor-bearing mouse model. To improve the specificity of the imaging probe, Chen *et al.* attempted to design Cy7-labeled RGD multimers and found

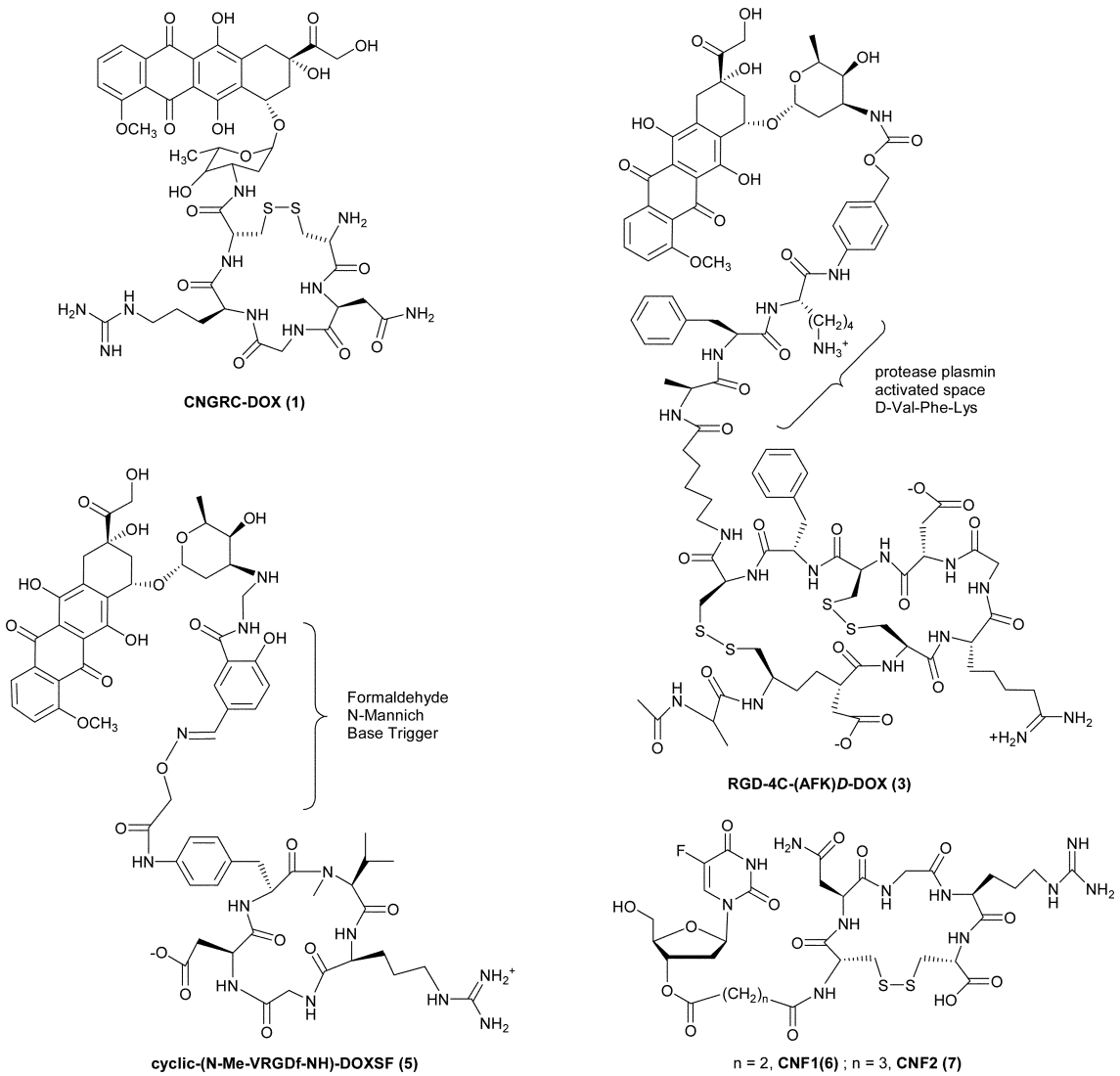


Fig. 1 Structures of tumor vascular-targeting anticancer prodrugs.

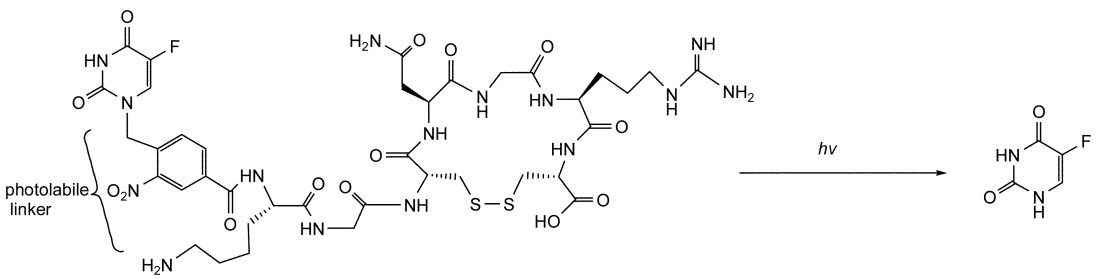


Fig. 2 Tumor vascular-targeting photoactivated prodrug **8** releasing 5-fluorouracil.

that the tetrameric RGD peptide probe Cy7-E{E[c(RGDyK)]$_2$}$_2$ (**11**) (see Fig. 3) showed the highest tumor accumulation and strongest tumor-to-normal tissue contrast in a U87MG tumor-bearing mouse.[27] Our group recently synthesized several dye-CNGRC conjugates, which could selectively label CD13/APN-positive HT-1080 tumor cells but not CD13/APN-negative MDA-MB-231 cells.[28] Such CD13/APN targeting probes may also have potential in tumor angiogenesis imaging.

Another novel type of protease-activated probe based on fluorescence resonance energy transfer (FRET) was recently developed as a tumor vascular-targeting probe. As shown in Fig. 4, autoquenched near-infrared (NIR) fluorescent dyes were bound to a long circulating macromolecule that could accumulate in a solid tumor through its leakage out of the tumor neovasculature. When such a probe arrives at the tumor tissue, it could be activated by tumor-associated protease to increase the fluorescence within the

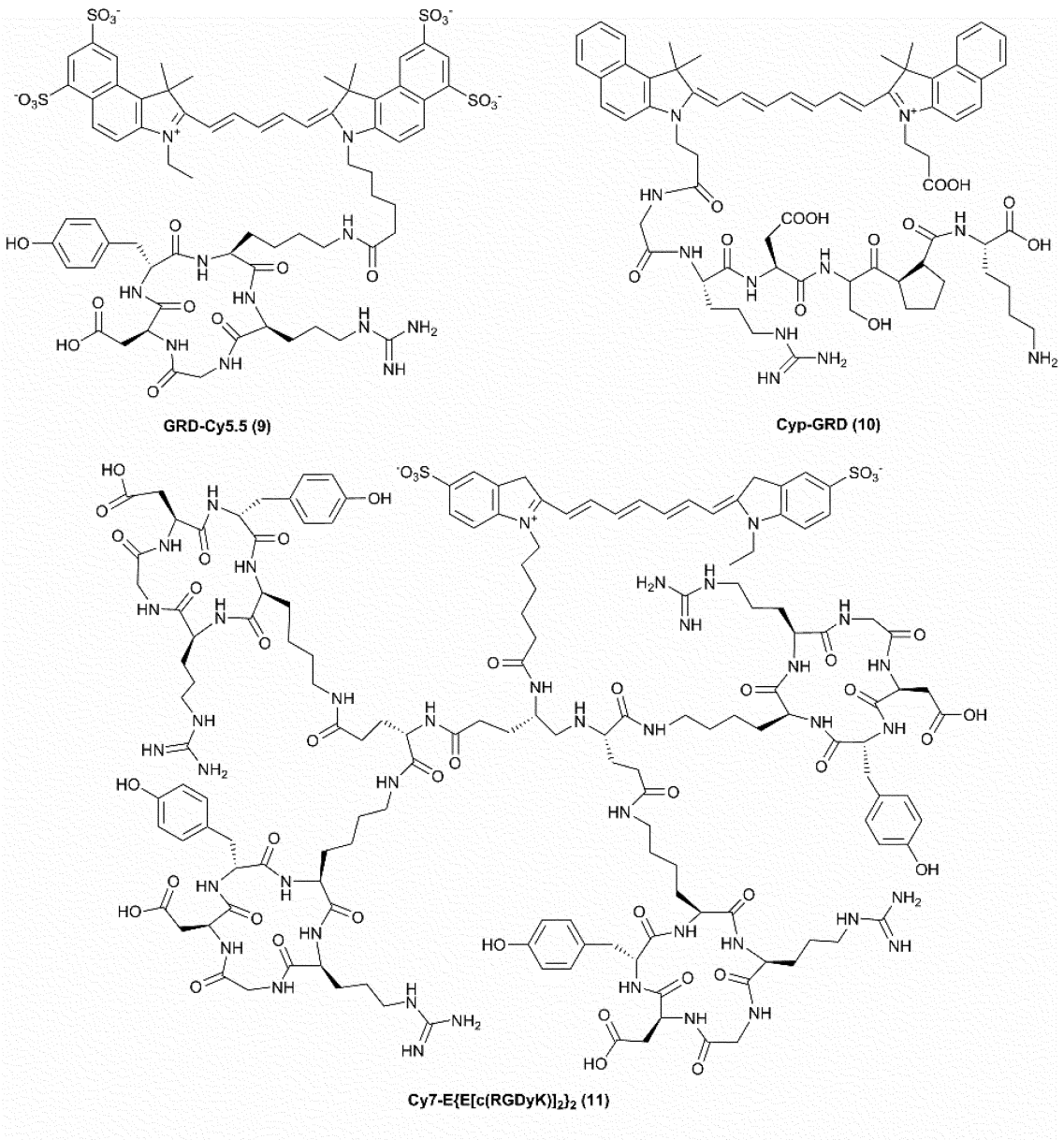


Fig. 3 Structures of tumor vascular-targeting fluorescent probes.

tumor, resulting in high signal-to-noise ratios for tumor imaging. A kind of enzyme-activated probe that is sensitive for cathepsin B and D, MMP-2, MMP-7, and MMP-9 has been developed for tumor imaging, and has been used to evaluate the effects of tumor anti-angiogenesis therapy,[29] and to visualize inflammation associated with atherosclerosis.[30]

3 Hypoxia targeting and imaging systems

Most cellular functions require the continuous and adequate supply of oxygen molecules from blood vessels. While stable oxygen supply is preserved in normal tissues by so-called oxygen homeostasis, inadequate oxygen supply to cells induces hypoxia, one of the pathophysiological characteristics of cardiac ischemia, inflammatory diseases, and solid tumors.[31] Tumor hypoxia is of especial importance because it is closely associated with the malignant phenotype of cancer cells, resistance to cancer therapies, and lower mortality rate of cancer patients.[31e,31f] In these contexts, the creation of functionalized drugs and imaging tools that work in hypoxic environments is imperative for cancer treatment and diagnosis.

3.1 Nitroazole radiosensitizers for hypoxic cancer cell treatment and imaging

The hypoxic and anoxic cells generated due to lack of oxygen diffusion are closely associated with the failure of radiotherapy. To overcome this oxygen effect on the treatment of cancer, certain compounds characterized by electron affinity have been identified to mimic oxygen in the radiosensitizing action on hypoxic tumor

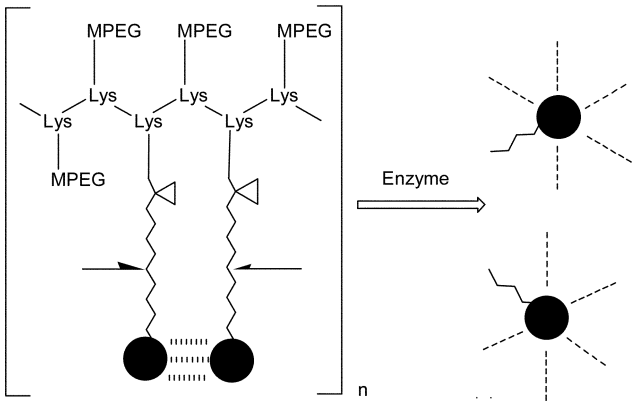


Fig. 4 Schematic representation of a protease-activatable fluorescent probe. Closed circles represent fluorochromes, which are autoquenched initially as bound to the poly-L-lysine backbone. With specific enzymatic cleavage of peptide spacers, fluorochromes are separated from the backbone and each other and markedly increase their fluorescence.

cells.[32] A number of studies have been hitherto carried out to search electron-capturing chemical agents that show radiosensitizing ability by the following mechanism: (1) a trapping of electrons by sensitizers, which are located near to the free radical centers of DNA generated by hypoxic irradiation,[33] (2) covalent bond formation between sensitizers and DNA to cause irreversible damage.

The first generation of radiosensitzers includes nitroimidazole and metronidazole (**12**) derivatives, which bear a hydroxyethyl side chain (Fig. 5).[34] This family of agents was confirmed to show effective *in vitro* and *in vivo* radiosensitizing activity exclusively under hypoxic conditions. The discovery of this family prompted the search for the related analogues with higher radiosensitizing ability. Consequently, the more active compound misonidazole (**13**) was discovered, and its cytotoxicity and functions were investigated.[35] Misonidazole **13** showed high potency when used with a single dose of radiation in a wide spectrum of animal tumors and appeared to be active against human malignancies.[36] The uniform response in various experimental studies led to extensive clinical trials, the results of which have been less promising than expected because of severe neurological toxicity.[37] Although further clinical trials of misonidazole **13** were given up mainly due to the occurrence of serious side effects, this prototype agent clarifies the possibility that radiosensitizers with electron affinity exhibit certain cytotoxicity toward hypoxic tumor cells upon irradiation.

The second generation of radiosensitizers possessing a 2-nitroimidazole skeleton was designed to improve the pharmacokinetic characteristics by modification of the side-chain structures. Among a large number of 2-nitroimidazole derivatives synthesized,[38] etanidazole (**14**) bearing a hydroxyethylamide side-chain, its fluorinated agent (KU-2285: **15**), and RP-170 (**16**) bearing a diol side chain showed sufficient radiosensitizing ability both *in vitro* and *in vivo*. Some of these second-generation radiosensitizers have been evaluated in clinical trials, which provided encouraging results including suppression of side effects such as neurotoxicity; however, these agents have not been applied widely to the clinical setting.

Hori and coworkers reported on novel bifunctional hypoxic radiosensitizers designed on the basis of the function of nitroazoles as hypoxia-targeting moieties. TX-1845 (**17**), TX-1846 (**18**), and the corresponding optically active agents TX-1898 (**19**) and TX-1900 (**20**) comprising 2-nitroimidazole and haloacetyl carbamoyl groups at the side chain were developed.[39] The haloacetyl group acts as an acceptor of intracellular nucleophiles such as mercapto and amino groups to form a covalent bond with DNA or proteins. Thus, these bifunctional haloacetyl carbamoyl compounds have both radiosensitizing and alkylating activity functionalities, exhibiting 100 times higher hypoxic radiosensitizing activity than conventional 2-nitroimidazole derivatives

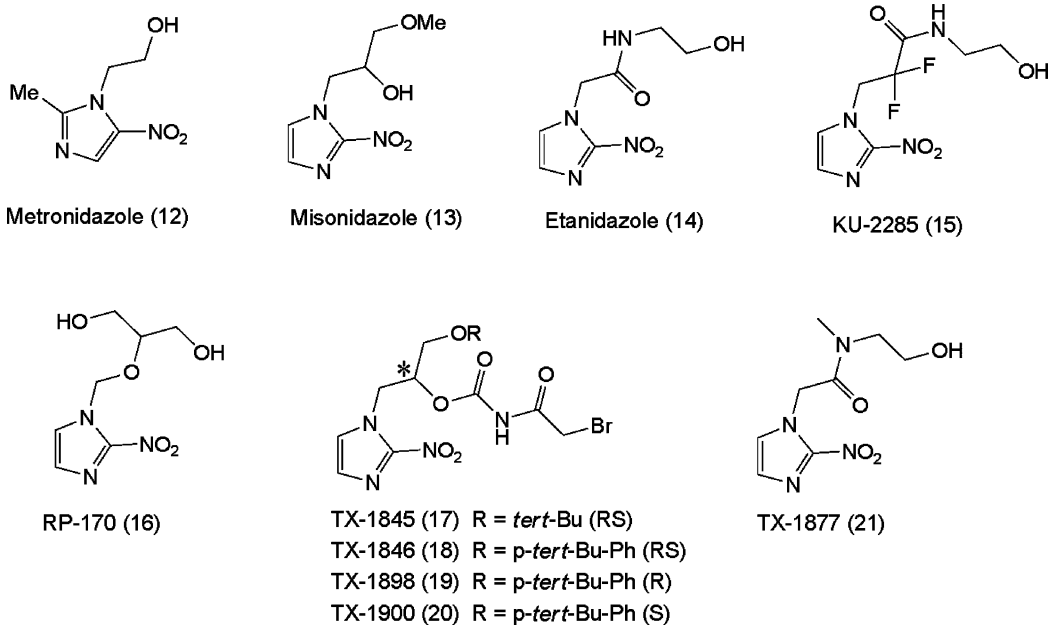


Fig. 5 Structures of nitroazole radiosensitizers.

and antiangiogenic activities in the chick embryo chorioallantoic membrane (CAM) assay. Hori and coworkers also developed another nitroimidazole derivative TX-1877 (**21**), which is conjugated with an acetoamide unit.[40] TX-1877 **21** shows higher inhibitory activity against metastasis and angiogenesis, and causes greater enhancement of macrophage infiltration. In addition, TX-1877 alone shows *in vivo* antitumor activity in the absence of radiation, although the detailed mechanism is unclear.

Endogeneous non-protein thiols (NPSH) play a crucial function in determining the response of biological cells to several types of radiation. The reduced form of glutathione (GSH), which is a typical endogenous NPSH and exists abundantly in cells, is known to protect intracellular molecules from radiation.[41] It is therefore likely that depletion of GSH in tumor cells is an effective target for radiation therapy. We have proposed a series of nitroazole radiosensitizers containing an α,β-unsaturated carbonyl group (**22**)[42] or propargylic sulfones (**23**)[43] in the side chains, which are able to capture GSH in tumor cells by alkylation (Fig. 6). These agents exhibit efficient NPSH-depleting ability in hypoxic cells, which enhances the hypoxic-cell radiosensitization *in vitro* relative to well-documented nitroimidazole radiosensitizers.

The nitroimidazole skeleton on the radiosensitizers can act as a hypoxia marker through bioreductive formation of hydroxylamine derivatives followed by the covalent bonded adduct formation with intracellular nucleophiles in hypoxic cells. In the light of these reaction characteristics, nitroimidazole derivatives have been applied to the imaging of hypoxic tumor cells.^{18}F-fluorinated misonidazole (^{18}FMISO) was recently designed as a hypoxia probe that can be imaged by PET. Yeh and coworkers identified an ^{18}FMISO labeled tumor to muscle retention ratio (TMRR) for the detection of tumor hypoxia in nasopharyngeal carcinoma (NPC).[44] The PET imaging of nasopharynx and neck by ^{18}FMISO showed a significantly higher TMRR in NPC than in normal tissue, indicating that the TMRR is a clinically useful index for identifying tumor hypoxia in NPC. Rajendran and coworkers compared PET imaging results between ^{18}FMISO with ^{18}F-labeled fluorodeoxyglucose (^{18}FDG) used for metabolic imaging to demonstrate significant discrepancies in the imaging patterns.[45] This result is not surprising, because hypoxia is not necessarily correlated to glucose metabolism.

3.2 Hypoxia targeting and imaging by non-nitroaromatic functional groups

In addition to 2-nitroazole radiosensitizers, new drugs and imaging tools for application to hypoxia have been developed with various approaches. Brown and coworkers reported on the design of a benzotriazine-*N*-oxide, tirapazamine (TPZ: **24**), as a hypoxic cytotoxin.[46] TPZ **24** suppresses neurotoxicity because of the absence of a nitro group in the chemical structure. To account for expression of its cytotoxicity, various activation mechanisms were proposed.[46d,46e] One plausible mechanism is shown in Fig. 7. TPZ is activated to generate radical anions through bioreduction under hypoxic conditions, followed by protonation to produce an active neutral radical intermediate. The resulting radicals abstract hydrogen atoms from intracellular DNA, leading to a potent cytotoxicity. A recent study revealed that TPZ **24** also induces topoisomerase II poisoning, resulting in DNA double strand breaks.[47] In contrast to the activation under hypoxic conditions, anion radical intermediates generated by bioreduction of TPZ exhibit reducing reactivity toward molecular oxygen to form original TPZ and $O_2^{-\bullet}$ under aerobic conditions, leading to a suppression of the net reaction. Thus, TPZ exhibits the cytotoxicity in a hypoxia-selective manner, *i.e.* 50–300 times more potent cytotoxicity toward hypoxic cells relative to aerobic cells. Consequently, TPZ has been recognized as a leading hypoxic cytotoxin and its clinical trial is under way.

Indolequinone derivatives are other representative cytotoxins that target hypoxia or exploit the over-expression of reducing enzymes in tumors.[48–51] These functional molecules are activated by intracellular enzymes that reduce quinone compounds. Among the reducing enzymes, NADPH:cytochrome P450 reductase and b5 reductase induce one-electron reduction of quinones to form the semiquinone radical anion ($Q^{-\bullet}$), whereas NQO1 (DT-diaphorase) results in two-electron reduction *via* hydride transfer to form the hydroquinone.[49] The one-electron reducing enzymes activate quinone derivatives in a hypoxia-selective manner, thus protecting normal aerobic tissues. In contrast, oxygen-independent activation of quinones occurs by treatment of two-electron reducing enzymes. Among the indolequinone derivatives, EO9 (**25**) possessing an aziridine ring has been developed as a bioreductive alkylating

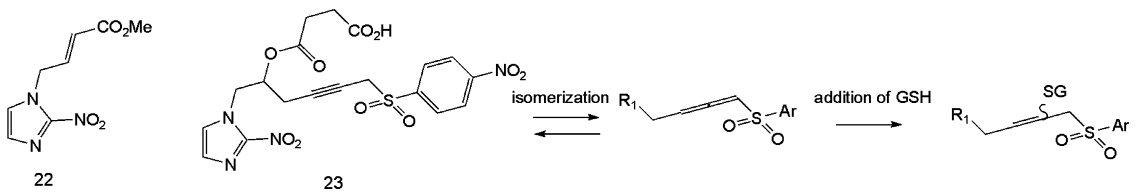


Fig. 6 Structures of radiosensitizers possessing GSH depletion ability and plausible reaction mechanism.

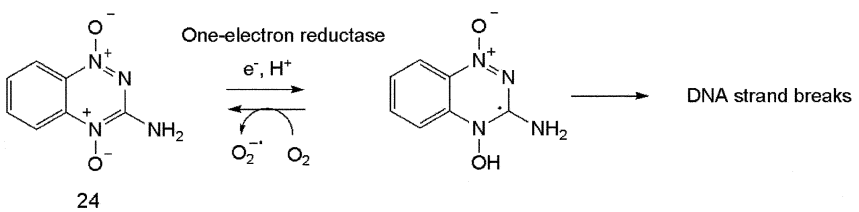


Fig. 7 Structure of tirapazamine (TPZ) and the mechanism for expressing cytotoxicity in hypoxia.

agent related to the naturally occurring antitumor agent mitomycin C (MMC) for the treatment of tumors.[50] The reducing enzyme NQO1 overexpressed in tumor cells or NADPH:cytochrome P450 transforms EO9 to an electrophilic intermediate in several tumor cells, causing cytotoxicity by alkylation.

Indolequinone derivatives have been identified as the effective eliminating substituents that work through bioreduction and radiolytic reduction, which encourage their applications to prodrug development. Such reductive activation of prodrugs with indolequinone derivatives to release the drugs accompanies the concomitant formation of electrophilic iminium cations, which potentially involve DNA alkylation or other mechanisms of cellular damage. In view of these reaction characteristics, prodrugs possessing an indolequinone structural unit may result in a synergic cytotoxicity that is attributable to both the original drug and the electrophilic iminium species released upon reductive activation.

Naylor and coworkers recently reported on an aspirin prodrug possessing an indolequinone structure (**26**).[48e] They demonstrated efficient reductive release of aspirin from the prodrug **26** and noted that the spatial position of the drug on the indolequinone structure seriously affects the drug release efficiency. Threadgill and coworkers reported reductive release of isoquinolin-1-one, a potent inhibitor of poly(ADP-ribose) polymerase (PARP), from prodrugs bearing indolequinone[51a] and nitro heteroaromatic groups.[51b] In addition, various phantom drugs were incorporated into indolequinones and details of their activation mechanisms were investigated.

More recently, we designed a prodrug of camptothecin (CPT) that is a potent inhibitor of DNA topoisomerase (topo I).[52] CPT stabilizes the covalent binding of topo I to DNA, which leads to irreversible and lethal strand breaks of DNA during its replication. Thus, CPT shows high antitumor activity, although the clinical application to cancer treatment is limited because of unfavorable properties such as non-specific toxicity. We attempted to develop a prodrug of CPT by conjugation of an indolequinone unit with CPT through an *N*,*N*′-dimethyl-1-aminoethylcarbamate linker to obtain prodrug IQ-CPT (**27**) (Fig. 8). IQ-CPT had lower cytotoxicity than its parent compound CPT, whereas IQ-CPT showed higher hypoxia-selective cytotoxicity toward HT-29 tumor cells than did CPT, as a result of releasing the original CPT in a hypoxia-selective manner.

These reaction characteristics of indolequinone derivatives prompted us to propose a hypoxia imaging by molecular probe comprising a reducing indolequinone structure. We designed an indolequinone derivative conjugated with a fluorescent coumarin (IQ-Cou: **28**) (Fig. 9). Two coumarin chromophores are conjugated with an indolequinone unit by a 2,6-bis-(hydroxymethyl)-*p*-cresole linker to produce IQ-Cou. The indolequinone unit of IQ-Cou undergoes one-electron reduction to liberate three functional components, in which spontaneous cyclization of a free amine intermediate occurs to generate phenol derivatives followed by

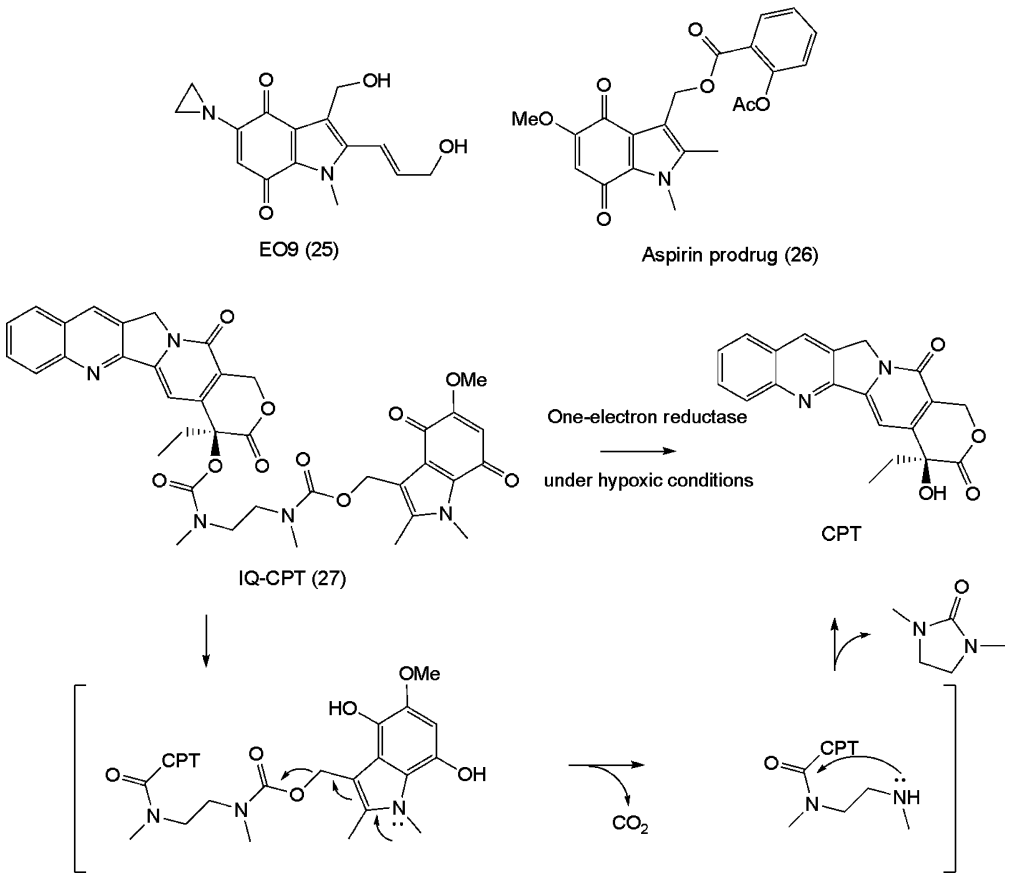


Fig. 8 Structures of prodrugs possessing an indolequinone skeleton and the mechanism for drug release upon one-electron reduction in hypoxia.

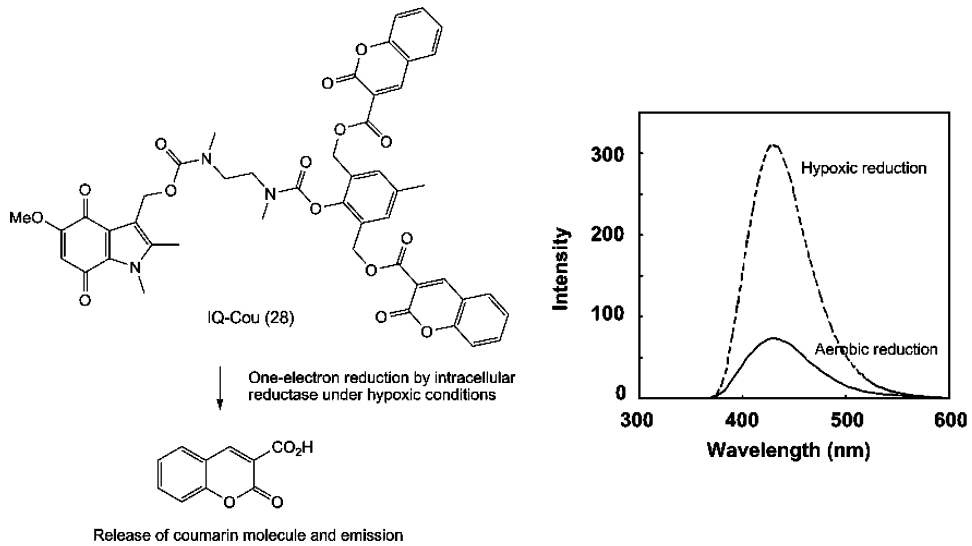


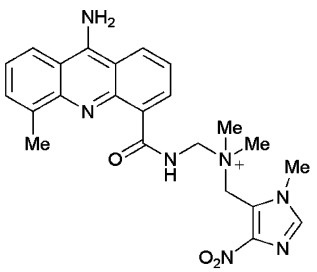
Fig. 9 Structure of indolequinone-coumarin conjugate (IQ-Cou) as a hypoxia imaging probe and fluorescence spectra of IQ-Cou upon treatment by reductase under hypoxic or aerobic conditions.

subsequent 1,4-quinonemethide rearrangement. IQ-Cou itself is non-fluorescent because the fluorescent excited singlet state of the coumarin unit is quenched efficiently by the indolequinone unit located in close intramolecular proximity. Upon one-electron reduction of the indolequinone unit, the coumarin chromophore is eliminated freely from the fluorescence quenching action of the indolequinone unit, which produces intense fluorescence. We also confirmed that IQ-Cou shows intense fluorescence in hypoxia upon incubation with the cell lysate of the human fibrosarcoma HT-1080 cells. Thus, IQ-Cou has unique properties that are favorable as a fluorescent probe for hypoxia-specific cellular imaging.

3.3 Hypoxia targeting radiation-activated antitumor prodrugs

As described above, solid tumor tissue contains hypoxic cellular areas of extremely low oxygen concentration that are resistant to conventional therapies including radiotherapy.[53] Production of reactive oxygen species by ionizing radiation is remarkably diminished in hypoxic areas, thus decreasing the efficacy of radiotherapy. A representative strategy for radiation-activated prodrugs is to use radiation-induced fragmentation of nontoxic or less-toxic prodrugs to release cytotoxic drugs. Radiolytic reduction-activated prodrugs comprise a cytotoxin and an electron-affinity moiety, which undergo reduction to trigger fragmentation by hydrated electrons (e_{aq}^-) generated upon radiolysis of water under hypoxic conditions. In contrast, in normal cells, oxygen molecules scavenge the reducing species of e_{aq}^- at a near diffusion-controlled rate and also reoxidize the one-electron reduced intermediates of the prodrugs. Because hypoxia is virtually unique to tumor cells[54] and the release of cytotoxin occurs only in hypoxic cellular regions within the radiation field, radiotherapy using this type of antitumor prodrug may have high efficiency, good selectivity, and adequately diminished side effects.

A family of compounds considered as radiation-activated prodrug candidates is nitro(hetero)cyclic methyl quaternary ammonium (NMQ) salts,[55] such as *N*,*N*-bis(2-chloroethyl)-*N*-methyl-*N*-[(1-methyl-4-nitro-5-imidazoyl)methyl]ammonium chloride (4-NIQ-HN2, **29**) and *N*,*N*-bis(2-chloroethyl)-*N*-methyl-*N*-[(1-methyl-5-nitro-1-pyrrolyl)methyl]ammonium chloride (5-NPQ-HN2). These were developed originally as bioreductive prodrugs[56] and were later shown to be reducible with one-electron stoichiometry by radiolysis to produce a DNA alkylating agent through a benzyl-type radical (Scheme 1a).[57] This family of prodrugs have potential features such as hypoxia-selective cytotoxicity, deactivation of nitrogen mustards, and high water solubility. To improve the modest cytotoxicity of mechlorethamine (HN2), a potent DNA alkylator of aminoacridine carboxamide (AMAC) was incorporated into NMQ compounds (4-NIQ-AMAC, **30**) (Fig. 10).[58] Irradiation of the prodrugs in anoxic buffer or culture medium released AMAC, although the yield of AMAC was lower



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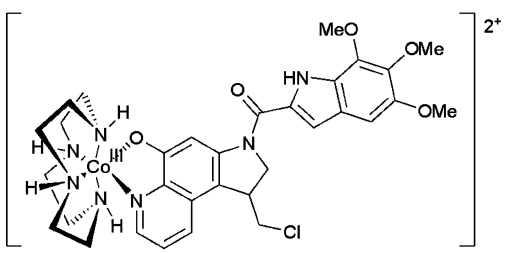

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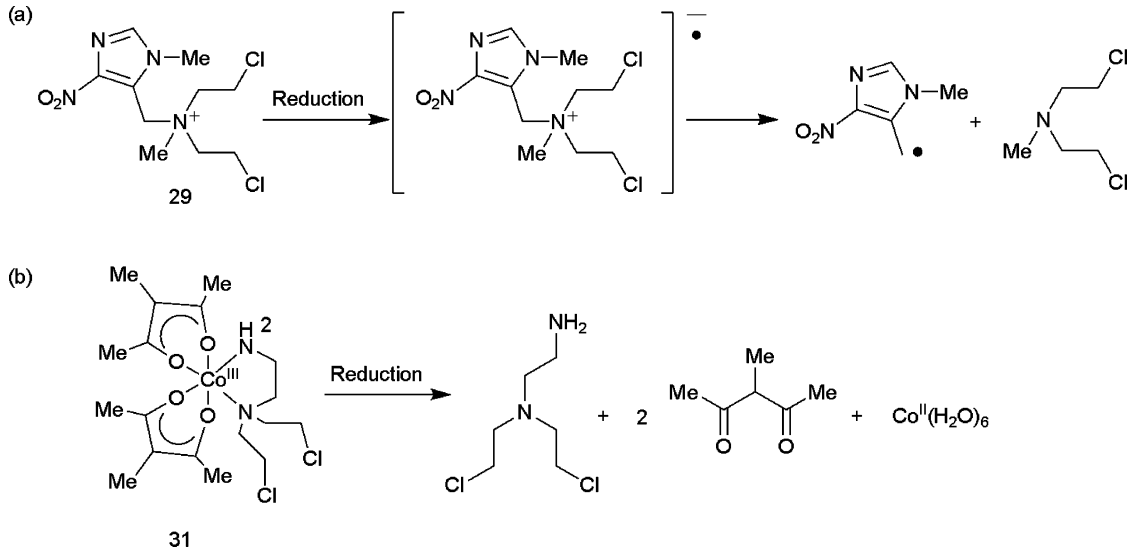
Fig. 10 Structures of radiation-activated antitumor prodrugs.



Scheme 1 Radiolytic reduction induced release of antitumor drugs.

than that of the HN2 analogs.[57a] The prodrugs also produced unpredictable toxicity *in vivo*, suggesting that nitrogen mustard may be released non-specifically,[56,57a] which has restricted further development of these compounds as radiation-activated prodrugs.

A second example is the cobalt(III) transition metal complexes, which were also investigated initially as bioreductive prodrugs to provide an inert framework for transportation of cytotoxins that allow cellular uptake and a cycle of reduction and reoxidation. The hypoxic environment of tumor cells prevents reoxidation and the reduced product of the high-spin Co(II) complex is much less stable than its predecessor, the d^6 low-spin octahedral Co(III) complex, therefore, it releases the coordinated ligand.[59] The bidentate mustard complex SN 24771 (**31**), in which the auxiliary coordination positions are occupied by acetylacetonato,[60] is activated *via* one-electron reduction by e_{aq}^- to release nitrogen mustard (Scheme 1b),[61] but is too unstable for *in vivo* use. A new series of Co(III) complexes comprising *N*-donor polyazamacrocyclic auxiliary ligands plus a bidentate cytotoxic effector ligand has been prepared to vary the reduction potential of the Co(III) metal center.[62] For example, SN 27892 (**32**) is a prodrug bearing tetradentate ligand, 1,4,7,10-tetraazacyclononane (cyclen), and a synthetic analog of the DNA minor groove alkylator duocarmycin SA. Under hypoxia, SN 27892 shows an efficient release of the cytotoxin with a clinically relevant radiation dose of 2 Gy and metabolic stability in mice, but no antitumor activity in RIF-1-bearing mice was observed when given before or after the irradiation.[63]

In 1992, we showed the first concept for radiation-activated prodrugs designed to release the antitumor agent 5FU by radiolytic one-electron reduction.[64] This concept arises from the dimerization of 5FU by electrochemical oxidation in an anoxic solution and the reverse reactivity of the product dimer to regenerate 5FU upon γ-irradiation of an oxygen-free aqueous solution. Hydrolysis of the dimer into 5FU did not occur at pH < 8.0, indicating a potential use of this dimer as a prodrug that can be activated in the radiotherapy of hypoxic tumors. 1-(5′-Fluoro-6′-hydroxy-5′,6′-dihydrouracil-5′-yl)-5-fluorouracil (**33**) was prepared in 70% yield by electrolytic oxidation of an Ar-purged aqueous solution of 5FU.[65] The initial step of the dimerization is the anodic one-electron oxidation of 5FU into the corresponding radical cation, followed by successive deprotonation at N1 to form the allyl-type radical which then undergoes a head-to-tail coupling. Radiolytic reduction of the dimer hydrate **33** by e_{aq}^- regenerates 5FU, along with 1-(uracil-5′-yl)-5-fluorouracil (**34**).[66] The pulse radiolysis study demonstrated a one-electron reduction mechanism by which a radical anion in the form of an electron-adduct at the 5FU moiety is generated as the initial intermediate during radiolytic reduction, followed by F^- elimination from the radical anion and hydrolytic splitting of N1–C5′ linkages to regenerate 5FU.[66] Further one-electron reduction can occur competitively from the radical [**33**(**-F**)•] into a byproduct **34**. The reduction mechanism of pyrimidine dimer has provided a novel strategy of radiation-activated 5FU prodrugs for the treatment of malignant hypoxic solid tumors (Scheme 2).

To achieve higher efficacy of radiation-activated prodrugs, our group developed a series of 5-fluoro-1-(2′-oxocycloalkyl)uracils (Fig. 11).[67] The compounds **35–43** bearing a 2′-oxo group were one-electron reduced by e_{aq}^- and released 5FU in 47–96% yields, whereas compounds **44** and **45** without the 2-oxo substituent had no activity toward the reductive C1′–N1 bond splitting. A similar mechanism was proposed for prodrug activation that e_{aq}^- produced by hypoxic irradiation is incorporated into the C5–C6 double bond of 5FU to form the π* radical anion, which is thermally activated to the σ* radical anion, followed by hydrolytic

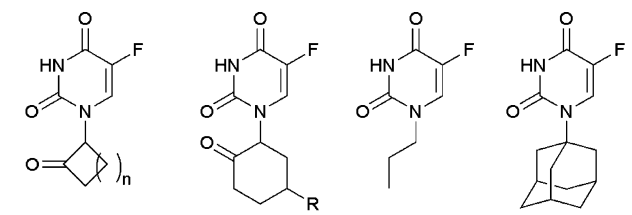
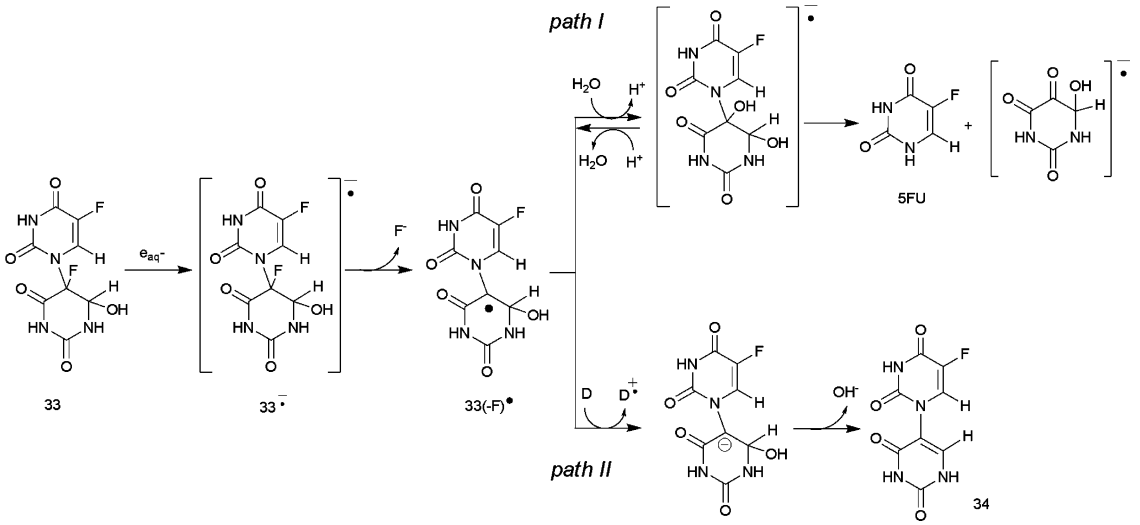


Fig. 11 Structures of N1-substituted 5-fluorouracil derivatives.



Scheme 2 Mechanism of reductive splitting of 5FU dimer hydrate **33**.

cleavage of the C1′–N1 bond and release of 5FU. Detailed studies on the quantitative structure–activity relationship using X-ray crystallography and molecular-orbital (MO) calculations suggest several important features of the prodrugs leading to effective release of 5FU: the 2′-oxo group provides ($\pi^* + \sigma^*$) LUMO + 1 by mixing of the π^* orbital of the 2-oxo substituent and the σ^* orbital of the adjacent C1′–N1 bond. The relatively small energy gap between LUMO and LUMO + 1 promotes intramolecular electron transfer from LUMO as localized at the C5–C6 pyrimidine double bond to LUMO + 1. Structural flexibility allows the dynamic conformational change to achieve a higher degree of ($\pi^* + \sigma^*$) MO mixing. Five- and six-membered ring compounds **37**, **38** were among the best substrates for 5FU release, resulting in nearly quantitative yields (96% and 93%, respectively). It is likely that the two compounds **37**, **38** have a moderately flexible structure that would be suitable for gaining maximum overlap between the π^* and σ^* orbitals. We evaluated the *in vitro* and *in vivo* activity of 5-fluoro-1-(2′-oxopropyl)uracil (**35**) as a prototype compound of radiation-activating 5FU prodrugs.[68] Upon hypoxic irradiation, **35** showed a significant cell-killing effect towards murine SCCVII tumor cells, and the degree of cytotoxic effect was consistent with authentic 5FU. In contrast, cytotoxicity was negligible in nonirradiated cells or in cells treated aerobically. Although a pharmacokinetic study showed that **35** was converted into 5FU *in vivo* as well as *in vitro*, growth delay assays using SCCVII tumor-bearing mice increased the tumor growth time only slightly.

We have explored an alternate design for radiation-activated prodrugs **46–50** containing 5-fluorodeoxyuridine (5FdUrd) as a more potent antitumor agent (Fig. 12).[69] The synthesized compounds with a 2-oxoalkyl group at their N3 position released 5FdUrd in 49–78% yields upon hypoxic irradiation in a manner similar to that of 5FU-releasing prodrugs. As investigated with laser flash photolysis, a 5FdUrd derivative **49**, forming a radical anion state 70 times more stable than the other compound **46**, was more efficient in releasing 5FdUrd. This clearly demonstrates that prodrugs generating stable radical anions are more favorable for 5FdUrd release. Although this class of prodrugs is activated *in vitro* as efficiently as the prodrugs of 5FU, clear *in vivo* effects were not detected.[70] The 5FU and 5FdUrd prodrugs have failed

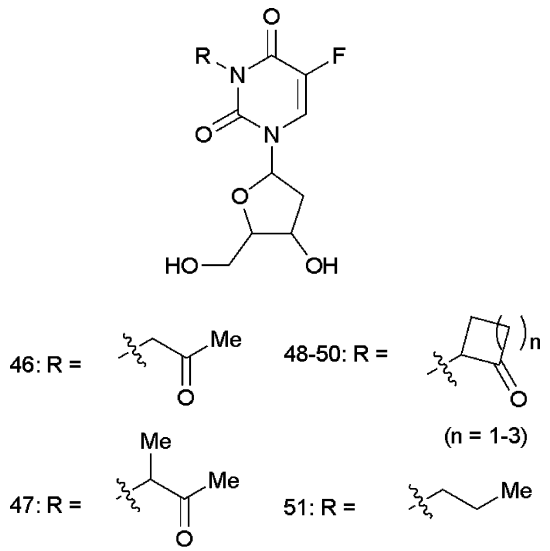
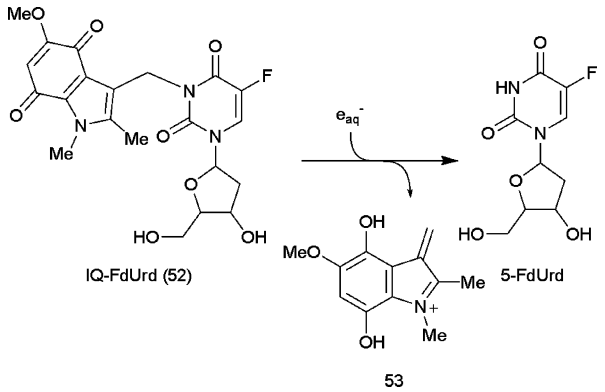


Fig. 12 Structures of N3-substituted 5-fluoro-2′-deoxyuridines.

to give the sufficient antitumor activity *in vivo*, but the strategy is still promising for targeting hypoxic cancer cells. Incorporation of more potent anticancer agents instead of 5FU and its derivatives into prodrugs may provide agents with greater *in vivo* effects that should be potent enough for clinical application.

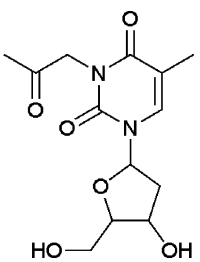
We have recently developed another type of prodrug comprising 5FdUrd plus an indolequinone moiety at the N3 position (IQ-FdUrd, **52**).[71] Hypoxic irradiation caused IQ-FdUrd to undergo one-electron reduction and release 5FdUrd as the sole product in a dose-dependent manner. Mechanistic studies on the fragmentation of IQ-FdUrd using laser flash photolysis suggest that the corresponding semiquinone radical anion decays predominantly by bimolecular disproportionation to generate the iminium cation (**53**) along with 5FdUrd (Scheme 3).[72] IQ-FdUrd **52** showed enhanced cytotoxicity upon hypoxic irradiation, as evaluated in the radiation-resistant EMT6/KU murine tumor cell line. Of most interest is that the estimated concentration of released 5FdUrd based on the *G* value for the 5FdUrd formation was much less than



Scheme 3 Radiolytic reduction of IQ-FdUrd to release 5FdUrd.

that expected from the IC_{50} value of 5FdUrd toward EMT6/KU cells. The results show that this type of prodrug has synergic cytotoxicity, which we envisage as arising from the strong cytotoxic effect of electrophilic iminium cations.

We also proposed 2-oxoalkyl caged oligodeoxynucleotides (ODNs) that can be activated through the removal of 2-oxoalkyl group upon hypoxic irradiation.[73] Several caging mechanisms[74] by which the recognition properties of nucleobases are temporarily blocked to control transcription upon light irradiation have been proposed recently.[75] Our new caged ODNs with a 2-oxopropyl group at the N3 position of thymidine ($d^{oxo}T$, **54**) are designed for binding to the nucleic acids or proteins to be modulated by irradiation with ionizing radiation (Fig. 13). Radiolytic reduction of the caged ODNs in an aqueous solution gives the corresponding uncaged ODNs preferentially under hypoxic conditions without producing detectable amounts of any other decomposed products. Recovery of the hybridization property of the caged ODNs after irradiation was confirmed by enzymatic digestion assay. Caged 18-mer ODNs (**55**) that have $d^{oxo}T$ in the middle of a *Swa I* recognition site were pre-irradiated and then incubated in the presence of ODN **56**, which is complementary to the uncaged analogue of ODN **55**. The enzymatic cleavage occurred in the ODN **55** irradiated in hypoxia but not for aerobically irradiated ODN, suggesting that the hybridization property of ODN containing 2-oxopropyl-modified thymidine can be controlled by hypoxic irradiation. This class of caged ODNs appears to be a promising strategy for a new approach to the temporal or spatial control of gene expression, in which RNA is inactivated by a radiation-removable 2-oxopropyl group and inversely reactivated by hypoxic irradiation, as well as for development of radiation-regulated caged-antisense oligonucleotides.



$d^{oxo}T$ (54)

18-mer ODN (55) 5'-d(TTTTT *ATT*^{oxo}*TAAATTTTTT*)-3'

18-mer ODN (56) 5'-d(AAAAAA*TTTAAATAAAAA*)-3'

Fig. 13 Caged ODN and the complementary sequence of the corresponding uncaged ODN.

4 Conclusion

Intelligent drugs and imaging systems with selective action in tumor tissues have widespread potential application to the treatment and diagnosis of cancer. In this perspective, we reviewed recent advances in the development of drugs and imaging tools that target tumor angiogenesis and hypoxia. The concepts are well established and have been demonstrated in numerous model systems.

The foremost task in the design of drugs is further suppression of unfavorable properties, such as non-specific toxicity against normal tissues by refining the target specificity. To overcome the problems of nonspecificity, a combination of current systems with other nanotechnologies, such as drug delivery systems (DDS) using nanoparticles and nanocarriers, may be effective. Because nano-scale molecules are hyperpermeable into tumor microvessels, these DDS methods may improve the targeting delivery of drugs or prodrugs and their selective accumulation into tumor tissues.

With regard to imaging systems of tumors, the use of NIR light for imaging is one key strategy for *in vivo* optical imaging because deep-seated malignant tissues cannot be imaged by probes with shorter emission wavelengths. Optical imaging may break the conventional depth limitation, and the effective depth is expected to reach more than 10 cm with the use of NIR probes. The development of noninvasive molecular imaging technologies may produce a revolution in early detection diagnosis and personalized therapy of cancer.

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