Review

Imaging beyond the diagnosis: image-guided enzyme/prodrug cancer therapy

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The ideal therapy would target cancer cells while sparing normal tissue. However, in most conventional chemotherapies normal cells are damaged together with cancer cells resulting in the unfortunate side effects. The principle underlying enzyme/prodrug therapy is that a prodrug-activating enzyme is delivered or expressed in tumor tissue following which a non-toxic prodrug is administered systematically. Non-invasive imaging modalities can fill an important niche in guiding prodrug administration when the enzyme concentration is detected to be high in the tumor tissue but low in the normal tissue. Therefore, high therapeutic efficacy with minimized toxic effect can be anticipated. This review introduces the latest developments of molecular imaging in enzyme/prodrug cancer therapies. We focus on the application of imaging modalities including magnetic resonance imaging, position emission tomography and optical imaging in monitoring the enzyme delivery/expression, guiding the prodrug administration and evaluating the real-time therapeutic response in vivo.

Keywords  molecular imaging; cancer; enzyme; prodrug; therapy

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Introduction

The success of chemotherapy in the clinic is limited by insufficient drug concentration in tumors, lack of targeting specificity, systemic toxicity, and the evolution of drug resistant in cancer cells [1,2]. Several strategies have been developed to minimize the systemic toxicity while maintaining the therapeutic efficacy. One of the most promising is the enzyme/prodrug therapy [3] in which a prodrug-activating enzyme is delivered or expressed in cancer cells following which a non-toxic prodrug is administered systemically. The enzyme pre-located in tumor converts the prodrug into an active anticancer drug, achieving high concentrations in the tumor while the normal tissue can spare of the toxicity due to the lack of the enzyme. For such a strategy to work clinically there are certain requirements. First, the enzyme should be non-human or expressed at very low concentrations in the normal tissue and should have high enzymatic activity. Second, the prodrug should be a good substrate for the enzyme but should not be activated in non-tumor tissue. Third, the prodrug should be non-toxic, but the activated drug should be highly toxic and can be actively taken up by adjacent cells for a ‘bystander cell kill’ effect, and ideally should not leak out into the systemic circulation.

Generally, there are three major categories of enzyme/prodrug strategies: (i) gene-directed enzyme/prodrug therapy (GDEPT) in which the genes encoded prodrug-activating enzymes is delivered into tumor tissue firstly. After the expression of the therapeutic enzyme, the prodrug is injected [4,5]; (ii) targeting group-directed enzyme/prodrug therapy (TDEPT) in which the prodrug-activating enzyme labeled with positive tumor-targeting domain such as antibody, peptide or small molecular ligand is delivered into tumor tissue followed the prodrug administration [6,7]; (iii) vasculature permeability-dependent enzyme/prodrug therapy (VPDEPT) in which the intratumoral delivery of the enzyme is realized through the higher permeability of tumor vasculatures compared with the normal vasculatures as well as the prolonged circulation lifetime of the macromolecular enzyme [enhanced permeability and retention (EPR) effect] [8,9].

In the strategies of TDEPT and VPDEPT, the non-toxic prodrug will be converted into toxic drug by the therapeutic enzyme prelocated in the tumor, but the normal tissues can spare of the toxicity due to the lacking of the enzyme. However, conversion of the prodrug by residual enzyme in normal tissues may lead to toxicity if the prodrug is injected too early, meanwhile the active drug concentration may be too low to kill the cancer cells if the prodrug is injected too late due to the low concentration of active enzyme resulting from the clearance and intratumoral proteolysis. Therefore, determining the optimal time window for prodrug injection is of utmost importance for the success of TDEPT and VPDEPT. Traditionally, the
optimum time window for prodrug injection is determined by measuring the *ex vivo* enzymatic activity ratio between the tumor and normal tissues (T/N ratio) obtained at selected time points after the enzyme injection [10]. However, the time with the highest enzymatic T/N ratio is difficult to be pinpointed with a few selected time points. Given the variable and heterogeneous nature of tumor vasculature [11], it is also difficult to generalize the time course of enzyme delivery and clearance. Furthermore, the determination of the *ex vivo* enzymatic activity in different organs at different time points is highly time and labor-cost, and the *ex vivo* results usually cannot truly represent the *in vivo* situation because the enzyme activity will be lost in experimental processing such as tissue homogenizing and extraction. Therefore, non-invasively imaging the delivery of a prodrug-activating enzyme and quantifying the biodistribution of enzyme in selected organs *in vivo* would be ideal to time the prodrug administration.

In the GDEPT strategy, the gene-encoded non-human prodrug-activating enzyme is delivered to the tumor followed the administration of a non-toxic prodrug. The outcome is that the prodrug is converted into toxic drug in tumor tissues transfected with the suicide gene and kills the cancer cells, while the normal tissue can spare of the toxicity due to the lack of the gene expression. However, the successes of gene-directed cancer therapy are hindered by the poor capacity of gene delivery, the inability to homogeneously transfrect every tumor cell, and the lack of tumor-specific gene transduction [12,13]. The ability to non-invasively monitor the location, magnitude, and duration of the therapeutic gene precisely would significantly increase the therapeutic efficacy and minimize the systemic toxicity of GDEPT by injecting the prodrug when the suicide gene expression ratio between tumor and normal tissues reaches its maximal value.

Besides timing prodrug delivery, the non-invasive molecular imaging technologies also provide the opportunities to real-timely monitor the therapeutic response during the treatment. Traditionally, the therapeutic efficacy of cancer treatments is determined by recording the tumor volumetric variation. However, this value is a morphological and lagging behind parameter that will not provide the physiological responses with high temporal and spatial resolution during the treatment. The non-invasive imaging modalities can fill an important niche here to real-timely track the therapy responses at the cellular/molecular levels, which is helpful to adjust the therapeutic protocol timely to achieve the optimal treatment efficacy and minimized side effects.

In this review, we present the recent developments in the image-guided enzyme/prodrug strategies, in which the enzyme delivery/expression can be non-invasively monitored by magnetic resonance imaging (MRI) and positron emission tomography (PET). Moreover, the latest progresses in the non-invasive image-guided therapeutic efficacy evaluation are also discussed.

**MR and optical imaging-guided enzyme/prodrug cancer strategy**

With its non-invasive characteristics and exquisite spatial resolution, MRI is one of the most powerful imaging techniques available in diagnostic imaging [14], and preclinical results can be easily translated to the clinic. However, MRI suffers from less than optimum sensitivity of detection [15,16]. Optical imaging, on the other hand, is highly sensitive and capable of detecting minute amounts of light-emitting materials in heterogeneous mediums, but its image resolution is poor because of the intrinsic absorption and light scattering of heterogeneous tissues, and it is not easily translated to the clinic [17]. Notably, the advantage of MRI (high spatial resolution) and optical imaging (high sensitivity) can compensate the shortages of the counterparts [18], and the multimodal MR and optical imaging are widely used in the diagnosis of tumor [19,20], atherosclerosis [20,21], inflammatory arthritis [22] as well as the stem cell tracking [23].

In the pioneering work of Bhujwalla et al., a prodrug-activating enzyme cytosine deaminase (CD) was labeled with the L6 antibody to target human lung adenocarcinoma H2981 tumor xenograft. This work demonstrated the feasibility of non-invasively tracking the conversion of the non-toxic prodrug 5-fluorocytosine (5-FC) to active anti-cancer drug 5-fluorouracil (5-FU) *in vivo* using the fluorine magnetic resonance spectroscopy (*19F MRS*) and fluorine magnetic resonance spectroscopic imaging (*19F MRSI*) [24]. However, due to the low sensitivity of *19F MR*, it is unable to image the distribution of the antibody conjugated therapeutic enzyme in tumor and normal tissues. To advance the image-guided enzyme/prodrug strategy, Li et al. reported a multimodal image-guided strategy in which the prodrug-activating enzyme bacterial bCD was covalently conjugated with an imaging reporter carrier polyl-lysine (PLL) that was functionalized with T₁-weighted MR contrast agent Gd³⁺-DOTA and fluorophore rhodamine. The resulting bCD-PLL conjugate (Fig. 1) demonstrated low cytotoxicity, fast cellular uptake rate and high enzymatic stability in human breast cancer cell cultures [8,9]. *In vivo* T₁-weighted MR imaging [Fig. 2(A)] and T₁ map [Fig. 2(B)] showed that bCD-PLL extravasated into the tumor interstitium as soon as 10-min post-administration after intravenous (i.v.) injection and significant MR contrast in the tumor area was apparent up to 24 h. *In vivo* and *ex vivo* optical imaging [Fig. 2(C, D)] verified the intratumor delivery of bCD-PLL and the fluorescence ratio between tumor and muscle (T/N ratio) attained its maximum value at 24 h post-injection [9].
The accumulation of bCD-PLL in tumor can be explained by the EPR effect due to its average hydrodynamic size of 12 nm, which is a good size to penetrate the tumor vasculature but not the normal vasculature [25]. The conversion of prodrug to active drug at the tumor site was dynamically monitored by \textit{in vivo} 19F MRS (Fig. 3), which clearly demonstrated the intratumoral conversion of 5-FC to 5-FU at 24 h post-injection of 5-FC [9]. Importantly the image-guided enzyme/prodrug cancer strategy demonstrated satisfactory therapeutic effect and tolerable systemic toxicity in small animal model. When single dose of prodrug was administrated at 24 h post-injection of therapeutic enzyme, the time point with the maximum T/N ratio of enzyme, the average tumor volume doubling time increased from 7 to 14 days. Furthermore, \textit{ex vivo} histological studies clarified that a large piece of necrotic area was demonstrated in tumor section [Fig. 4(A)] [9], while no obvious toxic effect was shown in main organs, including liver and kidney at 8 days after the treatment.

The multimodal image-guided enzyme/prodrug strategy demonstrated satisfactory therapeutic effect and minimized systemic toxicity. Notably, in the prototype of bCD-PLL, the bCD might be replaced by any prodrug-activating enzyme, meanwhile the imaging reporter functionalized in PLL can be replaced by radioactive PET or SPECT tracers to further improve the imaging sensitivity. Additionally, positive tumor-targeting moiety such as small molecular ligand or peptide can be modified into PLL to target tumor subpopulations. Furthermore, the strategy can be extended to image-guided targeting of radiation or chemotherapy repair enzymes in combination with prodrug enzyme treatment to further increase the therapeutic efficacy.

**PET-guided gene-directed enzyme/prodrug cancer strategy**

Lack of tumor-targeting specificity and the resistance to treatment are the main shortages of the conventional chemotherapy and irradiation therapy. One approach to overcome these problems could be GDEPT in which the transgenes-encoded therapeutic enzymes are specifically delivered to the tumor tissues following the administration of the non-toxic prodrugs. In the tumor the non-toxic prodrugs are converted into toxic metabolites by the expressed enzyme [26]. With the advanced characteristics of GDEPT,
challenges need to be addressed including dynamically monitoring the location, magnitude, and duration of the suicide gene expression as well as real-timely evaluating the therapeutic response in tumor tissue. The establishment of imaging moiety to non-invasively track the gene transduction, time the prodrug administration, and evaluate the therapeutic efficacy is therefore the most effective way to address these challenges [27]. In various imaging modalities, position emission tomography (PET) demonstrates the high sensitivity, improved spatial resolution, and the ability to non-invasively quantify the metabolism of the radionuclide-labeled pharmaceuticals in vivo [28]. Recently, PET-guided GDEPT has achieved significant progresses in the preclinical and clinical studies [29,30]. In this review, we track the recent developments of the marker enzyme and the reporter probes used in the PET-guided enzyme/prodrug cancer strategy.

Generally, three different types of PET reporter gene (PRG)/reporter probe (PRP) systems for the in vivo imaging purpose have been developed including receptor-ligand system, ion-transporter-based system, and enzyme–substrate system. In the receptor-ligand systems, the most investigated is the dopamine D2 receptor (D2R) and its substrate 3-(2′-[18F]fluoroethyl) spiperone (FESP) [31]. Due to the high association affinity between D2R and FESP (IC50 = 1.5 nM), the expression of D2R gene can be monitored by imaging the radioactivity of FESP bound with the D2R. In the ion-transporter-based systems, the sodium/iodide symporter (NIS) is a membrane glycoprotein that uses the trans-membrane sodium gradient maintained by the sodium/potassium ATPase to co-transport iodine and sodium into thyroid cells [32,33]. After the systemic administration of the radioactive iodide, the accumulation of iodide by the expressed NIS protein can be visualized by PET.

In the most prominent enzyme–substrate system, herpes simplex type-1 thymidine kinase (HSV-tk) is used as the prodrug-activating enzyme and its substrate gancyclovir (GVC) is used as the prodrug [34]. In this GDEPT, the gene-encoded HSV-tk is firstly transduced to the tumor, then a non-toxic prodrug GVC is administered systemically and it is phosphorylated by the HSV1-TK protein to GVC mono-phosphate (GVCMP) in tumor. The GVCMP can be further metabolized by the endogenous enzymes to the GVC tri-phosphate that exerts its cytotoxic effects intracellularly both by inhibiting cell DNA polymerases and by competing with dGTP for incorporation into DNA during the S phase of the cell cycle. For the imaging purpose, the substrate GVC can be replaced by its radio-labeled derivatives such as [18F]fluoro-ganciclovir ([18F]FGCV). The monophosphates of these radio-labeled substrates cannot be further metabolized or penetrate the plasma membrane and hence ‘trapped’ into the cells. Therefore, the cellular retention of radioactivity is used as an indicator for the suicide gene expression [35] and the HSV1-tk-dominated enzyme-substrate system can be applied in both cancer therapeutic and diagnostic purposes.

The sensitivity of the tk-mediated GDEPT can be further increased by (i) designing TK-related enzyme with higher enzymatic activity to the currently available substrates, (ii) designing substrates that have lower affinity for the cytoplasmic mammalian TK and a higher affinity for the HSV1-tk families. HSV1-sr39tk gene was generated by semi-random mutagenesis of the HSV1-tk gene [36]. Compared to HSV1-tk, HSV1-sr39tk demonstrated increased enzymatic activity to the substrate [18F]GCV in...
C6 glioma cell cultures and tumor xenografts [37]. Notably, HSV1-sr39tk was less efficient in phosphorylating thymidine than HSV1-tk, which further increases the signal T/N ratio by reducing the accumulation of phosphorylated substrates in cells without expression of the suicide gene. Gambhir et al. [36] also demonstrated that C6 cells expressing HSV1-sr39tk accumulated approximately twice as much $[^{18}F]$fluoro-penciclovir ($[^{18}F]$FP-CV) as HSV1-tk-expressing cells, and microPET imaging demonstrated greater sensitivity with HSV1-sr39tk compared with HSV1-tk. Therefore, HSV1-sr39tk is a better reporter gene compared with HSV1-tk for the imaging purpose.

The radio-labeled substrates of HSV1-TK/HSV1-sr39TK system generally comprise the pyrimidine nucleosides and the acycloguanosine derivatives. The pyrimidine nucleoside analogs include 2′-deoxy-2′-$[^{18}F]$fluoro-5-iodo-1-β-D-arabinofuranosyluracil ($[^{18}F]$FIAU), 2′-$[^{18}F]$fluoro-5-ethyl-1-β-D-arabinofuranosyluracil ($[^{18}F]$FEAU) and 2′-deoxy-2′-$[^{18}F]$fluoro-5-methyl-1-β-D-arabinofuranosyluracil ($[^{18}F]$FMAU) (Table 1). The acycloguanosine analogs as the substrates for imaging gene expression include $[^{18}F]$fluoroganciclovir ($[^{18}F]$FGCV), $[^{18}F]$fluoro-penciclovir ($[^{18}F]$FP-CV), 9-[3-$[^{18}F]$fluoro-1-hydroxy-2-propoxy)methyl]guanine ($[^{18}F]$FHPG) and 9-[4-$[^{18}F]$fluoro-3-(hydroxymethyl)butyl]guanine ($[^{18}F]$FHBG) (Table 2). In various radio-labeled substrates (Table 3) used in clinic, $[^{18}F]$FHBG is a relatively poor substrate for mammalian tks, resulting in its desired low background accumulation in cells without expressing the HSV1-tk/HSV1-sr39tk genes. In addition, preclinical and clinical studies demonstrated that $[^{18}F]$FHBG has the desirable in vivo characteristics, including high stability, rapid blood clearance, low background signal, high bio-safety, and acceptable radiation dosimetry in animal models and human volunteers [38,39]. For above reasons, HSV1-sr39tk/$[^{18}F]$FHBG system has been widely used to track the location, magnitude, and duration of the reporter gene in the image-guided GDEPT.

Currently, the hurdles for successful application of gene therapy in clinic lie in the heterogeneity of tumor tissue, the limited transduction efficiency of current vectors, and

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<th>The chemical structures of pyrimidine nucleoside derivatives as the radioactive substrates of the PRG HSV-tk/HSV1-sr39tk</th>
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<td>Compound</td>
<td>$X$</td>
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<td>f1</td>
<td>2′-deoxy-2′-$[^{18}F]$fluoro-5-iodo-1-β-D-arabinofuranosyluracil, $[^{18}F]$FIAU</td>
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<th>The chemical structures of the acycloguanosine derivatives as the radioactive substrates of the PRG HSV-tk/HSV1-sr39tk</th>
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<th>Table 3</th>
<th>The application of the PET reporters used in the image-guided GDEPT</th>
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<td>$[^{18}F]$FLT</td>
<td>Endogenous tk-1</td>
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<tr>
<td>$[^{11}$C]MET</td>
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<td>$[^{18}F]$FDG</td>
<td>Endogenous hexokinases</td>
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the difficulties for the real-time evaluation of the therapeutic response [12,13]. To solve above problems, Jacobs et al. [40] developed a series of non-invasive imaging protocols that allow (i) a non-invasive determination of viable target tissue, which might benefit from gene treatment paradigm; (ii) the assessment of the transduced ‘tissue dose’ of a therapeutic gene expression in vivo; (iii) the evaluation of the gene-directed therapeutic response at the molecular or cellular levels after the prodrug injection. With the help of these imaging modalities, increasing therapeutic efficacy and minimizing systemic toxicity could be achieved by timing the prodrug injection when the suicide gene expression ratio between tumor and normal tissues reaches its maximal value. Importantly, the therapeutic responses can be real-timely evaluated through the assessment of the expression level of the exogenous HSV1-tk and endogenous tk as marker for viable and proliferating tumor cells. In this work, amplicon vectors cdIREStk39gfp containing two therapeutic genes including bacterial bcd and mutated HSV-1 tk (HSV1-sr39tk) fused to green fluorescent protein (GFP) were established. The bcd was used as a therapeutic gene and HSV1-tk39 was used as a PET marker as well as a therapeutic gene. The combination of bcd and HSV1-tk39 demonstrated the synergistic anti-tumor activity and GFP was used as the fluorescent marker to track the gene expression in cell culture. Before the gene delivery, the position, density, and volume of the viable glioma tumor was non-invasively assessed by the multitracer PET imaging including Figure 5 Imaging viable target tissue suitable for gene transduction before the gene-directed enzyme/prodrug strategy Whole-body PET from rats harboring human Gli36dEGFR glioma tumor xenografts. Acquisition of [18F]FDG-PET, [11C]MET-PET, and [18F]FLT-PET was done on subsequent days. Homogeneous radiotracer uptake was observed in the small tumors. Heterogeneous radiotracer uptake was observed in the medium- and large-sized tumors. The whole-body images also clearly depicted the excretion of FDG and FLT through kidney and bladder and MET by hepatic metabolization.

Figure 6 Identification of viable target tissue and assessment of vector-mediated gene expression in a mouse-bearing human glioma xenografts Row 1, the viable tumor tissue is displayed by [18F]FDG-PET; note the signs of necrosis in the lateral portion of the left-sided tumor (arrow). Rows 2 and 3, following vector application into the medial viable portion of the tumor (arrow), the tissue dose of vector-mediated gene expression is quantified by [18F]FHBG-PET. Row 2, an image acquired early after tracer injection, which is used for coregistration; Row 3, a late image is used for quantification of the therapeutic gene expression level.
2-18F-fluoro-2-deoxy-D-glucose ([18F]FDG), methyl-11C-l-methionine ([11C]MET), and 3'-deoxy-3'-18F-fluoro-l-thymidine ([18F]FLT) (Fig. 5) [29]. [18F]FDG and [18F]FLT image the metabolic level of glucose and the activity of tk-1, which indicates the cellular density [41] and the proliferation activity [42] of the viable cancer cells, respectively. Meanwhile the [11C]MET images the expression of the amino acid transporters that reflects the development of the tumor neovascularization [43].

After the in vivo gene delivery, the HSV1-sr39tk transduction location as well as its ratio between the tumor and normal tissues was dynamically determined by the [18F]FHBG mediated PET imaging (Fig. 6) [29]. Mean cdIREStk39gfp expression was measured as 0.37 ± 0.3% ID/g in the in vivo transduced tumors and 1.22 ± 0.83% ID/g for stably transfected positive control tumors at 48 h after the gene delivery [40]. The therapeutic efficacy was evaluated by the assessment of the expression level of the exogenous HSV1-sr39tk by [18F]FHBG as well as by quantifying the difference of [18F]FLT accumulation (in % ID/g) before and after therapy (Fig. 7) [29]. Significant differences in [18F]FLT accumulation before (3.38 ± 3.65% ID/g) and after therapy (0.06 ± 0.19% ID/g) were obtained in stable cdIREStk39gfp-expressing positive control tumor. Meanwhile, for the in vivo transduced tumors, the [18F]FLT accumulation reduced from 1.91 ± 1.12% ID/g before the treatment to 0.42 ± 1.31% ID/g. Standard volumetric assay further verified the therapeutic efficacy. The human Gli36dEGFR glioma xenografts transduced in vivo with HSV1-cdIREStk39gfp gene vector followed the combined prodrug therapy with ganciclovir and 5-FC showed significant growth delay (68% of the tumors) and even complete disappearance (18% of the tumors) compared with the negative control.

The development of image-guided GDEPT demonstrates the feasibility to non-invasively track the in vivo gene-transduction status, time the prodrug administration, and evaluate the therapeutic efficacy. Importantly, the multi-tracer-mediated PET imaging shows the promise to avoid the therapy in vain by determining the ineffective gene transduction and low tissue dose of the therapeutic gene before developing the treatment plan. The imaging protocols therefore will significantly facilitate the translation of the GDEPT in clinic with high safety and reliability.

**Conclusion and perspectives**

Image-guided enzyme/prodrug cancer therapy is a newly developing technology, and its benefits including non-invasively monitoring the delivery/expression of the therapeutic protein in vivo, minimizing the systemic toxicity by timing the prodrug injection as well as evaluating the real-time therapeutic response at the molecular/cellular level make it very promising to be translated into clinical research. The future developments of the image-guided enzyme/prodrug cancer therapy may focus on humanizing the therapeutic enzymes to reduce their immunogenicity, labeling the gene vector with imaging contrast media to non-invasively visualize the gene vector distribution in vivo, and developing imaging PRPs with high affinity to the exogenous suicide enzyme but with low affinity to the endogenous protein, which will facilitate the development of safe and efficient enzyme/prodrug cancer strategy for clinical applications.

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