**In vivo** quantifying molecular specificity of Cy5.5-labeled cyclic 9-mer peptide probe with dynamic fluorescence imaging

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**Abstract:** We quantified molecular specificity of Cy5.5-GX1 **in vivo** with dynamic fluorescence imaging to better understand its kinetic properties. According to whether or not free GX1 was injected and when it was injected, twelve of BGC-823 xenografted mice were randomly divided into three groups and underwent a 60 minute dynamic fluorescence scanning. Combined with a principal-component analysis, the binding potential (Bp) of the probe was determined by both Logan graphical analysis with reference tissue model (GARTM) and Lammertsma simplified reference tissue model (SRTM). The sum of the pharmacokinetic rate constants (SKRC) was quantified by the Gurfinkel exponential model (GEXPM). Cy5.5-GX1 specifically targeted tumor both **in vitro** and **in vivo**. We obtained similar quantification results of Bp (GARTM Bp = 0.582 ± 0.2655, SRTM Bp = 0.618 ± 0.2923), and obtained a good linear relation between the Bp value and the SKRC value. Our results indicate that the SKRC value is more suitable for an early-stage kinetic data analysis, and the Bp value depicts kinetic characteristics under the equilibrium state. Dynamic fluorescence imaging in conjunction with various kinetic models are optimal tools to quantify molecular specificity of the Cy5.5-GX1 probe **in vivo**.

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1. Introduction

GX1 (CGNSNPKSC), a cyclic 9-mer peptide, was identified by Min Zhi et al. in 2004 by using in vivo phage display technology [1], which binds specifically to the gastric tumor-derived vascular endothelial cells. Further research confirmed that GX1 was capable of effective specific targeting of the tumor vasculature and could be applied to anti-angiogenesis iatreusis of cancer, together with anti-neoplastic agents, as a novel tumor vascular marker [2–6]. At the same time, GX1 was successfully conjugated with a fluorescing cyanine dye, Cy5.5, for optical imaging in vivo by Kai Chen et al. in 2012 [7]. Static optical imaging results revealed that Cy5.5-GX1 is highly sensitive, rapidly tumor targeting and has fast muscle tissue clearance, thus it was an ideal probe for optical imaging in living subjects. So far, the study of evaluating pharmacokinetic properties of GX1 in vivo is deficient. More specifically, the binding potential (Bp) of the probe, which is a receptor density-related parameter obtained by early dynamic imaging data, has not been determined. Therefore, further investigation needs to be addressed to facilitate the clinical application of GX1.

As a novel noninvasive visualization method, optical imaging possesses many appealing advantages compared with other imaging modalities, for instance, it has no radioactive hazard and costs are relatively low [8]. For example, near-infrared (NIR) fluorescence imaging within the wavelength range of 700-1000 nm has caught immense attention due to its low tissue auto-fluorescence and absorption, which has highly improved tissue depth penetration and image sensitivity and minimized tissue background interference [9–11]. On the other hand, dynamic imaging followed by kinetic analysis provides multiple advantages in comparison to static imaging, since it offers necessary parameters such as peak time, clearance rate, binding potential and distribution volume etc. [12, 13]. In addition, when there is a non-specific binding phenomenon, kinetic analysis also separates a specific signal from a non-specific signal to improve the accuracy of the quantitative analysis [14].

On account of the above advantages, several quantitative studies based on dynamic optical imaging have been studied. Gurfinkel et al. presented a new exponential model and successfully performed a quantitative analysis on Cy5.5-c(KRGDf) via dynamic optical imaging in combination with the exponential model, and obtained the sum of the kinetic rate constant and the timescale associated with receptor turnover of these two meaningful kinetic parameters (SKRC) [15]. Moreover, Li Zhu et al. demonstrated for the first time that when the singular value decomposition (SVD) analysis was used to un-mix the specific signal component of the tumor before a kinetic analysis, dynamic fluorescence imaging could be obtained with analogous in vivo quantitative results to that of dynamic PET imaging [12].

In this study, we drew on a predecessor’s work and used three kinetic methods to analyze 2D dynamic fluorescence data of Cy5.5-GX1. Utilizing these three methods will not only be able to capture the kinetic parameters of interest, but also avoid blood sampling [16]. Analyzing the results of the Gurfinkel exponential model (GEXPM) provided in vivo information of receptor-ligand affinity and timescales estimation associated with receptor turnover. We also used the Logan graphical analysis with the reference tissue model (GARTM) and the Lammertsma simplified reference tissue model (SRTM) to calculate the binding potential of Cy5.5-GX1, which is proportional to the available receptor density by the tracer affinity and is the key parameter of interest [17, 18].

2. Materials and methods

2.1 Cy5.5-GX1 synthesis

Cy5.5-GX1 was synthesized by FanBo Co Ltm. (Beijing, China) and identified by HPLC and TOFMS. The peptide was freeze-drying processed and preserved at −20°C.
2.2 Immunofluorescent staining

Immunofluorescent staining was performed as previously described [19, 20]. Co-HUVEC, HUVEC, GES and BGC-823 cells were trypsinized, resuspended, counted and grown in culture media for 24 hours. The cells were fixed with acetone at 4°C for 10 min, permeabilized in PBS containing 0.3% Triton X-100 for 10 min, and blocked with 10% BSA for 30 min. Cy5.5-GX1 was diluted in PBS at 10 μg/ml, added to the slips and incubated overnight at 4°C. Furthermore, the cells were counterstained with 1 μg/ml of DAPI for 15 min at 37°C and mounted with an anti-fading agent. Samples stained with immunofluorescence were detected using a laser scanning confocal microscope (FV10i, Olympus, Tokyo, Japan).

2.3 In vivo BGC-823 xenografted mouse model

Athymic female nude BALB/c mice (4-6 weeks; 18-22 g) were obtained from the Laboratory Animal Center, FMMU. Nude mice were handled according to aseptic techniques and maintained in a specific pathogen-free environment on a 12 hour light/12 hour dark cycle, with food and water supplied ad libitum. Animal Care and all experiments were performed in accordance with the internationally recognized guidelines. About 200 ml BGC-823 cancer cells suspended in PBS (1*10^7 cells/ml) were implanted subcutaneously into the right shoulder of nude mice and grown for about 14 days when the tumor reached approximately 300 mm^3 [6]. Twelve mice were randomly divided into three groups. In the non-block group (N = 4), each mouse only received 1nmol Cy5.5-GX1, and in the other 2 groups (N = 4, respectively), each mouse received GX1 (20 mg/kg) at 1 hour or 24 hours prior to the Cy5.5-GX1 injection (1nmol/mouse, 1h-block group and 24h-block group, respectively).

2.4 Dynamic fluorescence imaging and image analysis

Dynamic fluorescence data were acquired by the IVIS Kinetic imaging system. A laser source of 660 nm was used to excite the fluorophore, and an emission filter of 710 nm was used for detecting fluorescence. Before the bolus administration of the fluorescent tracer, the first five background images were obtained. The tracer injection dose and exposure time were equivalent to 1 nmol (in 0.2 ml volume) and 1 s. In the process of injection and image acquisition, mice were anesthetized with isoflurane and body temperature was maintained by a thermostat-controlled thermal heater. BGC-823 xenografted mice were subjected to 1 hour dynamic imaging immediately following Cy5.5-GX1 intravenous injection. Serial fluorescent images were acquired every 6 s for the first 540 s, every 12 s for the second 900 s, and every 60 s for the last 2160 s. Moreover, fluorescent images of all three groups were acquired again at 24 hours post-initial injection.

Image pre-processing and analysis were conducted using Matlab 2014b (The MathWorks, Inc., Natick, MA) and regions of interest (ROIs) were drawn out by Living Image 4.4 software. For the quantitative analysis, tumor ROI were determined in the white light images and the muscle region of the same size contralateral to the tumor was selected as the muscle ROI (a region with no specific binding as a reference tissue) [7]. The mean value of fluorescent signals (*10^6 photon/cm^2/s) within each ROI was calculated after a background image was subtracted from each fluorescent image, subsequently the fluorescence activity versus time curves (TACs) of the tumor and muscle ROIs of the fluorescent images were determined.

Singular value decomposition (SVD) method was first used to process the raw fluorescence data to un-mix the specific signal component in the tumor tissue before applying GARTM and SRTM [21]. SVD analysis was performed on these 2-dimension spatial-temporal matrices formed by the TACs in the background, muscle and tumor ROIs to obtain a series of eigenvalues and eigenvectors. In order to separate the meaningful eigenvectors, the L-curve method was used to threshold the eigenvalues. Then, there was a one signal component in the background ROI (corresponding to auto-fluorescence), a two signal component in the muscle ROI (corresponding to auto-fluorescence and muscle uptake) and a three signal component in...
the tumor ROI (corresponding to auto-fluorescence, muscle uptake and tumor specific uptake). Finally, the raw muscle and tumor TACs were projected onto the corresponding identified signal components to recover the pure tumor and muscle TACs [12]. Note that the average background image was subtracted from each acquired fluorescent image after completion of dynamic optical imaging. In addition, Parameters of GARTM were obtained by linear regression analysis, and the remaining parameters were fitted using a nonlinear least-squares algorithm. Only the original fluorescence data of the beginning 20 min were selected to fit the GEXPM [15].

2.5 Kinetic models and parameter estimation

The model selected for the tumor tissue assumes that there are three possible environments for GX1, so the two-tissue (three-compartment) model was used to describe the GX1 tracer kinetics. It consists of (i) non-metabolized tracer in the blood plasma compartment ($C_P$), (ii) free and non-specific binding tracer (non-displaceable uptake) in the extravascular and intracellular space ($C_{nd}$), and (iii) tracer specifically binding with the GX1 receptor ($C_S$) [22]. Figure 1(A) shows one kind of a three-compartment model describing GX1 tracer kinetics in the tumor tissue and one kind of a two-compartment model describing GX1 tracer kinetics in the reference tissue, and both are suitable for GARTM and SRTM [12, 23]. Figure 1(B) presents one kind of a three-compartment model describing GX1 tracer kinetics in the tumor tissue for the GEXPM [15]. All $C_p$, $C_{nd}$ and $C_s$ in Fig. 1(A) and Fig. 1(B) represent the tracer concentration in arterial blood plasma, that in the portion of non-displaceable uptake (free or nonspecific binding) of the GX1 tracer in interstitial and intracellular space, and that in the portion of GX1 tracers specifically binding with the receptor, respectively. $K_1$ reflects the tracer extravasation rate into tissues, $k_2$ reflects the non-displaceable uptake tissue efflux rate from interstitial space into plasma, $k_3$ and $k_4$ reflect the rate of specific binding and dissociation between the tracer and the receptor; moreover, $k_{nd}$ reflects the rate of tracer elimination in plasma via other routes such as kidney filtration and liver metabolism, and $k_r$ reflects the rate of tracer accumulation in the second tissue compartment whether through binding with the receptor or internalization.

Based on the compartment model shown in Fig. 1(A), the corresponding equation of GARTM is defined in Eq. (1) [24]:

$$\int_0^t ROI(t)dt - \int_0^t ref(t)dt + \frac{k_{nd}^{ref}}{ROI(T)} + int'T \quad (t > t') \quad (1)$$

where $ROI(t)$ and $ref(t)$ refer to the un-mixed fluorescence activity of the tumor and reference tissues, $k_{nd}^{ref}$ refers to the tracer clearance rate from interstitial space in the reference tissue, and $DVR$ refers to distribution volume ratio. When the probe is injected for a certain period of time ($t^*<t$), the exchange between tumor tissue and plasma will reach an equilibrium state. Thus, a plot of $\int_0^t ROI(t)dt / ROI(T)$ (set to the y-axis) vs $\int_0^t ref(t)dt + ref(T) / k_2^{nd} / ROI(T)$ (set to the x-axis) is linear with a slope given by $DVR$ [24]. The binding potential is calculated as $Bp = DVR - 1$, which is a macro-parameter reflecting the binding affinity and available receptor density, and is also equal to $k_3/k_4$ [25].

Likewise, the SRTM can be expressed as Eq. (2) [17]:

$$ROI(t) = R_t ref(t) + \{k_2 - R_t k_2 / (1 + Bp_{nd})\} ref(t) * \exp\{(-k_2 t / (1 + Bp_{nd})\} \quad (2)$$

where $Bp$, $ROI(t)$ and $ref(t)$ are the same as before, and $k_2$ represents the tracer clearance rate from interstitial space in tumor tissue. $R_t$ is defined as $R = K_t / K_t^{ref}$, a macro-parameter, where $K_t$ and $K_t^{ref}$ represent the tracer extravasation rate into tumor tissue and reference tissue.
Except for that, according to the model in Fig. 1(B), GEXPM can be expressed as Eq. (3) [15, 26]:

\[ I(t) = A[1 - \exp(-\alpha t)] + B[1 - \exp(-\beta t)] \]  

(3)

where the raw fluorescence activity of the tracer in the tumor or muscle tissues is given by \( ROI(t) \), \( A \) and \( B \) are macro-parameters of the magnitude of the detected signal, and \( \alpha \) and \( \beta \) are also macro-parameters of the receptor-ligand affinity. Here, we are mostly concerned about the \( \alpha + \beta \) value of the tumor tissue, which represents the sum of the pharmacokinetic rate constants (SKRC) and is equal to \( K_1 + k_2 + k_r + k_{el} \).

3. Statistical analysis

Quantitative pharmacokinetic parameters determined from the dynamic optical data were expressed as means ± SD (\( N = 4 \)). Statistical analyses were all carried out with SPSS software (version 22.0 for Windows, Chicago, IL, USA). Statistical significance was evaluated by an unpaired Student \( t \)-test. \( P \) values less than 0.05 were considered statistically significant.

4. Results

4.1 Cell location and binding specificity of Cy5.5-GX1

Co-immunofluorescence staining was implemented to confirm cell location of Cy5.5-GX1 and to identify the binding specificity to human gastric cancer vascular endothelial cells. Wild-type HUVEC, BGC-823 and GES cells were used as controls. As shown in Fig. 2, confocal microscopy photographs showed that Cy5.5-GX1 was localized in the cytoplasm, particularly around the nuclei of Co-HUVEC. The result indicates that Cy5.5-GX1 could bind specifically to Co-HUVEC. On the other hand, significant fluorescence was not detected in GES and
BGC-823 cells, while Cy5.5-GX1 was observed in HUVEC with a reduced luminance. All fluorescent images indicate that Cy5.5-GX1 can specifically bind to Co-HUVEC and target gastric cancer vessels.

Fig. 2. Confocal immunofluorescent images of Cy5.5-GX1 expression and localization in Co-HUVECs, HUVECs, GES and BGC-823 cells (400 ×). Cells were stained with DAPI (nuclear staining) colored in green, and Cy5.5-GX1 colored in red.

4.2 Dynamic fluorescent images

Figure 3(A)-3(C) shows serial raw fluorescent images (i.e., no background subtraction) of a representative mouse from each test group. The corresponding TACs of tumor and muscle regions are displayed in Fig. 3(D)-3(F). As shown in Fig. 3(A) and 3(C), the tumor region was more luminous with respect to its contralateral muscle region (the reference tissue) from the fluorescent images in both the non-block group and 24h-block group, especially enhanced in the 24 hours after Cy5.5-GX1 administration. Whereas in Fig. 3(B), throughout all time points, there was no obvious fluorescent signal in the tumor site of the mouse that received free GX1 peptide 1 hour prior to the Cy5.5-GX1 injection. The corresponding TACs of those three test groups in Fig. 3(D)-3(F) showed similar results. Although all TAC values of the tumor tissues were higher than those of the muscle tissues, the difference was not significant when animals received Cy5.5-GX1 at 1 hour after free GX1 administration as a blocking agent. In addition, the high intensity fluorescent signal in the kidney suggested that Cy5.5-GX1 was excreted through the bladder.

4.3 Pharmacokinetic analysis

As shown in Fig. 4(A), simple SVD analysis was performed on the three ROIs of the tumor, muscle and background to get a series of eigenvalues and eigenvectors. After threshold processing of the log scale eigenvalue curves with a L shape, we identified the specific tumor uptake (corresponding to the third signal component in the tumor ROI) and the non-specific muscle uptake (corresponding to the second signal component in the muscle ROI) [12]. Original TACs of tumor and muscle ROIs were projected to the corresponding components to obtain the respective pure TAC of tumor and muscle ROIs. Figure 4(B)-4(D) display the recovered pure
TACs of tumor and muscle ROIs obtained by the SVD analysis of one representative animal of each group.

Fig. 3. Representative original fluorescent images (no background subtraction) at different time points generated from 1 hour dynamic optical imaging and 24 hours after administration of (A) non-block group, (B) 1h-block group, and (C) 24h-block group. White arrows indicate tumor locations, and all fluorescent images generated from 24 hours after probe injection (the third columns) are displayed on the same linear color scale to allow for qualitative comparison. D-F. Corresponding time activity curves of tumor and muscle regions.

To compute and compare the binding potential in these three test groups, GARTM and SRTM were performed on the dynamic fluorescence data with the SVD analysis. Figure 5(A) and 5(B) represents the corresponding linear and nonlinear fitting results from one representative animal of each group. As seen in Fig. 5(A) and 5(B) and Fig. 7(A), GARTM and SRTM were well fitted to the dynamic fluorescence data and provided similar results. The non-block group showed the highest $Bp$ value ($Bp = 0.582 \pm 0.2655, 0.618 \pm 0.2923$, respectively), followed by the 24h-block group ($Bp = 0.566 \pm 0.0476, 0.549 \pm 0.164$, respectively), and the $Bp$ value of the 1h-block group was the lowest ($Bp = 0.149 \pm 0.065, 0.161 \pm 0.089$, respectively). The $Bp$ values obtained from SRTM were statistically analyzed, and the results revealed that the $Bp$ values of the non-block and 24h-block groups were significantly different from the $Bp$ value of the 1h-block groups ($p = 0.01, 0.023$, respectively). However, the $Bp$ values between the non-block group and 24h-block group were not significantly different ($p = 0.638$).

The typical TACs of the first 20 min in tumor ROI and muscle ROI and the corresponding nonlinear fitting results, from a representative animal in each group, are demonstrated in Fig.
6(A) and 6(B). As shown in the picture, tracer uptake of the non-block group was the highest in both tumor and muscle ROIs, followed with the 24h-block group, and that of the 1h-block group was the lowest in both ROIs. In addition, tracer uptake in muscle ROI was also reduced when free GX1 was administered as a blocking agent.

Fig. 4. A. Representative white light image showing the ROI locations; ROI-1 refers to tumor ROI, ROI-2 refers to muscle ROI, and ROI-3 is background ROI. Pure time activity curves in tumor and muscle regions recovered by the SVD analysis from raw fluorescent dynamic images corresponding to the (B) non-block group, (C) 1h-block group and (D) 24h-block group.

Fig. 5. A. Representative results of GARTM fitted to 60 min dynamic optical data with the SVD analysis, which showed good linearity of normalized integrated ( intimacy) tumor activity vs. normalized integrated muscle tissue activity. Slopes of fitted lines refer to DVRs and $B_p = DVR$-1. The definition of the x-axis and y-axis as described above. B. Representative results of SRTM fitted to 60 min dynamic optical data with the SVD analysis. The red triangles represent the original experimental data from animals receiving the Cy5.5-GX1 alone, while the blue dots and green squares represent the original experimental data acquired from an animal receiving Cy5.5-GX1 1 hour and 24 hours, respectively, after the administration of the GX1 peptide. The black solid lines show the corresponding linear and nonlinear fitting results.

As shown in Fig. 7(B), the shifting trend of the probe uptake is further reflected in the fitted pharmacokinetic parameters, which is the $\alpha + \beta$ value, representing the sum of the pharmacokinetic rate constants ($SKRC$). For the non-block group ($\alpha + \beta = 0.125 \pm 0.0286$) and the 24h-block group ($\alpha + \beta = 0.115 \pm 0.0284$), the $\alpha + \beta$ values in the tumor ROIs were significantly higher than the 1h-block group ($\alpha + \beta = 0.04 \pm 0.0081$, and $p = 0.01, 0.02$, respectively). Similarly, there was also no significant difference between the $\alpha + \beta$ values of the non-block and 24h-block groups ($p = 0.566$). Furthermore, as shown in Fig. 7(C), the $B_p$ and $\alpha$
+ \beta \) values are respectively set to the X axis and Y axis, and after fitting the corresponding data points, there is a good linearity between these two parameters \((p = 0.0036)\). In addition, in muscle ROIs of all of the test groups, the \(\alpha + \beta\) values were also not significantly different \((\alpha + \beta = 0.0228 \pm 0.0067, 0.0239 \pm 0.0013, 0.0269 \pm 0.0023, \) and \(p = 0.715, 0.192, 0.328,\) respectively).

![Fig. 6. The raw time activity curves of the first 20 min derived from one representative animal of each group in (A) tumor ROI and (B) muscle ROI. The definition of the symbol is the same as before, and the black solid lines show the corresponding nonlinear fitting results.](image)

**5. Discussion**

By analyzing real-time imaging results of molecular probes, molecular imaging technology is able to quantitatively evaluate the receptor expression level of the ROI without being invasive. In particular, dynamic imaging with a kinetic analysis facilitates the separation of the actual specific binding component from total molecular probe uptake in the ROI, which will significantly promote the pharmacokinetic evaluation of the molecular probe [27, 28]. In this one, optical imaging, despite the existence of photon scattering and tissue attenuation, still by virtue of the advantages of low cost and high temporal resolution, is also widely used in the field of molecular image quantification in vivo [29, 30]. By applying dynamic optical imaging, TACs of tumor and muscle tissues can be obtained and depicted. Moreover, a kinetic analysis can describe probe uptake in tumor and muscle tissues by modeling as different compartments, according to the homogeneous probe concentration in arterial blood plasma, interstitial space or tumor cells. Therefore, by fitting the TACs of dynamic optical data to the compartment model, the kinetic analysis could quantitatively estimate the probe binding affinity for the receptors in vivo [23, 31]. In this study, we evaluated Cy5.5-GX1 kinetics by using dynamic optical imaging and kinetic modeling in a BGC-823 xenograft model.

By comparing immunofluorescent staining images and raw dynamic fluorescent images, Cy5.5-GX1 apparently possesses a specific affinity to tumor tissue since the GX1 receptor is overexpressed in BGC-823 cells. The probe specific tumor targeting was abrogated when free

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**Fig. 7.** A. Binding potential \((Bp)\) of Cy5.5-GX1 in each group, and binding potential was calculated as \(Bp = k_3/k_4\) reflecting the binding affinity. The results were derived from GARTM. B. \(\alpha + \beta\) values of Cy5.5-GX1 in each group of tumor and muscle regions, which represent the sum of the pharmacokinetic rate constants. C. The relevance between \(Bp\) and \(\alpha + \beta\) values from all 12 tested animals, together with the linear regression result.
GX1 peptide was injected 1 hour before probe administration, and could be recovered when free GX1 peptide preceded probe injection by 24 hours. This finding was further supported by the kinetic analysis results, where \( Bp \) values of the non-block group and the 24h-block group were four times higher than those of the 1h-block group. Accordingly, the \( \alpha + \beta \) value of each group also showed a similar trend. Therefore, we can speculate that the turnover time of the GX1 receptor expressed in BGC-823 tumor cells may be less than 24 hours. By the given experimental dynamic fluorescence data of free GX1 peptide injection with different time intervals before the probe injection, we may be able to determine the receptor turnover time.

With the absence of a corresponding GX1 kinetic quantitative study \textit{in vivo} as a reference, we utilized the mean value of \( k_2 \) obtained from the SRTM to approximate the value of \( k_2^{\text{ref}} \) in GARTM for a linear fitting. The SRTM and GARTM are well fitted to the measured dynamic optical data, and the binding potential values recovered using these two models are very close. In the present study, we only showed the GARTM results to illustrate the situation. In addition, introducing the SVD method improved the accuracy of \( Bp \) value estimation, which is an indicator of the available receptor density and tracer specific binding, because the TAC of the tumor after SVD treatment is more consistent with the tracer specific binding component in the tumor region, while the TAC of muscle after SVD treatment is more in line with the non-specific binding component in the muscle region [12].

Note that the \( Bp \) value appears to have the same trend as the \( \alpha + \beta \) value of the tumor regions, and further regression analysis confirmed it. This is because the GEXPM solely uses parameter \( k_t \) to reflect on the effect of tracer specific binding and other parameters are not related, so the relation between the \( SKRC \) value (the \( \alpha + \beta \) value) and the \( Bp \) value is inevitable. Although the GEXPM describes the one-way process of continuous accumulation of the probe on tumor cells, it may be more appropriate to reflect on the kinetics of the initial stage of the probe injection and that is why only the initial 20 min of the dynamic fluorescence data were used as the model fitting. Based on the definition of the binding potential, \( Bp \) is considered as an indicator of the equilibrium state of the tracer injection, which directly reflects the specific binding affinity of the tracer and available receptor density, so it can be more comprehensive to describe the probe kinetic characteristics with the \( \alpha + \beta \) value \textit{in vivo}. In addition, as shown in Fig. 6(B), tracer uptake in the muscle region slightly decreased after GX1 injection as a competitive ligand, which may be due to the presence of a small amount of GX1 receptors in this region, thus we ignored it in the quantitative analysis.

In this study, we evaluated the kinetic parameters of Cy5.5-GX1 by using dynamic optical imaging in conjunction with various kinetic models, and compared the differences between those models. Quantification kinetic parameters can be used to access GX1 receptor density in tumor regions. Therefore, costly preclinical studies for developing gastric cancer medicine would benefit from longitudinal monitoring of GX1 receptor binding in response to candidate compounds.

6. Conclusion

Dynamic fluorescence imaging in conjunction with the combination of various kinetic models would provide optimal tools for \textit{in vivo} quantification of the molecular specificity of a probe. At the same time, our results indicated that Cy5.5-GX1 was a promising optical probe for imaging tumor vasculature and to selectively deliver anti-cancer medicine to tumor sites \textit{in vivo}. Quantification study of Cy5.5-GX1 would promote future clinical applications of GX1.

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