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EDITORIAL

FAP inhibitors: are we really using the best method to evaluate the residence time?

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Fibroblast activation protein inhibitors (FAPIs) have revolutionized molecular imaging, particularly with the development of PET tracers targeting the tumor microenvironment [1]. These tracers exploit the overexpression of FAP on cancer-associated fibroblasts (CAFs), enabling highly specific imaging of tumors with strong stromal activity. PET/FDG imaging plays a crucial role in cancer diagnosis, staging, and monitoring treatment response, providing valuable insights into metabolic activity and tumor progression [2, 3]. FAPI-based PET tracers, such as ^{68}Ga-FAPI, offer superior diagnostic performance over conventional FDG-PET in various malignancies, including those with low glucose metabolism. Their ability to provide high tumor-to-background contrast has expanded their clinical applications to detect challenging primary and metastatic lesions [4]. With ongoing advancements, FAPI-PET tracers are poised to enhance both diagnostic precision and theranostic strategies in oncology. Despite their promising potential, FAPI-based PET tracers face limitations, including variable tumor uptake, rapid tracer washout in certain conditions and potential off-target activity in non-malignant processes such as inflammation or fibrosis, which can complicate diagnostic specificity [5].

In the paper "Head-to-head comparison of different classes of FAP radioligands designed to increase tumor residence time: monomer, dimer, albumin binders and small molecules vs peptides" by J. Millul et al., the authors provide a comprehensive comparison of strategies aimed at enhancing the tumor residence time of fibroblast activation protein-alpha targeting radioligands. They investigate a range of designs, including a monomer (FAPI-46), its dimerized form, albumin-binding conjugates and a peptide-based radioligand (FAP-2286), with the goal of identifying the most effective approach for radiotherapeutic applications. The study revealed distinct advantages and limitations across the tested strategies. Dimerized and cyclic peptide radioligands demonstrated superior tumor retention and radiation doses compared to monomers. For instance, peptide-based FAP-2286 achieved the highest tumor-to-background ratios, sparing healthy tissues and improving therapeutic indices, although its efficacy was highly dependent on tumor FAP expression levels. In contrast, albumin-binding conjugates showed variable results, with some exhibiting high background retention and limited specificity, raising concerns about potential off-target toxicity.

Residence time (RT) optimization is critical for improving tumor retention, biodistribution and therapeutic indices, making it a key focus in this study. To calculate the residence time, the authors combined *in vitro* and *in vivo* methods, supplemented by advanced dosimetry modeling. However, none of the applied methods directly measured this parameter.

The potency of radioligands was assessed *in vitro*, by competitive inhibition assays against isolated human FAP, providing initial insights into their binding properties, then-radioligands were tested on FAP-positive cell lines with varying expression levels at multiple time points (15 min to 4 h) to evaluate their internalization, retention and non-specific uptake.

In vivo, tumor and organ retention of radioligands were measured in xenograft models using gamma counters. Data were collected at various intervals (4–120 h post-injection) to observe the temporal dynamics of radioligand retention; high-resolution imaging allowed real-time tracking

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of radioligand uptake, washout, and specificity in tumors expressing low or high levels of FAP; the AUCs for tumor-to-organ ratios and therapeutic indices were calculated from biodistribution data to estimate overall radioligand effectiveness and safety and finally, dosimetry provided a quantitative measure of therapeutic and off-target effects for each radioligand. Overall, the authors claim the potential of residence time optimization for enhancing the efficacy of FAP-targeting radioligands and formulate hypotheses on enzyme kinetics. However, drug-target residence time was inferred from these assays, but not measured directly [6].

Tumor retention, tumor uptake and drug-target residence time are relevant concepts in the context of drug development being all related to drug efficacy, however they focus on different aspects and are not synonyms. In drug discovery, the concept of drug-target residence time has garnered significant attention over the past decade [7]. Initially introduced as an alternative perspective to traditional measures such as binding affinity or dissociation constant (Kd), residence time—defined as the reciprocal of the dissociation rate constant (Koff)—describes the duration a drug remains bound to its target. A longer residence time generally implies that the drug has a more prolonged interaction with its target, which can lead to more effective signaling modulation or inhibition. Residence time offers valuable insights into the pharmacokinetic (PK) and pharmacodinamic (PD) profiles of drugs [8]. Advocates argue that longer RTs are linked to prolonged target inhibition, reduced dosing frequency, and improved therapeutic selectivity, driving efforts to integrate RT measurements into drug optimization workflows alongside traditional metrics like binding affinity. Hao Lu and Peter J. Tonge, emphasize that RT is critical for maintaining drug efficacy, ensuring sustained target engagement even as plasma concentrations fluctuate [9]. However, critics caution against over-reliance on RT as a sole metric. Rutger H.A. Folmer points out limited empirical evidence supporting RT's superiority over binding affinity or potency in predicting drug success. The author argues that RT must be considered within a broader context, as drug efficacy depends on a complex interplay of factors, including absorption, distribution, metabolism and excretion. Folmer warns that isolating RT from these broader PK/PD considerations risks oversimplifying the determinants of drug effectiveness [10].

This dichotomy underscores the need for a balanced approach to drug design. While RT can provide valuable insights into target occupancy and therapeutic duration, its integration with traditional metrics and PK/PD modeling is essential to dissect the complexities of drug action.

Despite significant advancements in the development of fibroblast activation protein inhibitors, a critical gap persists in effectively measuring and optimizing drug-target residence time. Although many studies underscore the need to enhance RT to improve therapeutic indices and clinical outcomes, few have rigorously evaluated this parameter [11, 12]. The lack of widely accessible and straightforward biochemical assays capable of assessing RT limits drug screening efforts, making preclinical development both costly and risky [13]. The current absence of reliable *in vitro* methods for RT measurement significantly restricts the number of compounds that can be screened during the early stages of drug development. This limitation, in turn, contributes to a higher failure rate in the later stages of drug discovery, where the implications of inadequate RT become apparent.

When measuring residence time for enzyme targets, as in the case of FAP, the choice of the method depends on the enzyme-ligand interaction and on the experimental goals (Table 1).

These approaches are often combined to cross-validate RT measurements.

For most cases, kinetic enzyme assays or SPR are recommended as primary methods for directly measuring RT in enzyme targets, particularly in early drug discovery and optimization. ITC can offer valuable thermodynamic insights, while MDS simulations can provide a deeper molecular understanding of the enzyme-ligand interaction, helping to refine lead compounds. Combining these methods offers a comprehensive approach to optimize drug design.

The suitability of traditional residence time measurement methods for high-throughput screening varies due to scalability and resource constraints. Kinetic enzyme assays are the most adaptable for HTS, particularly when integrated with automation and fluorescence detection, though slow-binding inhibitors may reduce throughput. Surface plasmon resonance and isothermal titration calorimetry are less suitable for HTS due to their low throughput, high cost, and sample consumption. Molecular dynamics simulations are also impractical for initial HTS due to their computational intensity. Alternatives such as label-free biosensors, fluorescence polarization, and mass spectrometry-based assays are emerging as more HTS-compatible methods for RT measurement. While traditional techniques are better suited for follow-up studies, advances in biosensor technology and assay miniaturization may enhance HTS capabilities for RT screening in the future.

In a recent study, to measure the RT of a series of newly synthesized FAPIs, we explored the potential of the jump dilution assay. Utilizing recombinant human FAP, we concurrently measured drug-target RT and inhibitor's potency. While this method offers promise, to effectively use the jump dilution assay for evaluating the residence time of an enzyme-inhibitor complex, several critical assumptions must be met to ensure the accuracy and reliability of the results [21]. FAP enzyme fails to fulfill all the assumption,

Table 1 Methods for calculating drug-target residence time and their characteristics

Method	Description	Advantages	Limitations	Suitability for HTS	Refs
Kinetic enzyme assays	Measure enzyme activity changes over time to calculate dissociation rate constant	Direct, real-time data on slow- or tight-binding interactions	Reduced throughput for slow-binding inhibitors	High (with automation and fluorescence)	[14]
Surface plasmon resonance (SPR)	Measures real-time binding kinetics	High precision; suitable for both fast- and slow-binding interactions	High cost, sample consumption	Low	[15]
Isothermal titration calorimetry (ITC)	Detects heat changes during binding to infer thermodynamics and kinetics	Label-free; preserves natural conformation of target	high sample requirements	Low	[16]
Molecular dynamics simulations (MDS)	Simulate binding and dissociation pathways computationally	Provides detailed molecular insights into mechanisms affecting RT	Computationally intensive; impractical for large-scale studies	Very low	[17]
Fluorescence polarization	Detects changes in molecular rotation upon binding or dissociation	Compatible with miniaturization	Limited to small molecule targets; dependent on appropriate fluorescence labeling	High	[18]
Biolayer interferometry (BLI)	Label-free biosensor-based technique for kinetic measurements	Cost-effective alternative to SPR; suitable for certain biomolecular interactions	Less precise than SPR for some targets	Moderate to high	[19]
Mass spectrometry (MS)	Measures binding and dissociation through mass changes	High sensitivity; label-free	Requires specialized instrumentation	Low	[13]
Thermal shift assays	Measures protein stability changes upon ligand binding.	Simple and widely applicable; can infer RT indirectly	Provides indirect RT estimates; less precise for quantifying k_{off}	High (with automation)	[20]

thus we were able to observe the direct interaction between FAP and its inhibitors only within a limited timeframe, corresponding to linear kinetic behavior. This further highlights the methodological hurdles in reliably measuring RT, emphasizing the urgent need for assay development that balances accuracy with practical applicability. Moving forward, improving biochemical tools for RT evaluation will be essential to reduce the risks and costs associated with preclinical development. Developing high-throughput and reproducible RT assays will expand the range of candidate molecules for screening, ultimately increasing the likelihood of successful therapeutic breakthroughs in the FAPI field. Enhanced collaboration between academic researchers, industry and regulatory bodies will also be crucial to integrate RT measurement as a standard component of drug discovery workflows.

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Declarations

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