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Tumor drug penetration measurements could be the neglected piece of the personalized cancer treatment puzzle

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Abstract

Precision medicine aims to use patient genomic, epigenomic, specific drug dose and other data to define disease patterns that may potentially lead to an improved treatment outcome. Personalized dosing regimens based on tumor drug penetration can play a critical role in this approach. State-of-the-art techniques to measure tumor drug penetration focus on systemic exposure, tissue penetration, cellular or molecular engagement and expression of pharmacological activity. Using *In silico* methods, this information can be integrated to bridge the gap between the therapeutic regimen and the pharmacological link with clinical outcome. These methodologies are described, and challenges ahead are discussed. Supported by many examples, this review shows how the combination of these techniques provides enhanced patient-specific information on drug accessibility at the tumor tissue level, target binding and downstream pharmacology. Our vision of how to apply tumor drug penetration measurements offers a roadmap for the clinical implementation of precision dosing.

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Introduction

Precision medicine in oncology entails tailored drug treatment for individual patients. Personalized dosing regimens based on tumor drug penetration play a critical role in this approach. Technologies necessary for this endeavor, such as in vivo molecular, functional imaging and ex vivo mass spectrometry, have matured. Tools providing enhanced patient-specific information on drug accessibility at tumor tissue level, target binding and downstream pharmacology are crucial to understand exposure-response relationships and guide precision dosing to improve treatment outcome.

Precision dosing as a missing piece of the personalized cancer treatment puzzle

Precision medicine aims to use patient's genomic, epigenomic, specific drug dose and other data to define disease patterns that may potentially lead to an improved treatment outcome.[1] Advances in precision medicine have been especially apparent in the field of oncology. An increasing number of targeted and 'classic' cytotoxic agents can now be tailored to patient's cancer characteristics, such as targeting BCR-ABL translocation in chronic myelogenous leukemia (CML) or dose reduction in polymorphism in enzymes involved in drug metabolism to reduce the risk of toxicity (e.g. UGT1A1*28 in irinotecan-based chemotherapy). In addition, some progress has been

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made to identify biomarkers that predict response to these agents. The human epidermal growth factor receptor 2 (HER2, also known as *ERBB2*/*neu*) has been one of the most well-documented successes in targeted cancer treatment. The HER2 gene is amplified in 15-20% of breast cancer patients. The approval of HER2 targeted agents, led by trastuzumab in 1998, has improved outcomes in curative and non-curative breast cancers.[2] Despite the success of targeted agents such as those for HER2, improved long-term outcomes from precision medicine are still comparatively rare, with over 600,00 Americans expected to die of cancer in 2018,[3] and a predicted global burden of 13 million cancer deaths by 2030.[4]

A potential missing piece of the puzzle for precision medicine in cancer treatment may be an integrated "**precision dosing**" approach that tailors to patients' tumor characteristics, as well as the extent of drug penetration in tumor tissue. In current dose escalation study designs, a correlation is sought between treatment dosage or systemic (plasma) exposure and treatment response[5]. Most often, drugs are assumed to distribute relatively homogeneously in the tumor tissue. However, distribution of drugs into tumor tissue is in fact highly variable and may not correlate with dose or plasma concentrations. Such variability of drug penetration into tumor tissues may result in suboptimal treatment responses and yet its significance is often neglected.

Thus, an important parameter in precision dosing is **drug tumor penetration**, which can be assessed by measuring accessibility of the target and drug penetration in tumors at macroscopic and/or microscopic levels. At the macroscopic level, drug penetration is often heterogeneous within one single tumor lesion and even more so across different metastatic sites in the same patient. At the microscopic level, sanctuary sites may result in heterogeneity in drug concentrations leading to a proportion of neoplastic cells not receiving the required therapeutic dose. Anticancer drug distribution will be investigated at four levels, in the context of drug accessibility and downstream pharmacologic effects (**Figure 1**), bridging the gap between drug dose and the pharmacological link with a solid tumor's clinical outcome.

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In this review, we will focus on the state-of-the-art tools, including imaging techniques, that provide patient-specific information on drug accessibility at the tumor tissue level, target binding and downstream pharmacology in the context of precision dosing. While these four drug distribution levels resemble the "three pillars of survival" framework, described by others,[6] that includes drug exposure at the target site, target binding and expression of pharmacological activity, additional consideration of spatial drug distribution in tumor tissue is added here. These four levels and their key considerations are further described below.

1. **Systemic drug exposure.** Assessment of systemic exposure ensures that the drug achieves a blood concentration during treatment that in principle, permits optimal penetration and target binding into the tumor tissue, allowing selection of a dose with the best probability to reach the maximal receptor occupancy in the tumor.[7] Ideally, this would be the dose which results in the number of bound target receptors/proteins in the tumor close to the maximum attainable, which is not always the maximum tolerated dose. Below, we discuss separately assessment of systemic exposure from measurements at the site of action (tumor tissue).
2. **Tumor tissue drug penetration.** At the tissue level, state-of-the-art techniques may be used to visualize whether the drug is able to homogeneously distribute throughout the tumor. A range of factors e.g. vascularity, hypoxia or drug efflux transporters may influence drug penetration, depending on characteristics of the drug. For immunotherapies, where immune cells are the effectors, the presence of specific immune cells and/or ligands in relation/proximity to tumor cells may affect the immune function and subsequent outcomes.[8,9] These studies suggest that before onset of an immune response, the presence and co-localization of immune and tumor cells, should be assessed,[8,9] in addition to drug tissue penetration. Changes in the tumor microenvironment in response to drug treatment can be investigated. Lastly, for small molecules, the free fraction of the drug (i.e. the pharmacologically active fraction) may be different in tissues versus

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the circulation, and therefore, whenever possible, total and free drug concentrations should be measured.[6]

3. **Cellular/molecular target engagement.** At the cellular/molecular level, it is possible to assess the presence and accessibility of the target in the right conformation to allow drug binding, i.e. target engagement.[10] As such, drug binding and target availability should be assessed within and across tumor lesions. Furthermore, for some drugs, it is important to assess temporal changes in drug-target engagement. For example, when drug binding results in target internalization and the pharmacologic effect causes target down- or up-regulation.
4. **Expression of pharmacological activity.** Biomarkers that reflect downstream disease cascading effects or treatment effects can also be measured through target binding at the site of action. These distal measurements of pharmacodynamics can indirectly demonstrate that sufficient levels of target modulation are being achieved at the site of action, in addition to providing a bridge towards quantification of drug efficacy and/or resistance.[6]

A multitude of state-of-the-art technologies can be applied to assess the four levels of drug penetration as shown in **Table 1**. Each of these techniques differs in the information provided and its potential role to inform clinical decision making and to guide optimal treatment strategies. We will mostly discuss implications for large molecules, although many of these concepts hold for, and can be extended to, small molecules as well. Techniques for drug characteristics to improve drug penetration at each level (eg. association to albumin to prolong systemic half-life, convection-enhanced delivery (CED) to improve tumor tissue penetration or changing affinity to the target to enhance target engagement) are beyond the scope of this article.[11–13]

[insert Table 1]

As each of these four levels of biological organization are linked, information acquired from each component cannot be viewed separately. In addition, dosing recommendations require coupling quantification of heterogeneity in drug penetration

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and target engagement with a drug's pharmacodynamic link and long-term outcome. *In silico* methods can help bridge the gap from all these data into a more comprehensive understanding. Modeling and simulation can be applied to integrate available information on accessibility, target engagement, pharmacodynamic effects and outcomes at multiple scales.[41] In oncology, the previously proposed concept of model-informed precision dosing (MIPD)[42] can be extended to include data from drug penetration studies. When properly validated, these models can be used in principle to predict and individualize doses.

Studying systemic exposure: Illustration and tools

As mentioned previously, drugs need to achieve systemic concentrations in the blood that permit adequate penetration and target binding into the tumor tissues.[7] Assessment of drug concentration in blood or plasma alone (plasma pharmacokinetics (PK)) may provide valuable information to guide drug dosing. PK can be influenced by factors related to the patient, such as age, body weight, activity of drug transporters and metabolizing enzymes, and renal or liver function. Additionally, for monoclonal antibodies or other large molecules, target binding, immunogenicity, affinity for the neonatal Fc receptor (FcRn), nonspecific uptake followed by proteolytic degradation and catabolism and deconjugation determine the plasma PK-time profile.[43] Furthermore, these plasma PK profiles can be profoundly different depending on the drug dose administered to the patient. At a low dose, non-target specific (e.g. Fc receptor in liver) or non-tumor but specific (e.g. circulating target in blood) binding may decrease the drug's systemic exposure, leading to less drug target binding at the tumor site (the so-called antigen sink). Higher doses may saturate the non-specific binding sites, and lead to relatively high, non-dose-proportionate, increases in systemic exposure compared to low doses, an effect called target-mediated drug disposition (TMDD).[44] Therefore, evidence of non-linear clearance by assessment of plasma PK profiles of monoclonal antibodies and other high affinity drugs can sometimes be used as a tool to predict the maximum binding capacity of the accessible drug target.[7]

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State-of-the-art tools can be used to assess systemic exposure. During drug development, plasma PK profiles are routinely assessed using techniques such as LC-MS for small molecules or immunoassays for monoclonal antibodies. Thus, validated methods for measuring drug concentrations in blood should be available during drug development. Less commonly, positron emission tomography (PET) imaging with radiolabeled drugs has been applied to quantify systemic exposure and TMDD.
By way of example, a ^{89}Zr-trastuzumab PET imaging study[14] demonstrates the interplay between systemic and tumor exposure in the presence of non-specific binding resulting in large tissue sinks. This study showed that reaching a minimal systemic exposure is necessary to improve drug penetration into the tumor and engage the target.[14] At 10 mg (1mCi) tracer dose, rapid ^{89}Zr-trastuzumab clearance was observed in trastuzumab naïve patients, and only after administration of 50 mg of ^{89}Zr-trastuzumab (replenished with non-radioactive trastuzumab), tumor penetrance was observed using PET imaging, indicating the ability to overcome the normal tissue sink. In patients undergoing treatment with trastuzumab (up to 6 mg/kg) at the time of tracer injection, a dose of 10 mg tracer was sufficient to indicate tumor distribution, suggesting that during treatment, some saturation of (non-specific) drug's elimination pathways (e.g. via catabolism or plasma levels of extracellular domains shed by HER2) occurs. This also indicates that there are remaining free receptors in the tumor, (we discuss saturation of the tumor sink in the next section).

Among many reported studies, one example of using TMDD to optimize dosing in drug development is shown in the analysis of the early phase study of RG7356, an anti-CD44 humanized antibody, in patients with acute myeloid leukemia.[15] At low doses, non-linear PK was observed, which plateaued at 1200 mg, at which point the maximum binding capacity of the accessible target was reached. This dose, as opposed to the maximum tolerated dose, was used to define the optimal dose for further phase 2 studies.[15]

This methodology is directly applicable to drug development: In 2017, the EMA suggested in EMEA/CHMP/SWP/28367/07, revision 1, assessment of target saturation

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in early phase clinical trials and proposed that, instead of maximum tolerated dose, ”maximum exposure” is considered, that provides complete inhibition or activation of the target and no further therapeutic effect is to be expected by increasing the dose.

In contrast to the TMDD characterization in the ^{89}Zr-trastuzumab and RG7356 -examples, the PK profile of monoclonal antibodies in blood at clinical doses (at maximum binding capacity) may be prognostic of disease rather than predictive of the binding capacity. Post hoc analyses of trastuzumab in a phase III study in Gastric Cancer (ToGa) showed that patients in the lowest quartile of trastuzumab serum trough concentration (C_{trough}) had shorter overall survival (OS) than patients in the higher C_{trough} quartiles.[45] More recently, clearance of the immunotherapeutic monoclonal antibody, nivolumab (a PD-1 blocking immunotherapeutic antibody) was shown to decrease when patients responded to therapy,[16] and durvalumab (a PD-L1 blocking antibody) change in clearance over time correlated with the change in tumor size during therapy and with the decrease in non-specific protein catabolic rate in patients who benefited from therapy.[17] This correlation suggests that the steep drug exposure-tumor response relationship observed in these studies may be confounded by prognosis and treatment outcome.[16,17] Reasons for the correlation between PK and performance status of the patient is an active area of research and needs to be studied further. It may be related to cachexia, antibody cleavage by the tumor or systemic inflammation.[16] These monoclonal antibodies, when used in clinical doses, generally show linear and dose-proportional clearance.[16,17,46] Therefore, it is less likely that TMDD contributed to the disease specific PK. As a result, increasing the drug dose based on exposure at linear plasma PK in patients with poorer prognostic factors may not improve their outcomes. The HELOISE study - a randomized study of high dose trastuzumab (8 mg/kg + 10 mg/kg) versus standard of care trastuzumab (8 mg/kg + 6 mg/kg) - indeed showed that higher doses of trastuzumab in patients predicted to have low exposure (based on a ECOG score of 2) did not improve outcomes.[47]

Studying tumor tissue penetration: Illustration and tools

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Tumor tissue penetration is largely determined by a variety of factors in the tumor microenvironment.[48] This includes tumor vascular architecture, the composition and structure of the extracellular matrix, and stroma. Aggressive proliferation of tumor cells and associated overexpression of pro-angiogenic factors often leads to the development of poorly organized vascular and lymphatic architecture in tumors. As a result, the irregular blood flow and increased interstitial fluid pressure can severely affect drug distribution within the tumor and limit delivery of anti-cancer drugs to cells distal from the vasculature, depending on characteristics of the drugs. Stroma proteins in the extracellular matrix may give rise to a dense network of tumor matrix components that forms a physical barrier to tumor drug penetration.[49] Small, hydrophilic compounds with low protein binding are expected to diffuse rapidly from the cellular rim into less vascularized or necrotic tissues (as seen in caseous tuberculosis),[50] whereas drugs with high lipophilicity, poor solubility and high number of aromatic rings are more likely to bind and not diffuse.[50] For macromolecules, extravasation across the relatively permeable tumor vasculature is a limiting step for tumor penetration.[7,48]

State-of-the-art tools can be used to assess tumor tissue penetration. At a macroscopic level, information on vascularity and vascular-related drug uptake can be obtained using dynamic contrast-enhanced (DCE) MRI or PET/SPECT perfusion studies. In these non-invasive methods, molecules that pass through the blood vessel walls of the tumor and enter the extracellular extravascular space without penetrating the cellular membrane can provide information on vascular-related drug uptake (e.g. blood volume, blood flow, and/or vascular permeability).[51] In DCE MRI, gadolinium chelates are contrast agents that alter the relaxation time of water protons in tissues to create a contrast in imaging. In PET/SPECT, radiolabeled tracers such as ^{15}O-water, ^{13}N-ammonia for PET[52] and ^{99m}Tc-sestamibi for SPECT[53] are used. In addition, microdialysis a semi-invasive method can be used. During microdialyis a catheter is placed in the vicinity of the tumor to allow measurement of the extracellular, non-protein bound drug in accessible solid tumors at multiple timepoints post-dose.[54]

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At a microscopic level, large molecules or non-ionizable agents can be analyzed in a section of the biopsy by immunofluorescence (IF) or multiplexed ion beam imaging (MIBI), if secondary fluorescent antibodies or those contain isotopically pure elemental metal are available.[55,56] To assess the spatial distribution of unlabeled small molecules (and their metabolites) in clinical tissue samples, matrix-assisted laser desorption/ ionization – mass spectrometry imaging (MALDI-MSI) can be applied [57,58] to a section of a fresh frozen biopsies.[58,59] By multiplexing (multiple stains per section), other histological assays can be applied to detect other factors in the tumor microenvironment influencing drug penetration (e.g. CD31 immunohistochemistry (IHC) staining for blood vessels).

Recently it has become feasible to study microscopic drug penetration in *in vitro* cultured tumor cells in a 3D setting, resembling the *in vivo* architecture and tumor microenvironment of the tumor.[60] Such tumor-on-a-chip models are a subset of organ-on-a-chip models. Therefore, tumor-on-a-chip models can easily be combined with imaging (e.g. confocal microscopy) to study how microscopic drug penetration is influenced by e.g. interstitial flow, the leakiness of the endothelial barrier or the density of the collagen matrix. [60] As an example, a tumor-on-a-chip was used for screening optimal nanoparticle designs prior to *in vivo* studies.[61] In this study, the effect of nanoparticle size and interstitial flow rate on tissue accumulation, was confirmed in murine tumor models.[61]

In the clinical setting, optical imaging can be used to assess tissue penetration. In fluorescence-guided surgery, IF-labeled drugs are administered prior to surgery to delineate tumor margin and to visualize drug penetration in solid tumors.[62] The optical technique can also be used to visualize the penetration of the IF-labeled drug in the resected tumor. An advantage of using surgical material to understand drug penetration over a 2D biopsy section-based assay is that the latter may be representative only of the immediate tissue surroundings. Multiple sections obtained during fluorescence-guided surgery may provide macroscopic and microscopic 3D information. Although the information on microscopic drug penetration may not be applied directly to optimize dosing in the same patient, data from these studies may help dose optimization in

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patients with similar disease characteristics and contribute to the understanding of mechanisms of drug penetration.

An example of macroscopic quantification by microdialysis is provided by a study measuring methotrexate in the extracellular fluid of brain tumors of four patients with recurrent high-grade gliomas after high-dose methotrexate (12 g/m^2).[18] Methotrexate levels were considerably higher in two patients who showed contrast enhancing regions of the brain by DCE MRI compared to two patients with non-enhancing brain regions.[18] The results of this small study suggest that methotrexate penetration into brain tumors is variable and that combining drug measurements with DCE MRI can be applied to predict drug penetration as a function of tumor perfusion.[18] That being said, clinical studies using microdialysis are currently too small to provide information on whether drug penetration relates with clinical outcomes.

Another example of assessing spatial drug gradients at a microscopic level with potential clinical implications has been provided for ado-trastuzumab emtansine (T-DM1), an ADC that binds to receptors of cells in close proximity to the vasculature.[19,63] T-DM1 drug penetration images in a HER2-positive xenograft mouse model (**Figure 2 A-C**) show that, at clinically relevant doses, the binding of T-DM1 to the HER2 target occurs at a faster rate than diffusion across tissues, with the drug becoming immobilized immediately outside of blood vessels, a phenomenon commonly referred to as the "binding site barrier." Lowering the payload to antibody ratio (DAR), by co-administering unconjugated antibody (trastuzumab) with T-DM1 at the same payload dose level, caused a larger fraction of antibody to compete and occupy receptors. This in turn allowed binding and internalization of the toxic payload to receptors across a larger number of tumor cells highly expressing the HER2 target (**Figure 2 D-F**). This method of lowering the DAR to improve homogeneity in payload penetration among tumor cells has been shown to increase response in the animals.[19,64] Although this study visualizes limited penetration of a ADC with a highly potent payload when target expression is high, the best strategy to homogenize the penetration of ADCs in patient tumors should be studied further in patients and furthermore correlated to patient outcome.

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Two recent studies sought to visualize microscopic drug penetration in resected tissues after fluorescence-guided surgery. The accumulation of fluorescently labeled bevacizumab, an anti-vascular endothelial growth factor (VEGF) antibody, was found to correlate with the pathological Bloom–Richardson–Elston (BRE) tumor grade (a score that indicates cancer aggressiveness)[20] in resected breast tissues from 19 breast cancer patients. In another study, the level of fluorescent labeled cetuximab, an anti-epidermal growth factor receptor (EGFR), accumulated within the resected tumor tissues of nine patients with head and neck squamous cell carcinoma **(**HNSCC), was correlated with cytokeratin (a measure of tumor density), EGFR expression, and Factor VIII (vascular density).[21] The latter biomarkers show that biological characteristics of the tumor may influence antibody (peri-) tumoral distribution and target binding.

Immunotherapy poses new challenges for assessing drug efficacy. The key event for successful immunotherapies is the ability to attract both the drug and the immune cells into the tumor microenvironment; i.e. immune cells, as opposed to drugs, thus become the mediator of antitumor effect. Tumor expression of PD-L1 might be required for response to anti–PD-1/ PD-L1–targeted therapies, but also the accessibility of tumor-antigen specific (PD-1 suppressed) immune cells to the tumor space is another important driver of immune checkpoint inhibitor efficacy. In melanoma patients, the spatial distribution and co-localization of immune cells immediately adjacent to PD-1/PD-L1 expressing tumor cells correlated with outcome of anti-PD1 therapy.[8,9] The distribution was assessed using histological sections of tumor biopsies collected from patients before anti-PD-1 therapy with outcome of anti-PD-1 therapy.[8,9] Molecular imaging of immunotherapies may show the presence and accessibility of the target, but it's use is still in its infancy.[22] PET imaging with ^{89}Zr-atezolizumab in NCT02453984 or with ^{89}Zr-pembrolizumab in NCT02760225 prior to the start of immunotherapy, may be able demonstrate the value immune-PET imaging for patient selection. Furthermore, the presence of other immune suppressive cells in the tumor microenvironment may predict resistance to immunotherapy.[65] Multiple other cell types may contribute to tumor mediated immune suppression, including regulatory T cells (Treg), type 2 natural killer T

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cells, tumor associated macrophages (TAMs), tumor-associated neutrophils (TANs) and myeloid derived suppressor cells (MDSCs) and therefore may influence the efficacy of PD-1 based therapies.[65] Importantly, an imaging biomarker of cytotoxic T-cell activity may be more valuable for predicting response to cancer immunotherapy than biomarkers characterizing the entire immune infiltrate. Accordingly, PET imaging of radiolabeled granzyme B, a protease released from $CD8^+$ T cells inducing apoptotic death of target cells, is currently under development.[23] In conclusion, a combination of target engagement/activation imaging and assessment of spatial heterogeneity in PD-1/PD-L1 expressing immune cells and tumor cells in the tumor microenvironment at the microscopic level may advance the prediction of response to immunotherapies.

Studying target binding at a cellular/molecular level: Illustration and tools

When systemic exposure (in blood) has been optimized and the drug has been shown to penetrate tumor tissues, the subsequent step is to demonstrate target engagement. Depending on the mechanism of action, target engagement can occur either intracellularly or extracellularly. For targeted drugs requiring internalization to be effective, intracellular accumulation should also be assessed.

State-of-the-art tools can be used to assess target engagement. At a macroscopic level, *in vivo* whole-body imaging (e.g. molecular imaging) may help to determine the presence of the target in the tumor lesion as well as the heterogeneity of the target expression across all lesions. Non-invasive *in vivo* imaging can be combined with standard pathology methods to provide absolute expression level (receptors/cell).

Molecular imaging has been applied to study target engagement through the visualization of the target's presence and accessibility (incl. the right conformation). It has largely been based on radionuclide imaging in the form of SPECT and/or PET. Other techniques such as optical, spectroscopy or photo acoustic imaging are also in clinical development.

At a microscopic level, standard pathology procedures on a tumor sample (*ex vivo*) such as immunohistochemistry (IHC) or immunofluorescence (IF), gene, RNA or

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protein expression measurements, are used to determine the presence of targets for therapy. However, these measurements should be appropriately validated allowing some quantification of these markers, as described in section 8.

Target engagement imaging could allow optimal drug selection and drug dosing. One **example** of optimal drug selection is the ZEPHIR trial (NCT01565200). It is the first prospective clinical study that sought to explore the clinical utility of HER2 imaging as a predictive biomarker to optimize treatment selection in advanced HER2-positive breast cancer. The study examined if low/absent radiolabeled trastuzumab tumor engagement, due to lack of target accessibility and/or drug penetration, could predict poor treatment response to HER2 targeted therapy, in this case T-DM1.[24]

In the trial, ^{89}Zr-trastuzumab PET/CT (HER2 PET/CT) and ^{18}F-FDG PET/CT imaging were performed at baseline prior to T-DM1 initiation. In an IHC/FISH confirmed HER2-positive population, one third of patients were found to be ‘HER2-negative’ based on HER2 PET/CT. Accordingly, the median time to treatment failure of the latter group was 3 times shorter than the ones with a more homogenously positive HER2 PET/CT. **Figure 3** shows a patient in which ^{89}Zr-trastuzumab drug does not reach its anticipated target. This patient’s lung metastasis was tested HER2 positive by IHC, but HER2 PET/CT showed a lack of penetration into the biopsied tumor. Response imaging on ^{18}F-FDG PET/CT after 3 courses of T-DM1 showed progressive disease.

Two ongoing clinical trials (study NCT02117466 and NCT01691391) show that dosing based on imaging of target engagement is feasible. In these studies, the uptake of ^{89}Zr-cetuximab assessed at day 6 after treatment onset is tested as a potential predictive biomarkers for early benefit of cetuximab as an EGFR targeted drug in treatment with colorectal cancer. The first results show that interpatient variability in PK and tumor uptake of ^{89}Zr-cetuximab only allowed dose escalation of cetuximab in six of 44 patients with mCRC.[25]

Another example is the use of target engagement for drug selection in neuroendocrine tumors (NETs). Somatostatin receptor (sstr)-based molecular imaging has been used to

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detect NETs overexpressing sstrs (initially using SPECT and more recently also using PET) and for the selection of candidates for therapies directed against these receptors. Moreover, sstr imaging may also be used to optimize drug dosing (i.e. dosimetry) when radiolabeled somatostatin analogue based treatment (i.e. peptide-receptor radionuclide therapy (PRRT)) is considered in advanced, well-differentiated somatostatin expressing NETs.[66–68] In addition, PRRT can be used as theranostic,[10] using the same peptide labeled with diagnostic nuclide such as ^{68}Ga-, ^{111}In for imaging and ^{177}Lu or ^{90}Y for radiotherapy. However, the dosimetry approach is still under debate due to conflicting results in dose-effect relationships.[26,69] Most PRRT are still given according to a fixed activity administration scheme,[70] or use pretreatment scans to adjust dosing based on organ uptake to avoid toxicity.[71] In addition, randomized studies comparing fixed versus tumor image-driven dosing are lacking.

Measurement of expression of the target may not always translate into a correct prediction of target engagement due to many interfering factors. An example of a factor which precludes optimal target engagement prediction based on standardized target expression measurements comes from a comparison of HER2 epitopes. A recent study[27] using a quantitative IF technique demonstrated the importance of the binding epitope on the target, in HER2-positive breast cancer. A comparison was made between quantification of HER2 based on its intracellular domain epitope (ICD, one used for IHC in clinical setting) versus its extracellular domain (ECD, binding epitope of trastuzumab) and their relation to treatment outcome of adjuvant chemotherapy and trastuzumab. This comparison showed that ECD was the most important predictor for a favorable treatment outcome, rather than ICD.[27] This study shows that caution should be applied when opting to characterize binding to a target using molecular imaging tools binding an epitope different than the therapeutic. When available, optimizing the pathology platform for assessing target expression based on multiple epitopes or pathways can be used to optimize treatment selection, like for HER2 directed drugs against multiple epitopes or downstream pathways.

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The presence of high molecular weight mucins may mask the binding epitope on the target and thus impede target engagement. Transmembrane mucin MUC4 has been reported to hinder the accessibility and hence the binding of trastuzumab to HER2 ECD, thereby impairing sufficient binding of trastuzumab to tumor cells.[72] Therefore, reducing MUC4-masking with mucolytic drugs improved HER2-accessibility, resulting in a higher anti-tumor effect of trastuzumab in HER2/MUC4-positive xenograft models.[28] Similarly, altered glycosylation in cancer cells increases sialic acids and carbohydrate structures called "tumor-associated carbohydrate antigens" (TACAs) within the cell surface's sugar coating, or glycocalyx, which may prevent immune cells to trigger or evade immunological recognition. Targeting the glycocalyx by sialidase conjugation to trastuzumab has been shown to preclinically enhance the cell-mediated cytotoxicity preclinically,[73] and vaccines against TACAs are being developed.[74] Clinical studies are needed to show whether reducing mucin masking or targeting the glycocalyx is applicable in patients.

Uptake of drugs by tumor-associated macrophages (TAMs) could be another barrier to target engagement. Pegylated liposomes were taken up primarily by macrophages in the tumor, whereas the same liposomes containing anti-HER2 antibody on the surface distributed over HER2 overexpressing tumor cells with similar overall tumor tissue accumulation.[29] Therefore, the interest for TAMs in oncology is not limited to their role in suppressing anti-tumor immune therapy response, but extends to the fact that they may limit drug target binding through macrophage-directed drug clearance[75] especially of lipophilic drugs,[50] or by removal of immunotherapeutic antibodies from immune cells.[76]

Drug transporters add another layer of complexity in intracellular target engagement.[77] P-glycoprotein (P-gp, *ABCB1*) and breast cancer resistance protein (BRCP, *ABCG2*) have established roles in conferring multidrug resistance by limiting intracellular drug accumulation in tumor cells. For example, polymorphisms in these efflux transporters and an increase in messenger RNA expression correlated with relapse and survival in 263 Chinese intermediate-risk acute myeloid leukemia (AML)

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patients treated with anthracycline and cytarabine.[30] However, up until now it is unclear whether transporter-mediated drug efflux by P-gp and BCRP leading to reduced intracellular drug accumulation actually occurs in tumor cells in patients.[78] Imaging agents need to be developed to quantify drug accumulation in tumor cells in patients. Unfortunately, the development of inhibitors of specific drug transporters has failed to provide benefit in the clinic to date.[78] A more targeted approach to stratify patients based on multidrug transporter expression and/or function should be considered. Further research is needed to understand how transporter expression can be used to provide information on dose selection.

Studying expression of pharmacological activity following target binding: Illustration and tools

We distinguish between proximal target binding and subsequent distal expression of pharmacological activity (e.g. antibody target binding *versus* downstream signaling response). This distinction may clarify how the drug mechanism of action modulates the biological effects following successful binding and provide insight into the drug's downstream effects. This may inform the existence and extent of a pharmacological link with outcome. Pharmacological activity at the protein or RNA expression level or downstream pathway activation can be assessed using **tools** as described in previous sections.

In recent first-in-human studies of drugs directed at the androgen receptor (AR) such as enzalutamide[32] and apalutamide[31] - two non-steroidal anti-androgens - molecular imaging was used to determine the optimal biological dose. The uptake of ^{18}F-fluoro-5α-dihydrotestosterone (FDHT, an endogenous dihydrotestosterone analogue) reflects AR expression and binding capacity. Therefore, the pharmacodynamic biomarker ^{18}F-DHT in PET-CT imaging gauge pharmacodynamic response to these treatments.[31,32] Uptake of FDHT reached a plateau at a dose of 120 mg apalutamide[31] and 150 mg for enzolutamide[32], suggesting maximal AR binding capacity and thus achievement of the optimal drug concentration. The recommended—

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and later FDA-approved —dose of apalutamide based on this study was much lower than the maximum tolerated dose.[31]

16α-[18F]-fluoro-17β-estradiol (^{18}FES) imaging has been used to predict responders to endocrine therapies targeting the estradiol receptor (ER): the absence of FES uptake at baseline may predict endocrine treatment failure in patients with ER (+) breast cancer.[79,80] In a feasibility study assessing ER availability before and during fulvestrant treatment, incomplete reduction of the ER target was observed after fulvestrant administration in six of 16 metastatic breast cancer patients (38%).[36] In addition, FES was used as a biomarker to assess efficacy of novel ER treatments such as Z-endoxifen (the most potent of the metabolites of tamoxifen), RAD1901(a novel, oral, ER ligand) and GDC-0810 (a selective ER degrader).[33–35] For the latter, a phase 2 study was designed with an optimal dose of GDC-0810 selected using the ER target engagement measurements.[35]

HER2 imaging with ^{89}Zr-trastuzumab might be a surrogate of the efficacy of novel agents like the heat shock protein 90 (HSP90) inhibitor luminespib (NVP-AUY922). HER2 is a sensitive client protein of HSP90, and was shown to be depleted by HSP90 inhibition with luminespib in preclinical experiments.[81] ^{89}Zr-trastuzumab PET was used to determine the *in vivo* degradation of HER2 caused by the drug. The change between tumor uptake on baseline and early ^{89}Zr-trastuzumab PET after 3 weeks of treatment with this HSP90 inhibitor had a moderate positive correlation with change in tumor size on CT after 8 weeks of treatment in 29 lesions of five patients, showing that HER2 imaging can be used to assess target degradation and response to novel agents, such as luminespib.[37]

Another example of imaging pharmacodynamic markers is the measurement of platinum-adduct formation (the covalent binding of carboplatin/cisplatin or oxaliplatin to nuclear DNA by IF,[39] by LC-MS [82] or inductively coupled plasma-mass spectrometry (ICP-MS).[40] Platinum adducts in tumors are highly variable between patients and small

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studies show that these may be more predictive of treatment response than platinum exposures in plasma.[38–40]

The role of modeling and simulation to integrate multiscale information and provide dosing guidance

To understand the effect of drug penetration at all levels on treatment response, information obtained from multiple levels and at temporal scales needs to be simultaneously considered. Multiscale models integrating information of drug distribution in spatial and temporal scales will be needed to understand macroscopic and microscopic distribution of drugs and to optimally guide personalized dosing[41] (**Figure 4**).

Preclinical information derived from either in vitro or in vivo experiments such as receptor internalization rate, binding affinity and the affinity for drug transporters (measured in cell culture), can be paired with clinical plasma PK,[44] along with preclinical[64] and clinical imaging data[10,83] to simulate events at molecular, cellular, and tissue levels based on data from imaging, blood samples, and biopsies **(Figure 4, top).** The diversity in the tumor environment should then be linked to spatial heterogeneity in the cellular states across the tumor.[84] In addition, mechanistic information from preclinical pharmacology models can be used to further understand drug dose-drug penetration-drug activity relationships.[85] When experimental data containing spatial information are obtained, image processing can reconstruct the relative order, geometry, topology, patterns, and dynamics of the two-dimensional (2-D) tissue sections. A three-dimensional (3-D) tumor is created, which can then be used to simulate temporal growth and evolution **(Figure 4, middle).**[41] By integrating all drug penetration information into spatiotemporal models, **(Figure 4, bottom)** one can predict the dose needed for the optimal response (maximal binding of target receptors) using prior drug information and data from both the individual and a similar population of patients**.** In such spatiotemporal models, the microscopic and macroscopic spatial scales as well as the temporal scales should be considered.[41] These temporal scales

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may encompass milliseconds for molecular interactions, hours for PK changes, days for tumor growth and weeks to years for disease evolution.[41] In such a model, the effect of drug transporters and specific factors that hinder target engagement, or change target expression during treatment, can be tested at all spatiotemporal levels.

PK-PD modeling approaches provide a powerful tool to integrate time-dependent exposure and response data to predict treatment outcomes.[42] However, in most current PK-PD models, drugs are assumed to distribute homogeneously in the tumor tissue. To describe the spatial distribution of drugs in tumors, common mathematical models involving ordinary or partial differential equations (PDE) or agent based modeling (ABM) can be applied.[41] ABM is an increasingly popular modeling approach where individual discrete "agents" are simulated that can interact according to some pre-specified rules; agents can simulate spatial heterogeneity, by moving along a three-dimensional lattice, thus accounting for spatial and temporal information.

An example of a multiscale model is the Oncosimulator.[86] In this model, clinical, imaging, molecular and treatment schedules are combined to predict response to anticancer treatments and radiotherapy.[86] MRI images (T1 with contrast enhancement, T2 and T2 flair) of patients with nephroblastoma before treatment onset and after 4 weeks of chemotherapy were used to validate the model predicted tumor size changes. Model-predicted tumor sizes were compared with an automated segmentation of the MRI images of the tumors and with clinical experts' annotation.[86] Inclusion of models assessing the heterogeneity in tumor drug penetration in the Oncosimulator models may further improve the predictive value of the models. Other examples show how the spatial measurements at three levels (systemic, tissue and cellular level) can be combined. First, Ribba *et al.* used longitudinal data a from multiple state-of-the-art techniques to describe the tumor drug penetration of an immune-stimulatory drug Cergutuzumab amunaleukin (CEA-IL2v). The non-linear plasma concentration-time profiles and IL-2R positive cells in peripheral blood of 50 patients were described using a TMDD model.[87] A Krogh cylinder model (a model describing the spatial drug gradients from the tumor blood vessels) was used to describe the extravasation and diffusion of the drug, thereby predicting the expansion of the target cells in the tumor by immune

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activation. The predicted tumor drug penetration was validated using measurements of drug uptake in tumor lesions following administration of 89Zr-labeled CEA-IL2v at 3 sequential timepoints in 14 patients. The final model was used to identify a dosing regimen with an optimal antibody tumor uptake in patients. Quantification of radiolabeled drugs per tumor site was accomplished here using an uptake scaling factor at the level of the extravasation processes, the rate limiting process of drug uptake. In future studies, such a quantification can conceivably be expanded by using tumor vascularization and expression data to determine the temporal microscopic distribution and response in each lesion.[19] Such models could be extended by using information about the molecular aspects of the drug of interest. For example Checkley *et al,* used a cell cycle model and incorporated mechanisms of DNA damage and repair based on *in vitro* and *in vivo* tumor growth experiments to describe the effects of an ATR inhibitor (AZD6738) and ionizing radiation.[88] When information about mechanism of action and spatial drug distribution at all four levels are combined, models such as those we presented can bridge the gap between preclinical experiments and clinical observations. When these models are correlated with clinical outcomes, the model structures may have re-usability across drugs with the same mechanistical properties.[89]

Challenges / prospects

As all four levels of biological organization we described are linked, understanding each aspect will inevitably lead to a cascade of interactions. To make precision dosing a clinical reality, optimization of all these processes is needed simultaneously.

Assessment of **systemic exposure** can become an integral part of precision dosing when adequate PK assays and data analyses are used to estimate the individual PK-profiles in bloodNonlinear mixed effects models combine structural models with estimates of nested variability in clinical observations, enabling estimation of means and variances of the statistical distributions of model parameters. Such a population approach may be used to calculate individual PK- parameters in blood, limiting the need

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to design and plan for very specific sampling strategies. Moreover, this approach utilizes both individual PK parameters and population estimates to quantify non-linearity in drug clearance and target-mediated drug disposition (TMDD).

Assessment of **drug tissue penetration** can become an integral part of precision dosing when carefully timed tumor biopsies during treatment or alternative non-invasive techniques are available. A challenge when assessing drug exposures is that the biopsy should be performed at specific times after dosing , chosen to be relevant to the temporal evolution of the drug's action. In addition, in a significant proportion of patients biopsies cannot be performed, owing to difficult tumor (metastasis) locations and low percentage of cancer cells in some samples,[90] specifically when these biopsies are taken during an effective treatment. While the I-SPY 2 TRIAL shows that it may be feasible to take a biopsy during treatment,[91] non-invasive techniques for macroscopic visualization of drug penetration or combination with other techniques such as fluorescence-guided surgery may help collection of the optimal study samples. A limitation of techniques such as image intensities by MALDI-MSI, IHC and immunofluorescence is that the resulting images do not allow comparison between patients. These measurements rely heavily on the specific settings used and show large variability among different assessments.[92] Efforts towards quantitative MALDI-MSI measurements of drug have been demonstrated in preclinical samples;[93] however, drug detection still requires the MALDI-MSI methods be developed and optimized for each drug of interest. An example that standardization of MALDI-MSI is possible is provided in the application of MALDI to detect the presence of bacteria in infectious diseases.[94] If appropriately validated, estimated absolute levels (e.g. drug or proteins/cell) would provide a dramatic improvement in uniformity across labs and comparisons between targets.

Target engagement molecular imaging can be used to perform precision dosing when both feasibility and benefit are confirmed in prospective clinical trials. One of the limitations of target engagement molecular imaging using SPECT/PET is the fact that radiolabeled molecules will irradiate the patient for a certain time conforming to the

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decay of the chosen radionuclide. Using long-lived radioisotopes, which are required for large molecules with long circulation time, will therefore result in higher radiation dose to the patient. One approach to avoid this is to use smaller protein scaffolds (affibodies, diabodies and nanobodies). An example of the latter is given by ^{68}Gallium HER2 nanobodies,[95] single domain antigen-binding fragments, that exhibit rapid targeting and fast blood clearance, high solubility, high stability, easy cloning, modular nature compared to radiolabeled HER2 antibodies. These tactics may result in different distribution relative to the therapeutic drug.[96,97] Binding of a targeted drug is localized onto a specific domain (i.e. epitope). The target presence therefore does not guarantee target engagement. Therefore, opting for these approaches may in fact be more suitable for determining the expression of pharmacological activity (discussed in section 6) rather than target engagement. Current tools to assess factors that hinder target engagement and the downstream pharmacologic effect provides mechanistic insight when predicting target engagement. However, with the current knowledge gap and lack of pharmacological tools to eliminate these factors, we can only speculate how assessing drug transporters, e.g. MUC4, or other factors may help with dose selection in the future.

Feasibility challenges of executing target engagement imaging include the cost of implementing molecular imaging in the clinic (imaging equipment, radiolabeled probes, and personnel costs for the required expertise).[10,98] A close collaboration between the nuclear medicine department, clinical pharmacologists, and medical oncologists is needed to implement target engagement imaging. Also, when multicenter studies are performed, evidence that the final radiolabeled drug products and manufacturing processes are comparable between preparing institutions should be provided.[98] A multicenter trial like SAKK 56/07, where validated PET or MRI imaging technique are applied in multicenter trial for response evaluation show that molecular imaging can be applied in larger populations.[99] The widespread use and reimbursement of ^{18}Fluorodeoxyglucose FDG-PET in solid tumor diagnoses and assessment of treatment response shows that standardized, radiolabeled techniques are able to influence how we diagnose and treat patients.[100] Given the advances in target engagement imaging,

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one day target imaging may replace some of the current pathology techniques for treatment guidance. A clinical trial investigating this hypothesis is the IMPACT-MBC (NCT01957332). This study compares the impact of FES-PET and ^{89}Zr-trastuzumab–PET with the gold standard (tumor biopsy) on treatment decision and outcomes in newly diagnosed metastatic breast cancer. The results of this trial will show whether target engagement imaging improves treatment outcomes compared to standard pathology.

The last level is **expression of pharmacological activity** following target binding, which allows the classification of a drug's pharmacodynamics between proximal (direct measures of target engagement) and distal (indirect measures of effect) measurements. A disconnect between positive observed target engagement and negative expression of pharmacology may suggest a partial understanding of the interconnected pathways the drug is expected to modulate, and in turn an incomplete understanding of the underlying biology. Currently a variety of detection platforms and assays are used to determine pharmacological activity, making validation among platforms and between laboratories crucial. Degradation or instability of proteins and (micro)RNA may limit the interpretation of the data.[101] As an example, in the Analysis for Therapy Choice (NCI-MATCH), a national signal-finding precision medicine study that relies on genomic assays to screen and enroll patients with relapsed or refractory cancer after standard treatments, a next-generation sequencing (NGS) RNA and DNA assay was validated in multiple laboratories prior to study onset.[102] In contrast, the presence of PD-L1 by IHC, which is used to select or stratify patients for PD-1/PD-L1 related studies, is not yet standardized and different cut-off values and scoring systems are used. These factors may explain some differences in the correlation between PD-L1 expression and outcome seen among studies.[103] This suggests a need for standardization and more sensitive and specific diagnostic tests.[104]

To bring all sources of data together, **modeling and simulation** may be used to perform precision dosing after prospective validation and clinical implementation. Darwich *et al.* describe ample evidence to support the use of Model Informed Precision Dosing (MIPD) tools to derive therapeutic recommendations for individual patients, but

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also stress that there is little evidence of its use and impact within clinical care.[42] Although this review did not specifically address the issue of studying heterogeneity in drug penetration, many of the suggestions to improve clinical implementation apply. Examples of improvement are the need for extensive model validation; prospective clinical evaluation; the perspective of developing MIPD as a companion tool together with other diagnostic tools, such as imaging probes and other biomarkers in the early stage of drug development.[42] Preclinical information used in these mechanistic models to inform personalized cancer treatment may be biased due to lack of translatability between preclinical experiments and patients. Therefore, each assumption needs to be validated e.g. by performing sensitivity analyses or prospective validation. Furthermore, spatiotemporal models have not been extensively validated in the clinic, and many steps are needed before these models can provide individualized dosing information.

A vision to design prospective clinical trials including drug penetration measurements

Multiple tools have been identified to help inform optimal treatment strategies, (pre)clinical studies provide evidence that drug measurements are the key to successful personalized dosing, and the key challenges for clinical implementation have been defined, so the last step is to discuss optimal implementation of measurements at a systemic, tissue and cellular/molecular level into clinical oncology practice to create the premise forprecision dosing.

We envision that, before treatment, non-invasive imaging-based measurements using the radiolabeled drug can assess the presence of the target in the target lesion, and the heterogeneity in abundance of the target in all lesions (**Figure 5** left). In addition, measurements related to the tumor microenvironment (e.g. vascularity, hypoxia, tumor stiffness, immune cells) may provide information to predict drug behavior and allow optimal drug selection. During treatment, performing plasma PK sampling may guide optimal systemic exposure and help assessing the maximum binding capacity (**Figure 5** right). When a biopsy is available during treatment or surgical

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resection is performed, this tissue material can be used to visualize drug penetration at a microscopic level. Additional imaging during treatment may inform on drug-response and/or drug resistance, either through target binding, or downstream expression of pharmacology, or (preferably) both. The information gathered during treatment can be used to decide whether drug dosing should be deescalated or escalated. When this information is integrated with preclinical and prior knowledge using multiscale models, this may further support adaptation of the treatment decision (**Figure 5** bottom).

The use of image-based treatment selection and dose optimization as proposed in **Figure 5** needs to be supported by prospective studies to i) assess whether image-based or standard assessment guided treatment provide better outcomes and ii) assess whether drug doses can be modified according to intra-tumor drug measurements. Freidlin *et al*. provide the methodologies to efficiently incorporate such biomarker-driven enrichment strategies, with the most efficient example provided by enrichment designs,[105] in which only patients that show high target engagement, high tissue penetration and high systemic exposures in imaging studies are to be randomized over a new treatment versus standard of care. Furthermore, a Bayesian approach[106] can be used to integrate clinical and preclinical data (**prior information**) to optimally inform dosing and speed up decision making. Then, an adaptive design can be used to efficiently test optimized dosing strategies in patients.

Conclusions

Recent advances in technologies at a macro-and microscopic level improve the visualization and assessment of drug penetration in solid tumors at the systemic, tumor tissue and cellular or molecular level and the expression of pharmacological activity following target engagement. Individual (pre-)-clinical studies of tumor drug penetration measurements to date, although small and generally retrospective in nature, suggest that “precision dosing”, i.e. personalized dosing based on drug penetration in a solid tumor, may improve outcomes in patients. Unambiguous assessment of the benefits of precision dosing require clinical investigation of anticancer drug distribution in randomized trials at the four biological levels that we outlined, with the results being

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analyzed using integrative, mechanistic models including spatial and temporal understanding of drug penetration. This review shows, that in today's era of potent targeted drugs, precision dosing remains the missing piece of the current oncology precision medicine puzzle.

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Figure Legends

Figure 1: *The pathway of drug administration to the tumor response is affected by tumor drug penetration at four levels. (1) the systemic level (the concentration of the drug in the blood pool, which determines how much of the drug is available for tumor penetration), (2) the tissue level (e.g. is the drug able to distribute throughout the tumor tissue, as influenced by the tumor microenvironment), (3) the cellular or molecular engagement level (where the drug is able to engage and interact with its target at the cellular/molecular level, a proximal or direct measure of drug mechanism of action), and (4) the expression of pharmacological activity following target engagement (a distal or indirect measure of drug pharmacodynamics). All these levels will be affected by responses to treatment (bottom).*

Figure 2: *At clinically relevant doses, the binding of T-DM1 to HER2 expressing tumor cells is limited to the cells near functional blood vessels, and much higher doses are needed to provide a more homogeneous penetration, as shown at the microscopic level in a HER2 expressing xenograft tumor model (NCI-N87 xenograft). A) An immunofluorescence image of a tumor 24 hours following administration of 3.6 mg/kg of Alexa Fluor 680 tagged T-DM1 - a dose comparable to the dose used in patients- to nude mice bearing NCI-N87 flank tumors (green). Immunofluorescence staining with CD31-AF555 (red) shows tumor vasculature, and intravenous administration and visualization of Hoechst 33342 shows functional vessels (blue) using* multiplexed imaging*. B) HER2 expression (ex vivo staining with trastuzumab) in the same tumor section (white) and enlarged C), indicating the uptake in the tumor was only sufficient to target a few cell layers. Images D, E, F show the same visualizations 24 hours following administration of 3.6 mg/kg of Alexa Fluor 680 tagged T-DM1 and 10.8 mg/kg unlabeled trastuzumab (14.4 mg/kg total in a 1:3 ratio), indicating a more homogenous tumor penetration of T-DM1. This dose reached many cells but did not occupy all accessible receptors in the tumor. Much higher doses up to 32 mg/kg of a combination of T-DM1 and trastuzumab, in a 1:8 ratio (the latter to avoid ADC toxicity and improve penetration) were required in this animal model (with high HER2 expression, ~1 million receptors/cell) to reach all cells (data not shown).*

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Red = CD31+ staining, Green = 3.6 mg/kg T-DM1-AlexaFluor 680 (A-C) or 3.6 mg/kg T-DM1-AlexaFluor 680 + 10.8 mg/kg untagged trastuzumab (D-F), White = HER2 (trastuzumab labeling of histology slide), Blue = functional vessels (intravenous Hoechst 33342).

Figure 3*: Lack of correlation between HER2 assessed by IHC and ^{89}Zr-trastuzumab uptake in the same lesion. A HER2 positive tumor of a metastatic breast cancer patient with lung metastasis was visualized using (A) ^{18}Fluorodeoxyglucose (FDG) PET/CT, (a marker of tumor metabolism) but not with (B) HER2 PET/CT (non-significant tracer uptake). Pre-treatment biopsy of a right metastasis in the middle lobe (C) shows IHC 3+ staining (antibody recognizing the intracellular domain of the receptor). Response assessment (D) with FDG-PET/CT shows progressive disease after 3 courses of T-DM1.*

PET= positron emission tomography, IHC= immunohistochemistry, T-DM1= ado-trastuzumab emtansine

Figure 4: *Drug development typically proceeds by optimizing molecular properties of target engagement and access (e.g. biophysical binding and cell culture methods) followed by preclinical studies (ex vivo and in vivo measurements) and eventual human trials to determine clinical endpoints. Here we present a vision of how we can use in silico methods to help bridge the gap between these methods to a more comprehensive understanding (top). These same approaches can be used to integrate personalized data (imaging, plasma clearance, biopsies) with computational models containing preclinical and in vitro data to develop personalized dosing schemes (bottom).*

Figure 5: *A vision for incorporating tumor drug penetration imaging to guide precision dosing. Non-invasive and invasive measurements can be applied to optimize treatment selection (prior to treatment initiation, left side) and to monitor and optimize drug dosing (during treatment right side. See text for further details).*

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Levels	Aim	Tools		Potential clinical relevance	Examples
		Macroscopic level	Microscopic level		
1. Systemic exposure	Ensure optimal bioavailability in blood to reach the maximal binding capacity in tumor tissues	*Pharmacokinetic measurements in blood ✓ Immunoassays for large molecules ✓ LC-MS(MS)/HP-LC for small molecules ✓ (radio)-labelled drugs *Molecular imaging: ✓ PET/SPECT (non-invasive)		Optimize dose (to overcome the tissue sink)	* ^{89}Zr-Trastuzumab PET imaging and plasma PK to understand the tissue sink effect[14] * Plasma PK of RG7356, an anti- CD44 humanized antibody to define optimal dose for phase 2 study instead of MTD[15] * Linear plasma PK of nivolumab and durvalumab may reflect severity of the disease, and may not be useful to guide dose adjustments[16,17]
2. Tissue penetration	Assess tumor vascularization, immune infiltration and other factors in the tumor microenvironment	(Labeled drug)- molecular imaging: ✓ PET/SPECT ✓ DCE-MRI ✓ Angio-CT/ SPECT * Microdialysis *Optical imaging	*IHC/ immunofluorescence *MALDI-MSI *Multiplexed ion beam imaging	Optimize treatment selection and understand mechanism of action.	* Microdialysis of methotrexate[18] * Immunofluorescence imaging T-DM1[19] * Fluorescent labeled bevacizumab/ cetixumab-guided surgery[20,21] * Immunotherapies: radiolabeled PD-L1[22] or granzyme B PET imaging[23]
3. Cellular/molecular concentrations	Ensure the presence/accessibility of the target	Labeled drug- molecular imaging: * PET/SPECT Imaging barriers of target engagement * Genomics	Biopsy based assay to detect the presence of the target and the presence of factors that limit target: *IHC /Immunofluorescence	Optimize treatment selection	Macroscopic imaging: * ^{89}Zr trastuzumab and T-DM1[24] * dose escalation guided by ^{89}Zr cetuximab[25] * Somatostatin receptor imaging e.g. ^{177}Lu-Dotatate treatment[26] Interference factors: * ICD/ECD HER2 expression[27]

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					MUC4 and trastuzumab[28] * TAM uptake of lipidic nanoparticles[29] * ABCB1 polymorphism -anthracycline and cytarabine[30]
4. Expression of pharmacology	Ensure that sufficient target modulation has been achieved, assess drug efficacy and predict drug resistance	Molecular imaging: * PET/SPECT	*Imaging of pharmacodynamic markers * E.g. platinum adduct by immunofluorescence	Change treatments, and optimize dosing	* ^{18}F-fluorodihydrotestosterone androgen receptor imaging post apalutamide[31] and enzalutamide[32] * ^{18}FES imaging post RAD1901/fulvestrant/ Z-endoxifen[33–36] * ^{89}Zr trastuzumab HER2 response imaging post HSP90 inhibitor[37] * Platinum adducts after carboplatin administration[38–40]

Table 1*: State-of-the-art technologies that can be applied to assess specific aspects of drug penetration related to the systemic level, the tumor tissue level and the cellular or molecular level.*

LC-MS= Liquid chromatography–mass spectrometry, HPLC= High-performance liquid chromatography , PET= positron emission tomography, SPECT= Single photon emission computed tomography, PK= pharmacokinetics, 18FES= FES16α-[18F]-fluoro-17β-estradiol, IHC= immunohistochemistry, ICD/ECD HER2: intracellular or extracellular domains of the human epidermal growth factor receptor, T-DM1 = ado-trastuzumab emtansine

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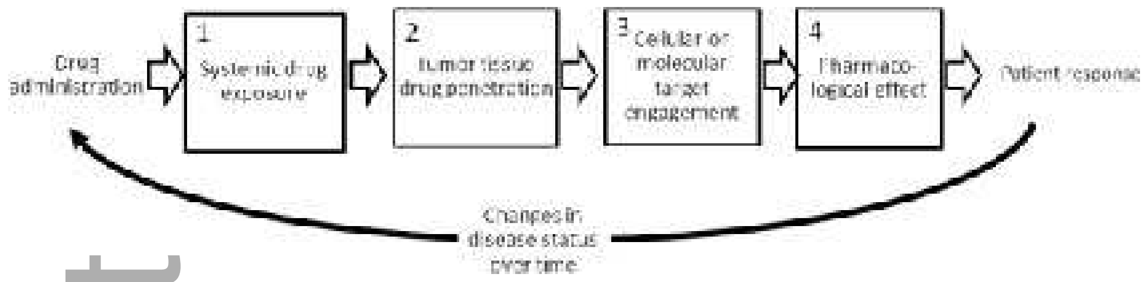

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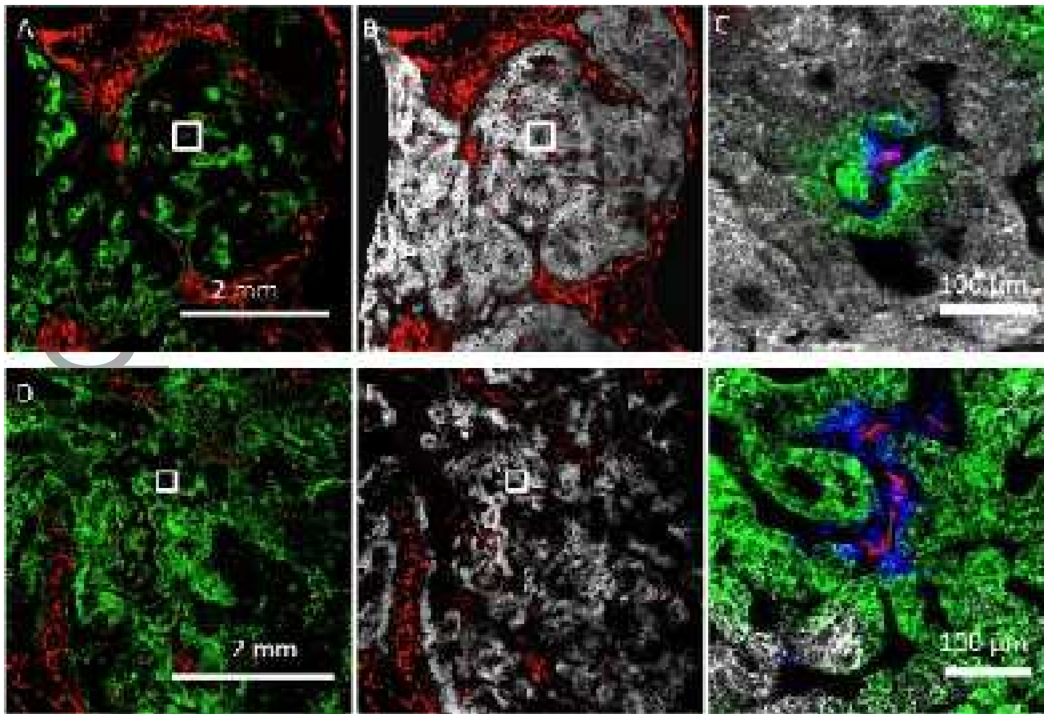

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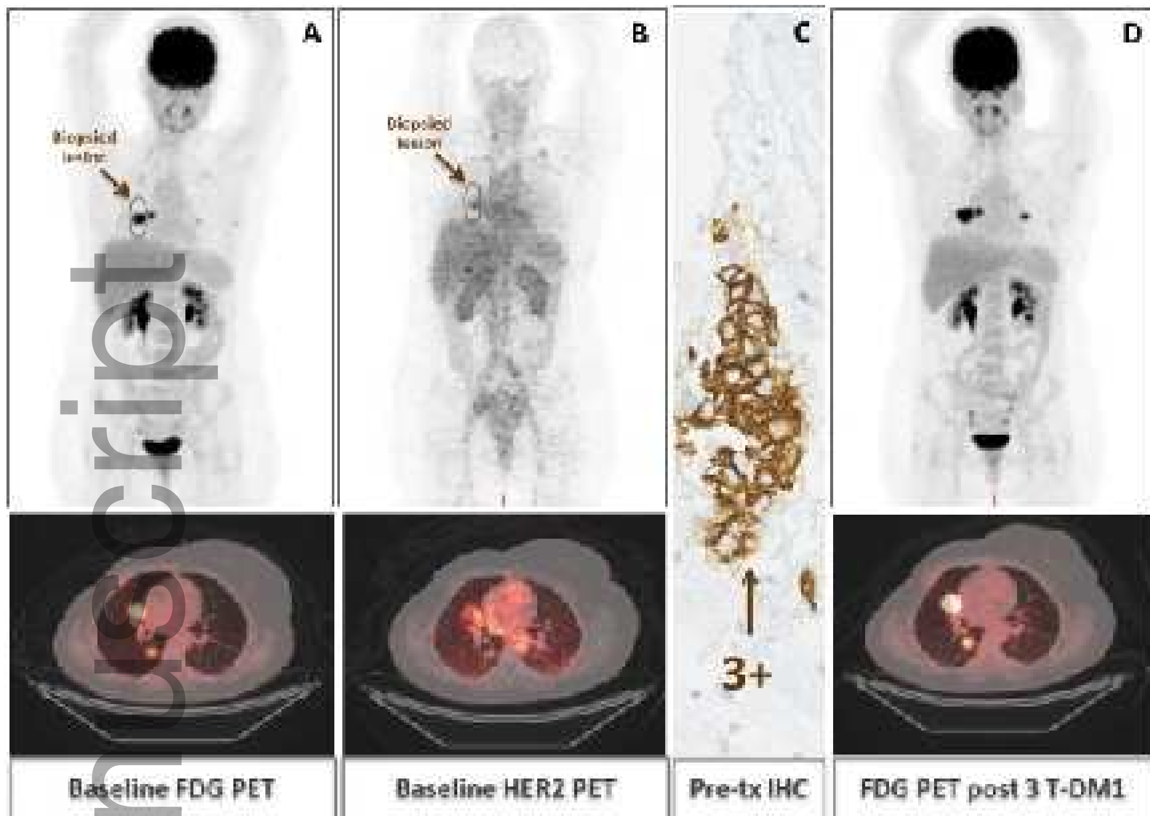

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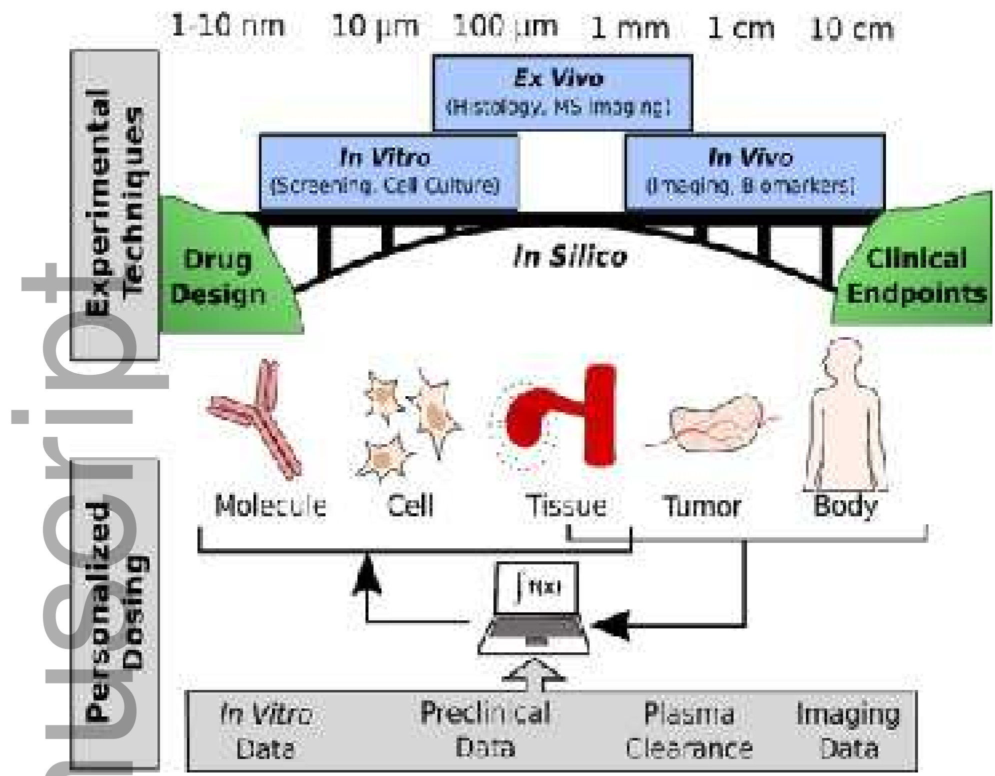

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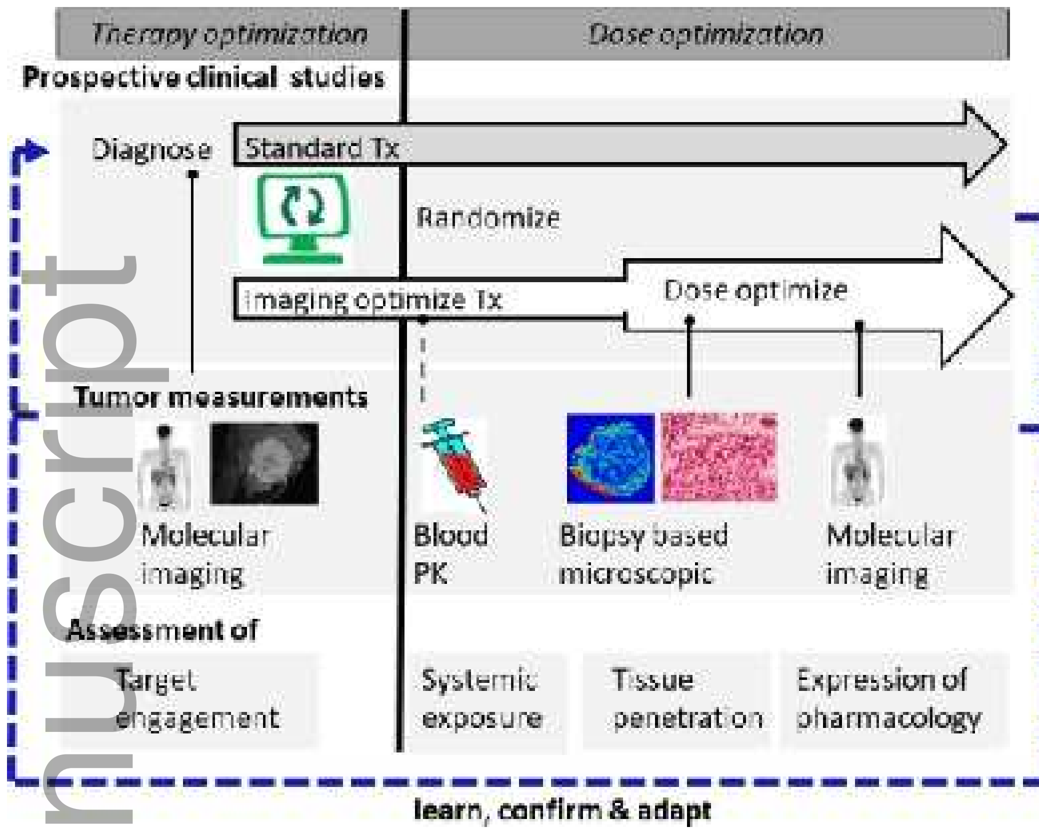

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