Eur J Drug Metab Pharmacokinet (2010) 35:15–22
DOI 10.1007/s13318-010-0003-z

ORIGINAL PAPER

In vivo target recognition with high-resolution imaging: significance for drug development

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Received: 5 January 2010 / Accepted: 22 April 2010 / Published online: 18 June 2010

Abstract In vivo target identification is basic for understanding mechanisms of drug action. Target identification requires cellular resolution. Extrapolation from blood bioavailability, low-resolution scans, radioassays, or in vitro tests regularly produce false-negatives and false-positives. Common ADME procedures disrealize organ complexities. While low-specificity high-capacity sites of deposition are easily recognized, high-specificity low-capacity receptor sites remain hidden. Serious limitations of target recognition are revealed in comparative studies with three methods: high-resolution microscopic autoradiography, radioassay, and whole-body autoradiography. With radioassays and whole-body autoradiography, many targets are simply undetectable. For example, high-resolution microscopic target information for vitamin D, gained 20–30 years ago, was widely ignored. The narrow calcium focus for this multi-target and multi-function hormone was perpetuated until recently through deficient results from conventional assays together with related expert bias. Thus, follow-up has been delayed on discoveries from the use of unconventional histopharmacology methods, pointing at important actions and therapies beyond systemic calcium regulation. High-resolution 'in vivo' target identification with associated functional characterization is useful not only for understanding mechanisms of action, but also for providing leads for innovative and successful drug development and prediction.

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Keywords Receptor · Histopharmacology · Imaging · Autoradiography · Arndt-Schulz rule · Hormesis · Vitamin D · ADME · Drug localization · Drug homunculus

"For the successful development of a drug it is important to know 'where it goes'." (Leake 1969)
"Don't homogenize the brain. The brain you are homogenizing may be your own." (Lloyd J. Roth) (Stumpf 2003)

1 Introduction

In vivo target recognition is crucial for understanding drug actions. Imaging efforts are aimed at recognizing sites of deposition and metabolism ['sites of loss' (Veldstra 1956)] and specific sites of action. Since target cell populations do not exist in isolation, but are associated with and functionally related to non-target cells, methods with cellular resolution are needed to distinguish one cell from another, in a way that separates specific target association from non-specific disposition. Target bioavailability cannot be assessed reliably from whole organs or dissected chunks, nor from whole-body imaging, and certainly not by extrapolating from blood bioavailability. Moreover, there is evidence that different target tissues of the same compound have different affinities and pharmacokinetics (Koike et al. 1998).

Surveys, computer models, and information not based on detail, i.e., without the necessary sensitivity and resolution of tools and mindset, are prone to misinterpretation. Methods can directly cause success or failure in drug development. There is evidence that reliance on common expedient methods can be inexpedient, misleading and costly.

Data about in vivo targets, target bioavailability, and target pharmacokinetics are incomplete or unavailable for

many, possibly most, drugs. There is no regulatory requirement for a target profile, albeit such data are helpful, even indispensible, for assessing effects, side effects, prediction, and toxicity. Efforts toward reliable and efficient methods were made in applied and basic research, but encountered difficulties with tissue surveys together with cellular resolution, for simultaneously preserving both tissue structure and in situ drug localization. These dual requirements posed almost insurmountable problems and pitfalls that resulted in many failed attempts. The complexity of this endeavor and related problems has been discussed (Stumpf 2003; Roth and Stumpf 1969). Current radioassays, whole-body autoradiography, nuclear imaging, and similar scanning procedures do not satisfy all of the basic requirements that include authentic preservation of in vivo localization, cellular–subcellular resolution combined with tissue surveys, unaltered chemical properties of labeled compound, and sufficient signal strength to permit both high- and low-dose studies. Over-interpretation of data with false-positives and false-negatives is an associated danger. While recognizing some utility of current imaging methods, their individual resolution and sensitivity vary and respective limitations must be kept in mind. None of these singular methods satisfies the three requirements: to reach the cellular–subcellular level, simultaneously provide detailed and holistic tissue orientation, and to keep the original compound and metabolites under investigation in their 'in vivo' place. Microscopic resolution without loss and translocation during tissue processing is required (see "Addendum" below).

The focus here is on lacking essential 'in vivo' information in contemporary pharmaceutical studies.

2 The dose

In as much as the dose makes the poison (Paracelsus 2003), the dose makes the medicine (Stumpf 2006). The Greek term pharmacon, meaning both drug and poison, reflects the inherent enantiodromia, the polarity which is expressed in the Arndt-Schulz rule of 1887, a fundamental and widely applicable concept, validated for pharmacology in carefully controlled experiments with sensitive methods (Calabrese 2004). As John Doull (2001) observed: 'the existence of hormesis—raises serious questions about the validity—to use a high-dose effect to predict a different low-dose effect', and 'both dose and time are independent variables in exposure.' Recognition of such important factors leads to the assessment and selection of appropriate methods and strategies. It raises serious questions about how useful and potentially misleading current procedures are (Monro 1994), which methods are helpful and which cannot be relied upon; and what kind of controls must be applied.

Sites of receptor binding with high specificity are frequently low-capacity sites, while low-specificity sites are considered secondary and high-capacity sites. In current ADME high-dose experiments, sites of loss (Veldstra 1956) can create false-positives and even obscure specific low-capacity sites. In low-dose experiments, specific binding sites remain undetected and create false-negatives (e.g. a non-existing blood–brain barrier).

3 The method

3.1 High-resolution imaging methods

Many imaging approaches are in use with varying resolutions. A review of all these is beyond the scope of this article. Imaging methods with high resolution are demanding and prone to artifacts, unless carefully executed. For complete target recognition, microscopic resolution is required. Many attempts have failed, producing erroneous claims due to results mixed with artifacts and thus discouraging general use in drug development. Increased resolution and sensitivity give rise to increased technical difficulty, requiring increased attention to detail. As such, drug localization efforts at the electron microscope level have not yet produced a workable method. Among current in (ex) vivo imaging methods for drug target identification, there are none that provide the high resolution and overviews commensurate with the information and confidence that has been achieved with Receptor Microscopic Autoradiography (Stumpf 2003). The image is the evidence.

For high-resolution localization of drugs a chosen method should feature the following aspects:

- Cellular-subcellular resolution of tissue structure associated with xenobiotic compound clearly visible in the same focal plane.
- Tags or carriers of the xenobiotic molecules under investigation must not change the chemical properties of the tagged molecules.
- A radiolabeled compound must be synthetically labeled and of high-specific activity and purity.
- High-specificity low-capacity binding sites can be recognized through reduced or eliminated binding of labeled compound in competition studies with high-dose unlabeled compound.
- Tissue treatment for microscopy should preserve in vivo conditions, i.e., maintain relationships between tissue constituents and administered compound, and avoid translocation and loss.

- The same preparation should provide high resolution details and low-resolution surveys.
- A new or modified imaging method—before its use and recommendation—should be tested and validated with two diffusible, non-covalently bound compounds with known cellular and organ target distribution.
- The experimenters should be knowledgeable in areas of cell biology, pharmacology and radio-biology, and capable of skillful attention to detail.

A study regimen may be followed to include different doses and times, expected and non-expected target tissues in limited pilots and follow-up on main experiments (Stumpf 2003).

3.2 High-resolution autoradiography with radio-labeled drugs

The availability of radio-tracers led to their successful use in biology and the development of different autoradiographic methods for studying the in situ fate of precursor molecules, hormones and drugs. Quantitative whole-body autoradiography with ^{14}C-labeled compounds, currently used in pharmacology, provides convenient in vivo surveys and important information on time-related organ distribution (Waddell 1973; Solon et al. 2002). However, this method does not distinguish unspecific deposition from specific target localization. Due to its low resolution and lack of sensitivity, the required dose levels are less suitable for the study of microdoses. Instead, microscopic resolution is necessary for target recognition. Therefore, thaw-mounting of thin sections on nuclear emulsion was introduced and then developed into receptor microscopic autoradiography that has been extensively applied (Stumpf 2003, 2005).

In microscopic autoradiography, nuclear emulsion is the detection medium for beta-particles. Nuclear emulsion provides high resolution and sensitivity by permitting the necessary close contact to the radiation source. Retention of the latent image allows storage of sparse signals through extended exposure times. This enables the finding of targets that are difficult or impossible to recognize otherwise. With thaw-mounting of thin sections, as is the case in receptor microscopic autoradiography, there is no need for separate handling of the radiation source and detector—thus, optimizing resolution and sensitivity. Receptor microscopic autoradiography provides both the histological image and the converted radiation signal into developed silver grains in the same focal plane. This allows convenient microscopic enlargement to the cellular and subcellular level and, at the same time, integrative low-resolution surveys, i.e., simultaneous recognition of molecular, cellular and tissue (organ) relationships (Stumpf 2003, 2005). Adherence to the steps specified in the tested procedure is paramount. Efforts to replace nuclear emulsion through digital imaging are underway, but have not yet succeeded in providing the necessary resolution and sensitivity.

A central imaging Laboratory for Histopharmacology, associated with current preclinical ADME routine and select studies of lead compounds, would provide a basis for establishing needed experience and efficiency.

3.3 Histopharmacology: vitamin D high-resolution imaging with receptor micro-autoradiography

While many convenient imaging methods are in use, none of them are suitable for in vivo high-resolution drug target localization at the cellular level. To create a target profile, cellular resolution is a conditio sine qua non. Simultaneous cellular detail and tissue survey information in the same preparation facilitates insight into functional relationships and aids integrative ‘systems pharmacology’. An example is the localization of radioactivity after i.v. application of a near physiological dose of tritium-labeled vitamin D. In the same preparation of the duodenum, the microscopic low-resolution survey shows both unspecific lumenal radio-labeled compound, probably excreted metabolites, and specific nuclear concentration in epithelium of villi and crypts (Fig. 1) (Stumpf 2003, 2008). With high-resolution imaging, cellular and subcellular detail distinguishes radio-labeled and unlabeled cell types. In the pituitary, the scattered labeled cells at the lateral anterior lobe can be characterized not only by their location, but with specific stains and antibodies (Fig. 2a, b). In the spinal cord and kidney, labeled target cells and unlabeled cells can be distinguished (Figs. 2c, d).

In another application after topical skin application of ^{3}H-vitamin D (not shown here), the route of absorption and related cellular distribution can be followed as it moves from epidermal cell layers with very high densities of radio-labeled compound to deeper dermal regions with successive lower densities. In the epidermal keratinocytes, there is high cytoplasmic concentration of labeled compound with low or absent nuclear concentration, while in keratinocytes of hair sheaths located in the dermis, there is nuclear concentration with absent cytoplasmic concentration. This latter distribution of radiolabeled vitamin D resembles and corresponds to the distribution after i.v. injection of a near physiologic dose. In the skin, low doses of vitamin D stimulate cell proliferation and differentiation (Tian et al. 1995), while high local doses diminish excessive cell proliferation in psoriasis (Tanghetti 2009) and high dose treatment even reduces tumor growth. Nuclear concentration and retention of 1,25(OH)$_2$ vitamin D$_3$ and related congeners clearly identify and correspond to specific target cells. The observed changes in dose-related

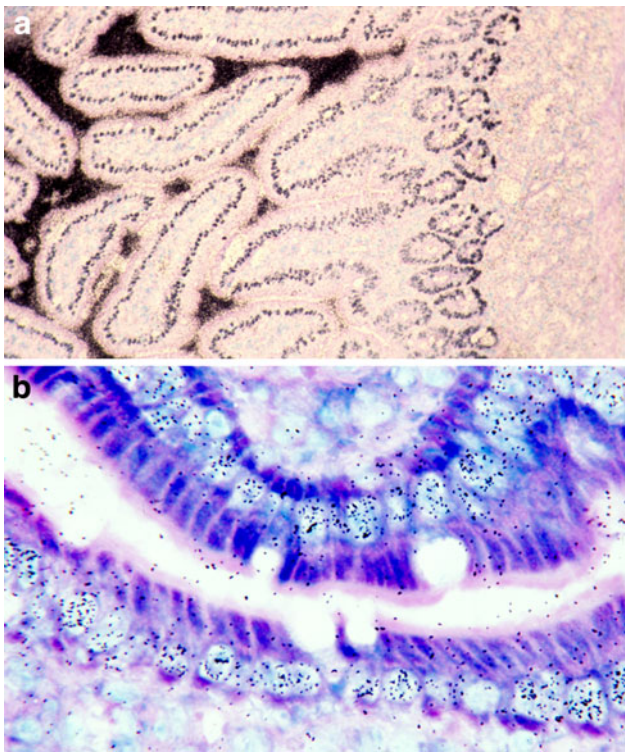

Fig. 1 High-resolution autoradiogram at different magnification after injection of ^{3}H-1,25(OH)$_2$ vitamin D_3 to rat. Duodenum. **a** Low magnification showing high level of radioactivity in intestinal lumen with apparent barrier to epithelium of villi. Radiolabeling of epithelial lining of villi and crypts, while cells of Brunner gland (*right*) are unlabeled. **b** High magnification of absorptive epithelium with nuclear concentration of radiolabeled compound. Goblet cells and stromal cells are unlabeled (Stumpf 2008)

alternate hormone depositions are noteworthy; a possible relevance to enantiodromic conversion, i.e., hormetic polarity of action, remains to be further investigated. Selective retention of the topically administered compound in intra- and extracellular surface coating granules of the stratum granulosum needs to be considered (Hayakawa et al. 2004).

3.4 Quantitative evaluation of cellular-subcellular densities of radiation signal (silver grains)

With high-resolution autoradiography, quantification is possible through computer-assisted counting of silver grains, attributed to specific cell types. If the silver grain yield (average number of disintegrations to yield one developed silver grain) is known, the number of silver grains can be converted to the number of molecules in a defined location (Stumpf et al. 1981).

The number of molecules and time needed to elicit a specific action in target cells is largely unknown. As seen in our autoradiograms, nuclear uptake and retention vary not only among individual target cells and target tissues, but are also modified by age, endocrine and metabolic status, dose, time, and other factors. For instance, in vivo occupancy of estradiol in uterine cells at 1 h after the injection of a near physiologic dose has been estimated between 12,000 and 14,000 per nucleus (Stumpf et al. 1981). Since many factors enter into the calculation, such measurements are difficult and results are valid only for specific conditions. For practical purposes, however, information on individual target cells may not be necessary. Semi-quantitative comparative data may suffice.

4 Controls

For controls of current expedient ADME procedures, an alternate method is not enough. The alternate method may contain the same kinds of deficiencies and artifacts as the primary experimental method. For instance, localization of a radio-labeled compound through non-invasive scanning, confirmed with equal data from whole-body autoradiography, may be flawed and deceiving due to similar low resolution and low sensitivity of both methods. Both may indicate a blood–brain barrier that does not exist, as in the case with vitamin D. Until recently, based on conventional ADME approaches, bone, gut, and kidney were considered main (classical) vitamin D targets, while the over 50 target tissues identified through histochemical approaches were largely ignored (Stumpf 1995). Amazingly now, during the past few years, the picture has reversed and it is claimed by some investigators that vitamin D receptors are expressed ‘in virtually every cell’. This contradictory change of opinion reflects the confusion of target identification through inadequate approaches. Noteworthy also: vitamin D, since lipid soluble, was assumed to be stored in fat tissue. However, instead we found it retained in ground substance of submucosae. Functions of skeletal and smooth muscle cells are highly calcium-dependent, therefore assumed targets for vitamin D, but we found neither nuclear nor membrane associations. However, pyloric muscle cells, atrial cardio-myocytes and spermatic cord muscle fibers showed typical nuclear concentration in autoradioradiograms, not recognized in conventional biochemical studies.

The example of vitamin D appears paradigmatic and representative for other compounds. Therefore, correlative and complimentary approaches should be considered in light of method limitations and related costly failures. The danger of over-interpretation of low-resolution data is always present. Together with Lloyd Roth, this author postulated that before recommending and applying a method for localization of a new drug, the method should be tested with two compounds for which the localization is known (Stumpf 2003). Resolution and sensitivity of an

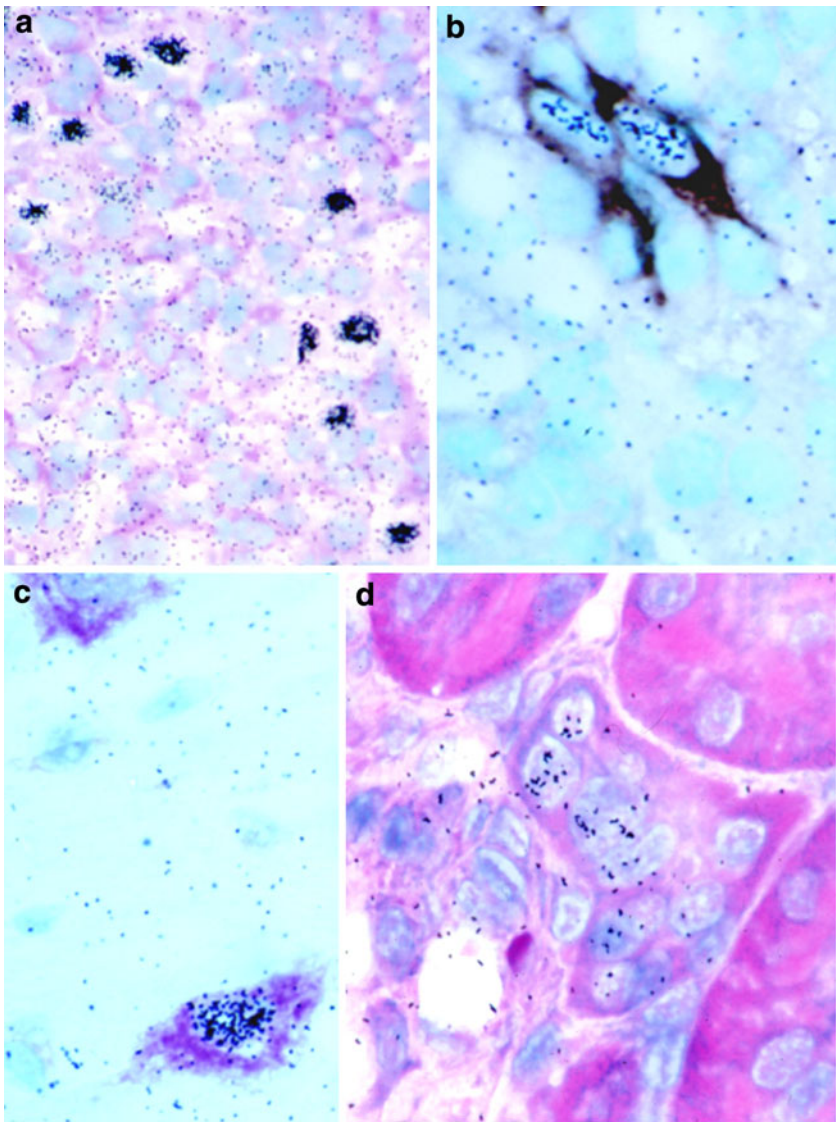

Fig. 2 High-resolution autoradiogram at different magnification after injection of ^{3}H-1,25(OH)$_2$ vitamin D$_3$ to rat. Pituitary (**a**, **b**), spinal cord (**c**), and kidney (**d**). **a** Low magnification of anterior pituitary showing dispersed strongly labeled cells among unlabeled or weakly labeled cells. **b** High magnification of combined autoradiography-immunocytochemistry with antibodies to thyrotropic hormone, characterizing these cells as thyrotropes with corresponding elevation of thyroid hormone blood levels after vitamin D treatment (Stumpf 1995). **c** High magnification of spinal cord (lamina IX), showing strong nuclear concentration in one motor neuron, absent in another one. Maps of target neurons in forebrain, midbrain, hindbrain, cerebellum, and spinal cord have been provided in contrast to negative data with radioassays (DeLuca 2008; Sandgren et al. 1991) and whole-body autoradiography (Stumpf 2003). **d** High-resolution kidney with nuclear labeling of macula densa cells next to a glomerulus; cells of adjacent proximal tubules are unlabeled (Stumpf 2003)

imaging procedure can be tested, e.g., with estradiol, which has specific binding sites not only in uterine, mammary, and ovarian tissues, but also in atrial muscle of the heart, keratinocytes of the skin, pituitary cells, neuron populations in cerebrum, brain stem, cerebellum, and spinal cord. Such studies have not been performed. Low-resolution scanning procedures that have not been validated are likely to furnish incomplete and more or less faulty pictures and concepts.

Validation based on comparisons of data from a low-resolution procedure, like radio-assay of excised organs or organ pieces, with data from a similar low-resolution approach, like whole-body autoradiography or nuclear imaging, must therefore be questioned as potentially misleading. Similarly questionable is any confirmation of in vitro data with data from alternate in vitro approaches. In vitro procedures require in vivo validation. According to the literature, relevant comparative tests of methods with different sensitivity and resolution are missing. New methods are applied without such prior controls.

5 Future perspective

ADME studies should not rely on radioassays, in vitro approaches, whole-body autoradiography and low-resolution imaging without information from additional in vivo high-resolution methods such as microscopic receptor autoradiography. Evidence indicates that in vivo target recognition can avoid costly oversights, facilitate low-dose studies, and enhance drug development as:

- a guide for functional and clinical follow-up,
- a control for validating data from in vitro experiments, and
- a complement to less sensitive scanning and imaging methods.

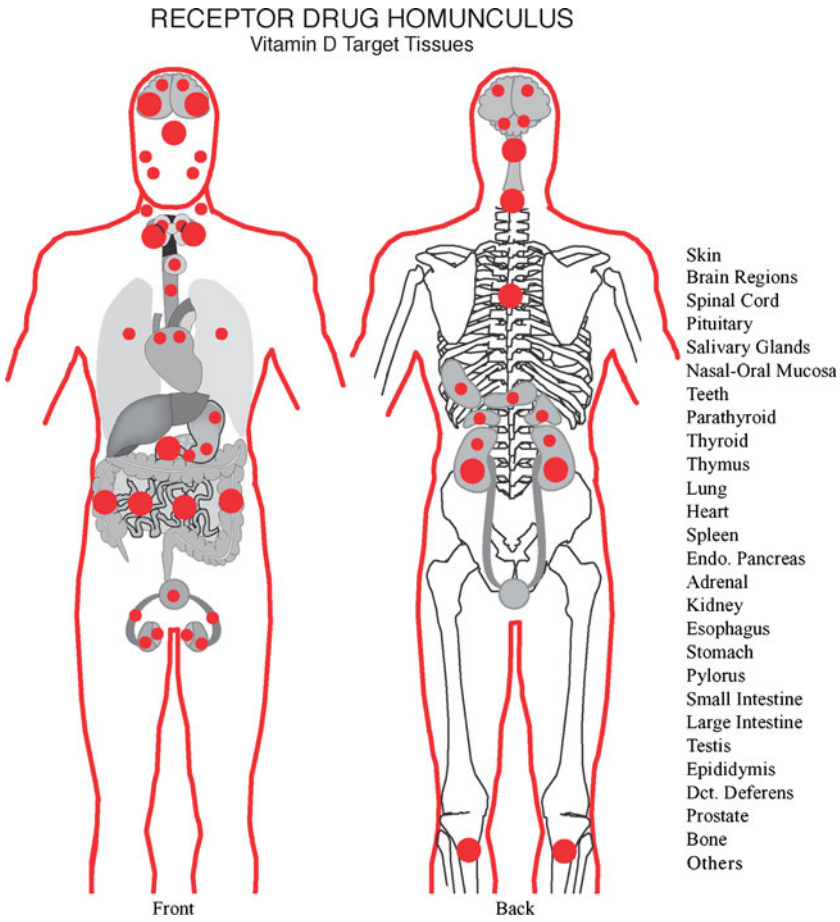

Fig. 3 Drug homunculus. Profile of vitamin D target tissues (*red*) assessed in rats and mice with receptor microscopic autoradiography. This holistic view provides a basis for quantitative and functional compound characterization (Stumpf 2005) a guide for potential therapies, a change of concept for the main biological role of vitamin D beyond calcium metabolism (Stumpf 2007)

Consider establishing a histopharmacology laboratory that provides resources and experience for various histochemical applications for target identification and characterization. An orientation towards enhanced recognition of tissue structure and organ composition will engender a higher and more efficient status of drug research. Possible obstacles are lack of appreciation and knowledge of cell biology by decision-making administrators, chemists, pharmacologists. High sensitivity-resolution methods are not for ad hoc use but require careful testing and experience.

Consider that expediency can be deceptive and costly. "We learn from history that expediency has rarely proved expedient" (B.H. Liddell Hart, Why don't we learn from history?). In drug imaging, the picture is the evidence. A valid target profile must be based on detailed high resolution in vivo data—not on expedient modeling from blood bioavailability or in vitro results alone. A high-resolution method is available. New high-resolution methods need to be developed. For now, high-resolution microscopic autoradiography can and should be applied to advantage for selected compounds.

Consider that most drugs have multiple targets. A 'drug homunculus' is a convenient way for representing and reviewing a compound-specific finger(body) print. As an example, the 'drug homunculus' for vitamin D (Fig. 3) demonstrates "the pattern of targets that can be reached with a single agent" (Drews 2000). In this manner, a new paradigm for the main role of vitamin D has evolved (Stumpf 2007). Appreciation of the full ranges of tissue detail, dose and time, opens the door for innovation—the road less traveled, and makes way for the untreated path.

6 Conclusions

- In vivo identification and characterization of target (receptor) sites is essential for advancing drug development and prediction.
- Current ADME and imaging methods do not provide the needed cellular and tissue resolution and are prone to creating false-positives and false-negatives. Extrapolations from blood bioavailability and from in vitro data can be misleading.
- High-resolution imaging provides data for finger(body) printing of a multi-target pattern, e.g. as a specific 'drug homunculus'.

- In vivo target recognition requires microscopic cellular–subcellular resolution in conjunction with integrative tissue surveys, for understanding mechanisms of action, for confirming and complementing biochemical and in vitro data, and for guiding functional and clinical follow-up.
- Receptor Microscopic Autoradiography is available as a tested method that satisfies requirements for high resolution in vivo drug localization. The need for radiolabeled compounds with high-specific activity, nuclear emulsion, and thin frozen sections, however, demands special care and experience.
- A central Histopharmacology Laboratory would serve as an efficient resource with experience.
- New high-resolution in vivo imaging methods should be developed, but must be tested before use with known diffusible compounds.

Addendum

Receptor Microscopic Autoradiography was developed for imaging of radiolabeled drugs with cellular-subcellular resolution. It is not 'in vivo' with a living animal—which would preclude cellular resolution—however, the in vivo status of drug localization and morphology of cell and tissue structure is preserved without loss and translocation. This is accomplished through rapid freezing without ice crystal destruction, and through cutting of 1–4 μm thin frozen sections and thaw-mounting them on nuclear emulsion slides. With this method, high-resolution and low-resolution surveys simultaneously permit detailed and integrative viewing in the same preparation.

Pristine preservation of the in vivo status was assured through control experiments with dry-mounted freeze-dried frozen sections and thaw-mounted frozen sections in extensive trial experiments to demonstrate preservation of tissue structure and constituents. Furthermore, for the validation of localization two diffusible compounds known localized, ^{3}H-estradiol and ^{3}H-mesobilirubinogen, as well as the extracellular space indicator ^{3}H-inulin, were used as test substances before recommending Dry-Mount and Thaw-Mount Autoradiography [Stumpf WE, Roth LJ (1966) J Histochem Cytochem 14:274–287; Jensen EV, Suzuki T, Kawashima T, Stumpf WE, Jungblut PW, DeSombre ER (1968) Proc Natl Acad Sci USA 59:632–638; Stumpf WE, Roth LJ (1965) Nature 205:712–713]. These methods, developed during the 1960s, have been widely applied, including quantification of target concentration and retention of radiolabel [e.g., Holderegger C et al (1981) Biol Reprod 25:719–724; Keefer DA (1982) Horm Metab Res 14:209–212; Koike N et al (1998) J Histochem Cytochem 46:1351–1358], with numerous discoveries and related new concepts [e.g., Stumpf WE (1998) Braz J Med Biol Res 31:197–206 (review); Stumpf WE (2007) Eur J Drug Metab Pharmacokinet 32(1):1–6].

Over the years, frozen section cutting and thaw-mounting have been facilitated through improved microtome-cryostats; quantification replaced manual counting of silver grains through computer programs. Altogether 'thaw-mount autoradiography' has been simplified and renamed Receptor Microscopic Autoradiography (Stumpf WE (2003) Drug localization in tissues and cells) to distinguish it from less suited methods.

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