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A strategic discovery roadmap towards high-quality leads and drug development candidates for kinetoplastid diseases. Part 3: from lead towards a drug development candidate

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Neglected tropical diseases such as leishmaniasis, Chagas disease, sleeping sickness and animal trypanosomiasis remain a significant global health challenge. This part of the roadmap outlines a streamlined path for progressing from lead identification to a drug development candidate, tailored to the specific needs of kinetoplastid infections. Besides the medicinal upscaling of synthesis, this review highlights key experiments in pharmacology in non-rodent species, toxicology, pharmacokinetics and pharmaceutics. These include but are not limited to early evaluation of safety using refined *in vitro* and *in vivo* methods to enhance predictive value, bioavailability and distribution to target tissues, and formulation strategies leveraging various delivery systems to optimize efficacy and safety. Environmental toxicity is also addressed proactively, for which *in silico* tools are presented. Collectively, this roadmap provides a practical, scalable approach to deliver high-quality drug candidates capable of addressing the urgent needs for kinetoplastid diseases (Figure 1).

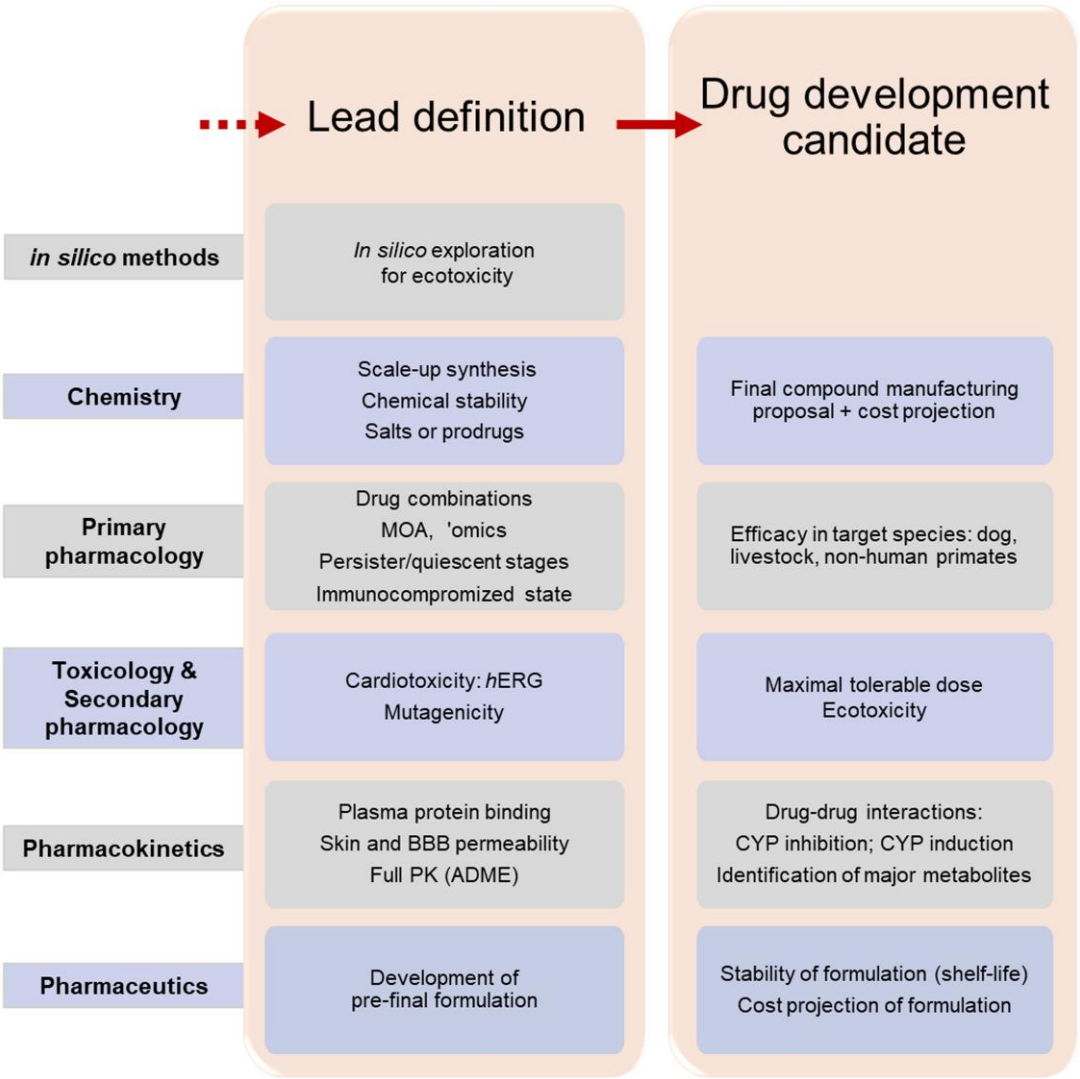

Figure 1. Schematic representation of the 'baseline' preclinical data package required during 'lead definition' and selection of 'drug development candidate', adopting a vertical (R&D stage) versus horizontal (discipline) tabular design. ADME, absorption, distribution, metabolism, excretion; BBB, blood–brain barrier.

Medicinal chemistry: upscaling and cost-effective manufacturing

Lead definition

The lead definition phase requires a scale-up of synthesis (>250 mg) to support generating an initial data package encompassing pharmacology, (eco)toxicology, pharmacokinetics and pharmacodynamics and pharmaceutics.

Chemical stability is usually assessed by analytical methods that are used for chemical identification, such as HPLC or GC, MS and infrared spectroscopy (especially Fourier transform infrared). Compound stability and the presence of impurities can be determined by specific prescribed tests and appropriate measures to avoid drug degradation, such as storage at lower temperatures, protection from light and use of appropriate storage containers should be considered at an early stage. Establishing a suitable analytical method is instrumental to pharmacokinetic (PK) studies.

Salts and prodrugs represent additional approaches for improving the physicochemical and pharmacokinetic properties of leads. Salts can improve solubility and stability, hence improving bioavailability. Prodrugs can improve absorption and increase selectivity by targeting specific cells (e.g. macrophages) or subcellular compartments (e.g. the phagolysosome) or rely on specific activation by parasitic enzymes.[1–3] These approaches facilitate the transition of a lead compound into a viable drug development candidate.

Drug development candidate

Entering the compound manufacturing phase requires focusing on developing scalable and efficient processes to produce the drug candidate in sufficient quantities for preclinical and clinical

studies. This involves optimizing synthetic routes to maximize yield, reduce steps and simplify purification while ensuring reproducibility. Although generally out of the scope of academic groups, key deliverables include sufficient amounts of GMP-grade material for clinical trials and detailed technology transfer documentation to support implementation at manufacturing facilities. Chemists also contribute to making a cost projection of producing the drug development candidate, which is crucial for its economic acceptance.

Primary pharmacology: broader characterization of the potency considering field application

Lead definition

In vitro drug combination studies are advisable since combination therapy is preferred for most kinetoplastid-related indications to prevent the adaptive emergence of drug resistance.[4] This approach has already been used multiple times to define interactions between new and existing reference drugs and typically relies on established chessboard assays.[5–8] The clinical application of drug combinations delays the onset of clinical drug resistance and may allow enhanced efficacy while decreasing the dosing schedules either in drug concentration or in treatment time. In *in vivo* studies, dose titrations of combinations can be explored on the basis of the results of monotherapy to obtain an overall efficacy of >95%.[6] Use of computer-based combinations for *in vitro* and *ex vivo* tests provides preliminary results avoiding the excessive use of animals.[6] Bioimaging has been useful not only to unravel biological peculiarities of trypanosomatids but also provides a powerful tool to determine the *in vivo* efficacy of antiparasitic drugs in real time.[9,10]

The identification of the mode of action (MOA) and resistance mechanisms via genomic, metabolomic and transcriptomic analyses also help to avoid cross-resistance.[11] By avoiding the introduction of drugs with similar MOA to existing drugs, treatment failure due to cross-resistance will be prevented. Moreover, *in vitro* and *in vivo* selection of resistance against new drug leads might help in elucidating the MOA and hint towards potential cross-resistance with other drugs.[12–16]

More advanced secondary-level animal models may allow to control defined parameters on disease progression, immune response and therapeutic interventions and test hypotheses derived from human clinical observations. Recent understanding of parasite biology has shed light on the occurrence of persister or quiescent parasites that affect the efficacy of drugs.[17–20] Specific *in vitro* and *in vivo* assays to evaluate efficacy against such quiescent/persister stages are therefore highly needed to identify suitable compounds and MOAs. When considering HIV-visceral leishmaniasis (VL) coinfections, models mimicking an immunocompromised state by immunosuppression with cyclophosphamide can be used to evaluate leads under conditions in which an accelerated treatment relapse can be expected.[21]

The implementation of sex-related inclusion criteria in animal studies would argue for the inclusion of both male and female animals. Although in principle both sexes can be used for models of cutaneous leishmaniasis (CL), VL, animal trypanosomiasis (AT) and Chagas, the use of females is often preferred. For instance, male mice are more prone to fight and mutilate their littermates over an extended duration of an infection/treatment experiment. This makes the use of males particularly challenging, for example, in the evaluation of drug efficacy against CL, in which the direct follow-up of skin abrasions and lesions is the main drug evaluation criterion. Aggressive behaviour can be reduced by offering environmental enrichment and various forms of refugia.

Drug development candidate

Whenever a promising 'lead' emerges after profiling in rodent models, its efficacy should ideally be tested in the intended target species, although this aspect is specifically considered during the clinical development phase. Working with larger animals is strictly regulated and involves elaborate ethical and legal requirements which may be far too expensive or unavailable to academia. Nevertheless, and whenever feasible, small pilot experiments may be advantageous to strengthen initial proof-of-concept in the target species and attract collaboration initiatives with public–private partnerships or industry partners that have capabilities to engage in formal clinical development.

Trypanosomiasis in livestock animals Target host species, such as cattle, goats, pigs and horses, have been used in AT research for assessing drug efficacy and pharmacokinetics (Table 1). While their use entails high maintenance costs and ethical considerations, they provide initial insights into the therapeutic approaches that would be effective under practical field application.

Canine models for Chagas and leishmaniasis Since dogs are an important reservoir for *Trypanosoma cruzi*, the Beagle dog was proposed as a model since it reproduces the clinical and immunological findings described in patients, including cardiomyopathy.[22–24] However, no correlation was found between the parasite load in tissues/myocardium and fibrosis at either the acute or chronic phase of the infection.[25] For this reason, studies using dogs for efficacy evaluation remain very scarce in the literature.[26,27]

Dogs are natural hosts of *L. infantum* and similarly to human VL, canine leishmaniasis is characterized by gross pathological lesions such as mild hepatomegaly, lymph node enlargement and variable splenomegaly, making dogs a useful model in drug evaluation.[28] Factors such as parasite virulence and stage, infecting dose, inoculation route and dog characteristics (age, sex, breed) strongly influence infection outcome.[29,30] High numbers of animals may be avoided when using young Beagle dogs (<1 year) produced by authorized breeders, in well-designed

Table 1. Overview of some livestock models for AT

Trypanosoma species	Animal species	References
T. b. brucei	Pigs	92
T. b. evansi	Canarian goats	93
T. b. equiperdum	Welsh pony mares (*Equus caballus*)	94
T. vivax	Girolando calves, Holstein Friesian cattle	95,96

experiments allowing adequate follow-up and reproducibility. We recommend intravenous (IV) inoculation of freshly obtained amastigotes (10^8/animal) of a virulent isolate[31] or, alternatively, stationary-phase promastigotes (>5×10^7/animal) to explore the chemotherapeutic value of a new drug candidate. For the diagnosis and post-treatment follow-up, IFAT is widely considered the 'gold standard' measuring specific anti-*Leishmania* IgG responses.[32,33] For parasite quantification and interpretation of drug efficacy, a PCR-based method combined with parasite back-transformation in limiting dilution assays can be recommended.[34,35]

Non-human primate models for HAT, Chagas and leishmaniasis Further down the preclinical drug R&D road, the use of non-human primates (NHPs) has been instrumental in advancing our understanding of Human African trypanosomiasis (HAT) and leishmaniasis as their physiological and immunological responses closely resemble those of humans.[36] Several models involving squirrel monkeys, baboons, chimpanzees and cynomolgus macaques have been explored (Table 2). Cynomolgus having acquired *T. cruzi* infection in an NHP breeding colony were used for assessing drug therapy.[37,38] NHPs develop cutaneous lesions when they are experimentally infected with *Leishmania* species such as *L. amazonensis* and *L. major*[39,40] or cutaneous and mucocutaneous lesions when infected with *L. braziliensis*.[41] Furthermore, rhesus macaques infected with *L. infantum* develop visceral immune alterations.[42] They offer insights that are highly translatable to human trials and might help to understand increasing treatment relapse rates.[43] However, the high cost, ethical considerations, and logistic challenges associated with NHP research limit their widespread use.

Toxicology: identification of potential liabilities

Lead definition

The use of laboratory animal models currently remains essential for preclinical evidence of safety and efficacy, and for developing clinical predictions of expected outcomes in target species.[43] The abandonment of the LD_{50} test in 2002 triggered the development of alternative test methods and updated legal regulations.[44] For example, the OECD recommends the up-and-down procedure as an effective method to determine acute (oral) toxicity in female rats. By sequentially administering a single dose of the test substance to fasted animals and adjusting the dose of the next animal (lower if the first animal dies or higher if the first animal survives), the number of animals used has decreased from ~100 to 5–9 animals.[45]

At a relatively early stage, a small set of (non-GLP) pilot tests evaluating cardiotoxicity and mutagenicity should be included as they may reveal a 'go' or 'no-go' decision on unfavourable test results. Cardiotoxicity is identified by assessing the compound's inhibition on the *h*ERG (human Ether-a-go-go Related Gene)-coded potassium channel, which is responsible for the potassium flux inside cardiac muscle cells. Although past attempts aimed at predicting the *h*ERG inhibition potential of drugs *in silico*,[46] most data are still based on *in vitro* fluorescence-based assays, voltage clamp techniques in mammalian cells transfected with the *h*ERG-gene and radioligand displacement assays.[47] More recently, two biomimetic HPLC property measurements are proposed in the screening for *h*ERG inhibition potential using the measured binding of compounds to alpha-1-acid-glycoprotein and immobilized artificial membrane.[48]

The mutagenicity pilot test set, i.e. the Ames test, with various commercial kits relying an *in vitro* reverse mutation assay using bioengineered *Salmonella typhimurium or Escherichia coli* strains is essential.[49] Additional *in vitro* assays that should preferably also be considered are the micronucleus,[50] the Comet[51] and the mouse lymphoma assay.[52]

The integration of *in silico* methods for ecotoxicity assessment early in drug development can improve this approach by providing rapid and cost-effective tools, such as the Estimation Program Interface Suite,[53] Toxicity Forecaster (ToxCast)[54] and Sequence Alignment to Predict Across Species Susceptibility (SeqAPASS)[55] based on chemical structure, *in vitro* toxicity data, and protein sequence alignment, respectively. By simulating and analysing

Table 2. Overview of some non-human primate models for trypanosomiasis and leishmaniasis

Parasite species	Animal model	References[a]
HAT		
T. b. rhodesiense	Chimpanzee	97
	Vervet monkey (acute, 1st stage)	98
T. b. gambiense	Vervet monkey (chronic, 2nd stage)	99,100
Chagas disease		
T. cruzi	Rhesus macaque (natural infection)	37
Leishmaniasis		
L. infantum and *L. donovani*	Vervet monkey, syke and baboon (asymptomatic/spontaneous cure)	101
	Aotus monkey, squirrel monkey and marmosets (fulminating VL)	102–104
	Rhesus macaque (systemic disease similar to human VL)	42,105
L. major	Vervet monkey, rhesus macaque (CL)	39,106
L. amazonensis	Rhesus macaque (CL)	40
L. braziliensis	Rhesus macaque (ranging between cutaneous and mucocutaneous manifestations)	41

[a]Limited selection, more references are available in the literature

potential environmental interactions computationally, these tools enable proactive identification and mitigation of environmental risks.[56]

Drug development candidate

Currently, the most common approach to determine *in vivo* toxicity of drug candidates is the maximum tolerable dose,[57–60] which represents the highest dose of a drug that can be administered without causing life-threatening side effects or overt toxicity. Toxic effects can be evaluated by changes in various blood parameters, histopathological analyses and behavioural observations[61]: ALT and AST levels as well as total bilirubin in the blood give clues about liver damage;[62–64] creatinine and urea blood levels are important biomarkers to assess renal function;[65,66] histopathology is essential in evaluating liver and renal toxicity[67–69] in addition to body weight, feed consumption and the presence of any toxicity-associated behaviour.[70–72] Maximum tolerable dose is mainly used in chronic toxicology studies to determine the appropriate dose, maximize the probability of detecting effects, interpret findings, and conduct studies following the 3Rs principles.[73]

Ensuring environmental sustainability alongside therapeutic efficacy requires consideration of associated ecotoxicity risks. Traditionally, ecological risk assessments are conducted late in the development process after significant investments of time and resources have already been made.[56] These assessments typically follow a tiered approach outlined in regulatory guidelines. In the initial phases, drug candidates undergo basic screening to assess their potential environmental impact, which involves a comprehensive assessment of a compound's physicochemical characteristics, usage, dosing, and excretion pathways to gauge environmental exposure.[74] If this initial assessment raises concerns, further testing may progress to more complex *in vivo* studies using animal models.[75] Although these studies aim to assess a drug's effects on various ecological endpoints, they are time-consuming, expensive and often involve ethical considerations regarding the use of animals.[76]

Pharmacokinetics: aiming for oral bioavailability and distribution to target tissues

Lead definition

An important PK parameter is plasma protein binding as it strongly affects the effective drug concentration at the pharmacological target site. Binding of the drug to blood plasma components, such as albumin, α-acid glycoprotein, lipoproteins (γ-globulin) and erythrocytes, significantly influences drug distribution rates between plasma and tissues and therefore influences the clearance (Cl) and volume of distribution. The gold standard *in vitro* methods to measure plasma protein binding are equilibrium dialysis, ultrafiltration, ultracentrifugation, LC techniques, capillary electrophoresis, spectroscopy, HPLC and calorimetric techniques.[77]

For drugs specifically intended for topical use, *in vitro* skin permeability assays should be conducted to support the animal disease model. The skin is composed of three main layers (epidermis, dermis and hypodermis) that may present a barrier to permeation. The most common technique to quantify drug permeation into the skin uses a Franz diffusion cell (FDC). This glass device consists of a donor and receptor compartment filled with a suitable medium separated by a membrane, either synthetic or biological skin. The drug formulation is applied onto the membrane in the donor compartment and samples are taken from the receptor chamber at different time intervals to measure the drug concentration. Depending on the experimental design and concentrations of the drug collected, several parameters can be calculated and evaluated to compare the different formulations,[78] including the use of different membranes (animal versus human or diseased versus healthy). In addition to measuring the concentration of the permeated drug, the membrane on disassembly of the FDC can undergo tape stripping whereby adhesive tape strips are sequentially applied and removed from the skin after drug application. Quantification of the amount of drug on each strip allows building a drug distribution profile within the stratum corneum. Drugs can be extracted from the remaining membrane to assess permeation into deeper skin layers, including the dermis that represents the tissue target for CL. This methodology is valuable during formulation development and optimization, and to compare formulations.[79]

To evaluate drug concentrations in the skin and brain *in vivo*, relevant for CL and HAT treatment respectively, microdialysis can be applied.[80] In this technique, a small probe is inserted into the skin or stereotactically in the brain and perfused with a liquid, allowing the continuous sampling of the extracellular fluid and real-time monitoring of drug concentrations. In contrast to using biopsies for drug extraction, this methodology probes the unbound and free drug fraction. Further imaging techniques such as Raman spectroscopy, confocal and fluorescence microscopy can provide spatial distribution of the drug. These techniques necessitate a drug tagged with a fluorescent or Raman-active marker that might modify its permeation behaviour. An alternative is the use of spatial LC-MS, however, this elegant technique does not apply to all drugs and sensitivity can be a limitation.

After snapshot PK studies on a small number of animals (*vide supra*), standard full PK studies are conducted especially during lead optimization to support final drug formulation efforts. These studies are still considered the gold standard during the final selection of a drug development candidate to fully characterize lead PK characteristics using the optimized formulations for development.[81] In full PK studies, drugs are administered either IV or *per os* to a limited number of animals ($n=3$) at different dosing regimens and subsequent blood samples are collected from each animal via serial blood sampling up to at least 24 h post-treatment. Plasma samples are analysed for each animal individually to assess inter-subject variability.

Drug development candidate

As patients sometimes are treated for several indications at once or a combination therapy of different drugs is preferred to treat one indication, it is necessary to evaluate potential hazardous drug interactions typically involving two key mechanisms: cytochrome P450 (CYP) enzyme inhibition and CYP induction.

Most importantly, CYPs 1A2, 2C9, 2C19, 2D6 and 3A4 are involved in the metabolic degradation of most marketed drugs and their inhibition is associated with a high risk for drug interactions.[82] High-throughput assays using recombinant CYPs and substrates that are metabolized by each CYP in fluorescent

metabolites are generally used to determine the inhibitory concentration of the drug that results in a 50% reduction of enzyme activity.[83] Conversely, CYP induction involves the stimulation of enzyme production by a drug, which can result in decreased plasma levels and reduced efficacy of co-administered molecules. CYP1A2, CYP2B6 and CYP3A4 induction assays are considered essential and rely on the induction of transcripts and enzymatic activity in primary human hepatocytes.[84]

For a drug development candidate, all major metabolites should be identified. Although both *in silico* tools and *in vitro* systems can be used to predict the presence of some metabolites (Part 2),[85] a correlation with *in vivo* PK data remains essential as many model systems struggle with some mechanistic and physiological limitations.[83]

Pharmaceutics

Lead definition

As part of the pre-final formulation evaluation, specific physicochemical tests are performed to ensure that drug and excipients are chemically preserved and that the intended physical form of the formulation has been achieved. Key tests for parenteral, oral and topical formulations are addressed (Table 3).

A solution formulation prepared from known pharmaceutical excipients following a simple protocol is acceptable for parenteral administration.[86] For IV formulations, special attention needs to be paid to drug precipitation on injection and to assess *in vitro* precipitation by using a serial dilution method.[86] When the solution does not meet the criterion of 'absence of precipitation' and the intended route is IV, more complex formulations such as nanosuspension or microemulsion should be considered. In the case of less exigent parenteral routes regarding potentially toxic formulations i.e. subcutaneous, intramuscular and intraperitoneal routes, (micronized) suspension in methylcellulose and water, with or without an added surfactant such as Tween 80, may be considered.[86]

It is important to consider that several physicochemical and biopharmaceutical properties of the drug may limit their effective delivery by oral administration. These properties include poor water solubility at both physiological and low pH, low membrane permeability, poor chemical and biological stability due to pH lability or enzyme metabolism and their recognition by efflux transporters. Further, some drugs can cause local irritation and nausea. These 'challenging' drugs require the development of advanced formulations to overcome such biological barriers.[87,88]

In vitro assays of oral formulation candidates hold great promise in predicting the *in vivo* performance and reducing *in vivo* studies and are intended to mimic the two crucial steps of the process of oral drug absorption: the step of drug dissolution and the drug permeation step. Dissolution tests alone are generally considered predictive of *in vivo* oral drug absorption for immediate-release, solid dosage forms and suspensions

Table 3. Critical physicochemical and biomimetic tests for drug delivery systems

Key test	Critical parameter	Criteria for 'not go'
Physicochemical tests		
Particle size distribution	Mean particle diameter (D)	D > 500 nm (nanoformulation for IV administration)
	Polydispersity index (PI)	PI >0.3 (nanoformulation for IV administration)
Chemical and physical stability	Content of API excipients and volatile solvents; drug encapsulation efficiency; physical phase; particle size distribution; rheological properties; pH	Significant change during storage
Biomimetic tests (IV route)		
Precipitation test upon serial dilution in 0.067 M phosphate buffer at pH 7.4[86]	Precipitation	Cloudiness or precipitation within 5 min after dilution
Stability of drug delivery system in plasma at 37°C	Transformation half-time (Size change or enhanced drug release)	Less than 10 min (in case of drug targeting)
Kinetic of drug release from the carrier in sink conditions against isotonic buffer or plasma at 37°C	Half-time of release	Less than 10 min (in case of drug targeting)
Biomimetic tests (oral route)		
Precipitation test on serial dilution in SGF[a] (distilled water containing NaCl 2 g/L, with pH adjusted to 1.2 with HCl)[86]	Precipitation (or chemical drug instability)	Cloudiness or precipitation within 5 min after dilution (or chemical drug instability)
Precipitation test on serial dilution in SIF (distilled water containing KH_2PO_4 6.8 g/L, with pH adjusted to 7.5 with NaOH)[86]	Precipitation	Cloudiness or precipitation within 5 min after dilution

[a]SGF, simulated gastric fluid; SIF, simulated intestinal fluid

containing highly soluble drugs, i.e. drugs from Biopharmaceutics Classification System classes I and III, with systemic action. On the other hand, when evaluating poorly soluble drugs, i.e. Biopharmaceutics Classification System class II/IV drugs or formulations that contain excipients that may affect drug absorption, additional *in vitro* permeation assays are required for predicting *in vivo* drug performance.[89]

As key recommendations for developing topical formulations, the composition and physical form must be carefully chosen to accommodate the physicochemical properties of each API, maintain adequate stability, provide appropriate skin tolerance and promote delivery of the API to its site of action to achieve the desired pharmacological effect.[90] As described previously, data generated from *in vitro* percutaneous permeation studies using FDC with excised human skin, together with tape stripping to assess drug penetration into the skin, give a good prediction of *in vivo* bioavailability and can be used to compare different topical formulations. Although the loss of stratum corneum in CL initially facilitates the entry of drugs through the skin, re-epithelialization and wound healing during treatment represent an additional challenge for topical treatment. Indeed, the formulation should be effective in all possible situations: intact, partially or completely damaged skin. Thus, in addition to intact skin, stripped skin may also be employed to investigate the importance of the stratum corneum as a diffusion barrier and also to simulate the loss of this barrier as observed in CL when lesions evolve into ulcers.

Drug development candidate

After the first proof-of-concept of *in vivo* efficacy, the drug formulation often needs to be further optimized and the most appropriate route should be identified, to offer the best therapeutic benefits. Priority is generally given to the development of an oral treatment as it will give more convenience for the patient. The IV route has the benefit of accurate dosing but is more exigent regarding the characteristics of drug delivery systems. Their dimension should be in the nano range to prevent embolization. Formulations that contain nanoparticles as drug carriers and delivery systems are particularly attractive for enhancing drug solubilization and absorption efficiency due to increased surface area and for targeting specific tissues or cells. Examples of nanocarriers include liposomes, nano- and micro-emulsions, solid lipid nanoparticles, micelles, polymer nanoparticles and drug–cyclodextrin complex.[91] The characteristics of these nanocarriers must be optimized based on physical and chemical criteria and considering their interactions with biological systems. The formulations should be subjected to evaluation of stability (shelf life) and cost projection.

General conclusions

The combined parts present a structured roadmap for progressing from early 'hit' identification to the development of high-quality drug leads and candidates targeting kinetoplastid-related neglected tropical diseases such as African trypanosomiasis, Chagas disease and VL and CL. Anchored in the practical realities of diverse R&D networks, it integrates key criteria across medicinal chemistry, pharmacology, toxicology, pharmacokinetics and pharmaceutics. Particular emphasis is placed on early de-risking, formulation optimization, and the use of predictive *in vitro* tools and state-of-the-art animal models to guide compound selection. While recognizing the challenge of harmonizing drug discovery efforts across settings, this roadmap emphasizes the importance of proper design and reporting of experiments, ethical responsibility through the 3Rs principle, and sustainability by accounting for environmental impact. The final goal is to enable efficient translation of new chemical entities into effective, patient-friendly therapies.

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