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Drug Discovery by Aptamers in Protozoan Infectious Diseases

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1. Introduction

In the last decades the need for novel diagnostic and chemotherapeutic tools became apparent. The increasing population and upcoming diverse disease patterns are leading to a synergistic effect, which emphasises the required demand for new types of pharmacological agents. Additionally, newly gained knowledge such as completing the human genome and on-going transcriptome and proteome projects identified novel targets for therapeutic intervention. However the upcoming number of diseases as well as their variety force medical science to accelerate the process of drug discovery by employing sophisticated methodologies for chemical synthesis of biological active compounds and time- and cost-sparing screening assays to develop novel drugs. These diseases are not only related to cancer or insights from the human genome project but become more prominent by time, since they are derived from human external sources such as infectious agents causing serious disease of mankind. For instance, approximately a quarter of the entire population is living in malaria endemic areas and is thereby daily exposed to lethal pathogens such as *Plasmodium falciparum* (WHO).

Due to novel chemical synthesis procedures, approaches using combinatorial libraries followed by high-throughput screenings for biological activity are the current method of choice to increase the procession time and decrease application costs in the search for possible drug candidates. Subsequently possible drug targets are identified by *in vitro* assays, which test their biological relevance, and validated in animal models. Rational drug design, natural product or combinatorial library approaches have been employed to identify possible lead compounds for drug development. In order to compensate for the high demand of new drugs novel high-throughput strategies for screening high numbers of compounds were developed, which also decreased the production time (Fernandes, 1998). Most promising drug candidates are often subject to chemical modifications to improve their synthesis parameters as well as their toxicity profile and stability under physiological conditions. This procedure has been employed for several generations of drug development which were afterwards verified in animal models before clinical studies were applied (Faria and Ulrich, 2002, Majumder et al. 2009). The design of specific inhibitors against proteins involved in disease mediation is one of the primary objectives in pharmacological research.

The identification level of possible drug candidates for therapeutic applications has been increased by using a variety of screening strategies for active compounds in combinatorial libraries. In general, the suitability of combinatorial library approaches for drug design is based on the probability that a member of a high quantity population consisting of different molecules and structures, like peptides, carbohydrates and/or oligonucleotides, binds to the respective target of choice. For the discovery of specific ligands with binding capabilities to a desired target peptide- and oligonucleotide-based libraries were of particular interest since the library and thereby identified binders can be replicated by enzymatic catalysis. The SELEX technique (systematic evolution of ligands by exponential enrichment), was developed in parallel by Tuerk and Gold (1990) and Ellington and Szostak (1990) and is based on an oligonucleotide combinatorial library containing a vast number (around 10^{13}) of different sequences and structural motifs for the *in vitro* selection of DNA or RNA molecules with binding specificity to their respective targets. The selected high affinity binders are called aptamers (from *aptus* (Latin) = to fit). Functional DNA or RNA molecules were identified as specific binders to a number of different targets including nucleotides (Sassanfar and Szostak, 1993, Meli et al., 2002), biologically active peptides and soluble proteins (Jellinek et al., 1993, Ruckman et al., 1998, Williams et al., 1996, Proske et al., 2002) and complex targets such as membrane-bound receptors (Ulrich et al. 1998) and blood vessels (Blank et al. 2001). However, due to degradation processes of nucleic acids in biological systems, aptamers were not ideal drug candidates. This limitation was overcome by chemical improvements in oligonucleotide synthesis which prolonged the stability of the molecules in biological tissues as well as their pharmacokinetics (Trujillo et al., 2007, Ni et al., 2011). New skills in oligonucleotide modification and lower synthesis costs extended the investigation of protein interactions from *in vitro* to *in vivo* applications and also the design of new compounds for pharmaceutical applications. The deriving aptamer properties are evoked by their respective tertiary structure providing unique features for these oligonucleotides to bind with nanomolar or even picomolar dissociation constants to their target proteins being higher than most of the natural occurring ligands or inhibitors. Moreover, aptamers possess numerous advantages over antibodies in many applications due to their non-peptide character and flexibility to interact with even to hidden targets with characteristics of small molecule binding. Further advantages of aptamers include the simplicity of synthesis and the ability of chemical modification of the nucleotides or backbones, their stability against thermal denaturation and nuclease degradation, the lack of immunogenicity and rapid penetration of tissues. Moreover, aptamers posses the ability to interact with functional protein domains such as ligand-binding domains mainly due to molecule-size advantages compared to antibodies or natural ligands (Ulrich et al., 2006).

As already outline above aptamers can be subject to a variety of changes to enhance their stability especially for *in vivo* applications. These modifications can be introduced by using T7 RNA polymerase which accepts, for instance nucleotides like 2′-F-pyrimidines as substrates (Pestourie et al., 2005; Ulrich et al., 2006). By adding phosphorothioate based nucleotides to DNA- or 2′-F or 2′-amino-substitution of 2′OH groups of riboses to RNA-libraries a nuclease resistant oligonucleotide library will be produced.

In contrast to 2′-amino-pyrimidine substitution – 2′-fluoro-pyrimidine modifications lead to higher thermal stability (Aurup et al., 1994; Cummins et al., 1995) resulting in a theoretically increased affinity of aptamers to their targets (Eaton et al., 1995). In order to improve pharmacokinetics and the bioavailability, high molecular weight or/and lipophilic moieties

like polyethylene glycol are fused to the respective ends of the aptamers increasing aptamers' half-life in plasma up to nine hours in contrast to a few minutes observed with unmodified aptamers (Willis et al., 1998). However, on the back site, aptamer applications face some diagnostic and/or pharmaceutical limitations in terms of their incapacity to pass biological membranes hindering intracellular aptamer applications (reviewed in Rimmele 2003).

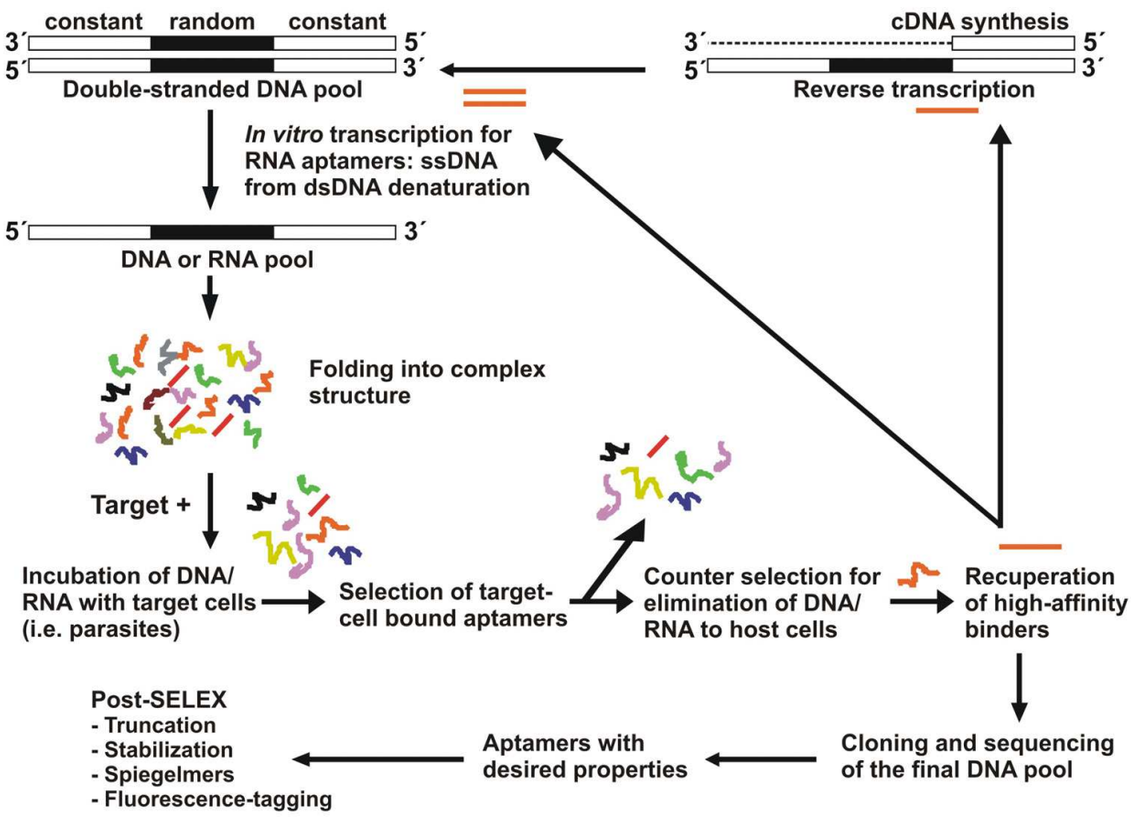


Fig. 1. *In vitro* selection of aptamers as high-specificity binders to target cell epitopes.

RNA or single-stranded (ss)-DNA-libraries for *in vitro* selection are obtained from a double-stranded DNA template containing a random region flanked by two constant sequences for PCR amplification (and *in vitro* transcription in case of RNA aptamers). Libraries are stabilized against nuclease activity by chemical modifications of the nucleotides. DNA or RNA libraries are generated by *in vitro* transcription or thermal denaturation followed by single-strand purification. The random oligonucleotide pool is exposed to its target cell (i.e. an erythrocyte expressing *P. falciparum* derived proteins on its cell surface), followed by elution of bound RNA/DNA molecules. Subsequently, these oligonucleotides are incubated with uninfected erythrocytes. All DNA/RNA molecules bound to these healthy cells are discarded, while unbound oligonucleotides are used for PCR amplification and generation of the DNA pool for the next selection cycle. The Cell SELEX process continues until the combinatorial oligonucleotide pool is purified to a homogeneous population binding with high affinity and specificity to their respective targets. Aptamers are identified by DNA sequencing and characterized for their binding affinities. Post-SELEX modifications are performed for optimization of aptamers for diagnostic and *in vivo* applications.

The modality of Cell SELEX, where aptamers are selected on the basis of binding to specific cell surface epitopes of disease cells, i.e. cancer cells or cells infected by pathogens (a positive selection step), followed by exposure of the library to a healthy somatic cell type, where all DNA/RNA molecules binding to common cell surface epitopes are discarded (negative selection step) has further broaden possible applications of the SELEX technique (see Figure 1 for details). The final library following various cycles of positive and negative selection ideally contains one or more aptamers binding to differentially expressed cell surface markers. In many cases, these aptamers were able to detect a differential expression pattern on the cell surface, also named a molecular signature of the cells, not expressed by other cells. These features have made aptamers potent tools for targeting cancer cells and therapeutic agents for delivering a toxic load or an RNAi construct interfering with cancer cell replication or metabolism. The combination of improved synthesis protocols towards industrial scale and novel chemical modifications techniques was beneficial for the development of aptamer-based tools for diagnosis and therapy. We foresee that aptamers as nucleic acid based drugs will soon become of major pharmacological relevance in both, diagnostics and therapeutics.

2. Applying the SELEX technique on protozoan parasites

Parasite-caused diseases affect millions of people in the entire world. Parasitic infections are not only restricted to humans, they also play a major role in animal health. Just a few years ago infectious diseases were classified as an issue of the past. However, due to the increasing level of drug resistance of pathogens the need for new treatments or even more important vaccines has become apparent. Unfortunately, these infectious diseases which are responsible for a level of mortality and morbidity are particular endemic in developing countries (Renslo and McKerrow, 2006). A variety of these parasitic diseases, including the so called neglected Chagas disease, leishmaniasis, sleeping sickness, schistosomiasis, elephantiasis, or onchocerciasis and of course malaria, are transmitted by vectors and, therefore, attempts to combat transmission vectors became prominent. Due to the continuously increasing human population and rising resistances of human pathogenic agents towards current treatment, there is an urgent need for novel diagnostic and therapeutic tools to tackle the foreseeable problems of the next generation. High attention is not only drawn to the development of new tools and their fast application but also to the commercial value in terms of their maintaining and application costs. In this chapter we summarize the current status of SELEX applications in human infectious diseases caused by protozoan pathogens.

3. SELEX applications in *Leishmaniasis*

The sandflies of the *Phlebotomus* genus are responsible for the transmission of the protozoan flagellate *Leishmania.* Infection with this parasite causes leishmaniasis, which can cause death in the absence of treatment. Specially, visceral leishmaniasis (kala azar), caused by *L. donovani/infantum* infection, is responsible for a high level of mortality if untreated. Muco-cutaneous leishmaniasis (*L. braziliensis* infection) or cutaneous leishmaniasis (*L. major/mexicana* infection) are not considered to be lethal, however the latter can result in unaesthetic stigmata (Herwaldt, 1999). The major intention of the treatment is prevention

from death after infection by *L. donovani*. Due to this high potency the antimonials Pentostam® and Glucantime® were the therapeutics of choice for antileishmanial therapy since decades. However, these drugs revealed a number of disadvantages in terms of their mode of administration, the long duration of treatment, the high cost and the serious side effects. Therefore, the traditional drugs Amphotericin B and pentamidine were resurrected although they are known for causing irreversible toxic effects. However, new formulations and adapted dosages made these drugs applicable (Berman, 1997, Berman, 1999). The first oral drug, miltefosine was introduced by the Indian government for treatment of visceral leishmaniasis in 2002. Despite cure rates of up to 98% (Roberts, 2005, Berman, 2008), the drug also evokes serious side effects such as vomiting, diarrhea and can cause abnormalities. In order to discover novel therapeutics to tackle leishmaniasis without such serious side effects, the SELEX technology has been applied on the parasite *L. infantum* which is responsible for the most severe visceral leishmaniasis. Gonzales and co-workers were focussing on peculiarities such as gene expression and organisation in *Leishmania* (Ramos et al., 2007 and 2010). *Leishmania* does not condense its chromatin during mitosis which might be caused by the respective histones. Although histones are extremely conserved proteins in all livestock, high sequence divergences have been identified in the N- and C-terminal protein regions as targets for diagnostic and/or therapeutic intervention. Ramos et al. developed aptamers against the recombinantly expressed histone antigens H2A and H3 and even, despite of the sequence homology of histone proteins, the generated aptamers were highly selective for the parasite proteins. Aptamers were also directed against the *L. infantum* kinetoplastid membrane protein-11 (KMP-11) which is a major component of the cell membrane of kinetoplastid parasites (Moreno et al., 2003). Since KMP-11 is a cytoskeleton-associated protein it is believed that its function lies in mobility or is related to the flagellar structure. The SELEX technique has also been employed in gaining cellular knowledge on how the tRNA import into the mitochondrion of *L. tropica* is achieved (Bhattacharyya et al., 2002). However, approaches towards the development of therapeutic have yet to be carried out due to missing biological activity of the selected aptamers or difficulties in accessing target proteins. Therefore, current applications of aptamers to *Leishmania* are mainly restricted investigative or diagnostic tools.

4. SELEX applications towards treatment of *Trypanosoma* infections

In the last century infectious diseases caused by the tropical protozoan parasites *Trypanosoma* became more prominent due to their medical importance. Infections are caused by the pathogens *Trypanosoma cruzi*, the causative agent of Chagas' disease occurring in Latin America, and *T. brucei ssp*., the African trypanosomes, which are responsible for sleeping sickness and Nagana in cattle. These infectious diseases are accountable for relevant health and socioeconomic problems in endemic countries such as Latin America, sub-Saharan Africa, and tropical and other subtropical areas in Africa and America. The current medication is known for its toxicity, poor activity in immune-suppressed patients and long term treatment combined with high costs. Moreover, vaccines are not foreseeable in the near future. Therefore, the current treatment is just relying on chemotherapeutics. The treatment of Chagas' disease is based on two nitroheterocyclic drugs, nifurtimox and benznidazole, which are administered as pro-drugs and become intracellularly activated. However, both drugs occasionally reveal serious side effects, but no other compound is near preclinical or clinical development for the treatment of Chagas´ disease. The human African

trypanosomiasis (also called sleeping sickness) is caused by *T. brucei*, which is transmitted to humans by infected Tsetse flies. Human sleeping sickness is triggered by two sub-species *T. b. rhodesiense* is responsible for acute infections and *T. b. gambiense* leading to more chronic infections. The parasites proliferate in the lymph, blood as well as in peripheral organs and invade the central nervous system, instigating serious neurological disorders (Barrett et al., 2003).

Without treatment the disease is lethal. In the human host, African trypanosomes sustain extracellularly and face the exposure to the human immune system. The parasites circumvent the human immune response mainly by antigenic variation of their variant surface glycoprotein coat (VSG) (Vickerman, 1978). However, the parasite also displays invariant surface proteins, but they are inaccessible to the host's immune response.

Prior to infesting the central nervous system by the parasites, medication is carried out with suramin and pentamidine against the *rhodesiense* and *gambiense* forms of the disease, respectively. Afterwards first-line treatment is mediated by melarsoprol, although the drug often induces serious, sometimes fatal side effects. Originally developed for cancer chemotherapy, eflornithine was also effective against the *gambiense* form. Eflornithine is an inhibitor of the ornithine descarboxylase (ODC) leading to a depletion of the parasites' polyamine levels (Docampo and Moreno, 2003). Interestingly, the drug shows similar affinity to both the mammalian and parasite's enzymes; however the trypanosomal ODC has a shorter half-life than its mammalian counterpart which makes the parasite's metabolism more susceptible resulting in growth arrested. The parasite does not differentiate into the non-dividing stumpy forms and become sensitive to host immune system, since these forms are not able to undergo antigenic variation of their VSG (Wang, 1995). Surface proteins, such as the VSG coat of the African trypanosomes, were also targeted by aptamers. Three aptamer families were isolated with binding affinities in the nanomolar and subnanomolar range (Lorger et al., 2003). Since RNA aptamers are subject to rapid degradation in serum they need to be modified in order to prolong their half-life from a few seconds to several hours (Ulrich et al. 2004). Chemical modification of the anti-*T. brucei* aptamer led to nuclease resistance (Lorger et al., 2003). However, as per definition the VSG coat is variable and thereby modified by the parasite in time to escape the immune response of the human host, attention was drawn to other surface proteins of *T. brucei*. In particular, invariant surface glycoproteins (ISGs), which comprise receptor complexes and transporters (Overath et al., 1994), were targeted by RNA aptamers using a combinatorial selection process on live trypanosomes. The selected RNA aptamers bound to a single 42 kDa protein located within the flagellar pocket of the parasite which might be ESAG 7, a transferrin receptor subunit (Homann and Goringer, 1999). These results were very promising since the aptamer bound to an invariant trypanosomal surface protein enabling the selective targeting of the non VSG coat. Even further, Homann and Goringer could demonstrate that at increased temperature, the terminal ends of the aptamer were degraded and became rapidly engulfed into the parasite and incorporated into the lysosome by vesicular transportation. Proof-of principle experiments were carried out with biotin labelled aptamers which were visualized by immune fluorescence microscopy using anti-biotin antibodies (Goringer et al., 2003). The obtained results demonstrate that the specific RNAs could be exploited as so called 'piggy backs' molecules to traffic aptamer-coupled drugs into the lysosomal compartment of the parasite. Such aptamers might have the

potential to act as novel drugs against African trypanosomiasis. SELEX applications were not only restricted to T. brucei; this technology was also applied on American trypanosomiasis caused by T. cruzi. As already outlined the causing agent of Chagas' disease depends on invasion of host cells to complete its life cycle. Thereby the parasite needs to build up parasite-host cell adhesion for initiation of the invasion process (Alves and Colli, 2008). Macromolecules on host cell surfaces such as laminin, thrombospondin, heparan sulfate, and fibronectin are believed to be involved in parasite-host cell contact (Ulrich et al., 2002, Simmons et al., 2006). Approaches have been undertaken to selectively interfere with parasite host cell receptors in order to inhibit the cellular invasion process of *T. cruzi*. The SELEX technology was employed to evolve nuclease-resistant RNA aptamers which block *in vitro* receptor-ligand interactions between *T. cruzi* trypomastigotes and epithelial monkey kidney LLC-MK(2) cells and thereby partially inhibit cell invasion by the parasite (Ulrich et al., 2002). Aptamers were identified with binding affinities in the nanomolar range to parasite receptors expressed by infective trypomastigote and not by insect epimastigote forms for the host cell matrix molecules fibronectin, heparan sulphate, laminin and thrombospondin (Ulrich et al., 2002; Alves and Colli, 2008). Reduction in the infection rate *in vitro* of up to 70 percent was observed at a low micromolar aptamer concentration.

5. SELEX approaches have also been undertaken to other protozoan parasites such as the malaria pathogens

The parasitic disease malaria represents one of the most serious threats to human health worldwide with an enormous impact on the mortality and morbidity, especially in sub-Saharan Africa. More than 2000 million people are exposed to the infection with malaria leading to estimated 500 million clinical cases and more than one million deaths per annum; mostly young children in Africa. A vaccine is not available and the control of the disease depends solely on the administration of a small number of drugs. Malaria is presently undergoing resurgence and the fight against *Plasmodium falciparum* -the most virulent species accounting for over 90% of deaths- has become a significant problem (Greenwood et al., 2008). Due to the high mutational rate of the parasite and its resulting rapid adaptation to environmental changes, its drug resistance and geographic distribution are increasing. Further aspects such as socio-economic factors, increasing migration patterns, failing health care systems and the rapid development and dispersal of the respective insecticide resistant forms of the mosquito vector are contributing to the problem. Currently, only one drug, Artemesinin, is still effective against the malaria parasite. However, the first evidence of drug resistance against Artemisinin and combinations has been reported at the Cambodian/Thailand border from where resistance against the most cost-effective drug, chloroquine, has also spread to the rest of the world (Wangroongsarb et al., 2011). Therefore continuous discovery and development of new drugs are urgently needed. In the past the discovery of novel antimalarials was mainly directed towards chemotherapeutic interventions and vaccines (Alonso et al., 2011). As far as drugs are concerned, major focus was drawn on the peculiarities occurring in the malaria parasite such as the folate metabolism, haemoglobin degradation and subsequently the polymerisation of heme into the chemically inert “malaria” pigment, hemozoin. Chloroquine is believed to interfere with the heme detoxifying biochemical pathway which leads to parasites’ death. In a recent

report aptamers were used to prevent hemozoin formation in a proof-of-concept study to demonstrate that oligonucleotide-based drugs are exploitable to modulate essential biochemical pathways in *P. falciparum* (Niles et al., 2009). Indeed hemozoin formation was significantly inhibited by heme-binding aptamers in parasite extract. Even further, a growth inhibitory effect could be shown in aptamer preloaded human erythrocytes (Niles et al., 2009). However, *in vivo* assays have yet not been performed in order to evaluate whether these aptamers are also applicable as drugs. In another approach RNA aptamers were generated binding to the major parasite derived surface protein embedded in the erythrocytic membrane – the *var*-gene encoded *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) (Barfod et al., 2009). *Pf*EMP1 is involved in cytoadherance to human cells, such as endothelial cells of blood vessels or in spontaneous binding of uninfected erythrocytes to *P. falciparum*-infected red blood cells (rosetting). The secretion of this protein is proposed to accentuate the symptoms of malaria by preventing the clearance of infected erythrocytes in the spleen (Buffet et al., 2011). While doing so the parasite would become susceptible to the human immune system. However the genome of the parasite encodes about 60 var-genes and the parasite is able to switch expression to another gene of this family, leading to a different *Pf*EMP1 protein. Due to antigenic variation the parasite can avoid interference with the host immune response (Flick and Chen, 2004). Recently, Barfod and co-workers selected aptamers against the recombinantly expressed N-terminal DUFFY-binding like domain (DBL1a) of the *Pf*EMP1 protein. Subsequently, the isolated aptamers were tested on cellular level by screening against populations of infected and non-infected erythrocytes (Barfod et al., 2009). The set of biological active aptamers inhibited the rosetting progress which is indicative for the functionality of the RNA ligands and promising for further evaluation of their activity *in vivo*.

6. Conclusion

Aptamers are able to identify individual epitopes within a complex mixture of potential targets and can also be used for ligand-mediated target purification (Nery et al., 2009, Ulrich and Wrenger, 2009). It has been demonstrated that aptamers evolved against human membrane proteins bind specifically to previously unidentified target proteins (Morris et al., 1998). The same methodology (deconvolution SELEX) was used to stain rat brain tumor vessels and identify the endothelial regulatory protein pigpen as aptamer-target protein (Blank et al., 2001). These works resulted in the conclusion that each cell type differs from others by its molecular signature exposed on the cell surface. The fact that aptamers can distinguish between small differences in the molecular signature of cell-surface antigens emphasizes the feasibility of the chosen technology. Even further highly specific aptamers distinguishing between host cell proteins are capable to selectively bind and thereby interfere with parasite-derived proteins in order to stop proliferation of these deadly human parasites.

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Drug Discovery and Development - Present and Future

Edited by Dr. Izet Kapetanović

ISBN 978-953-307-615-7
Hard cover, 528 pages
Publisher InTech
Published online 16, December, 2011
Published in print edition December, 2011

Drug discovery and development process aims to make available medications that are safe and effective in improving the length and quality of life and relieving pain and suffering. However, the process is very complex, time consuming, resource intensive, requiring multi-disciplinary expertise and innovative approaches. There is a growing urgency to identify and develop more effective, efficient, and expedient ways to bring safe and effective products to the market. The drug discovery and development process relies on the utilization of relevant and robust tools, methods, models, and validated biomarkers that are predictive of clinical effects in terms of diagnosis, prevention, therapy, and prognosis. There is a growing emphasis on translational research, a bidirectional bench to the bedside approach, in an effort to improve the process efficiency and the need for further innovations. The authors in the book discuss the current and evolving state of drug discovery and development.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Carsten Wrenger and Henning Ulrich (2011). Drug Discovery by Aptamers in Protozoan Infectious Diseases, Drug Discovery and Development - Present and Future, Dr. Izet Kapetanović (Ed.), ISBN: 978-953-307-615-7, InTech, Available from: http://www.intechopen.com/books/drug-discovery-and-development-present-and-future/drug-discovery-by-aptamers-in-protozoan-infectious-diseases

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