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Review

Virtual Screening and Zebrafish Models in Tandem, for Drug Discovery and Development

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Abstract: Introduction: The combination of Virtual Screening (VS) techniques with in *vivo* screening in the zebrafish model is currently being used in tandem for drug development in a faster and more efficient way. **Areas covered:** We review the different virtual screening techniques, the use of zebrafish as a vertebrate model for drug discovery and the synergy that exists between them. **Expert opinion:** We highlight the advantages of combining virtual and zebrafish larvae screening for drug discovery. On the one hand, VS is a faster and cheaper tool for searching active compounds and possible candidates for therapy than in *vivo* screening when processing large compound libraries. On the other hand, zebrafish larvae form a vertebrate model which allows *in vivo* screening of large amounts of the compounds. Importantly physiology and chemical response are mostly conserved between zebrafish and mammals. The availability of the transgenic and mutant zebrafish lines allows an analysis of a specific phenotype upon treatment along with toxicity, off-target effect, side effects, and dosage. Advantages of VS, *in vivo* whole animal approach screening, and the screening combinations are also reviewed.

Keywords: drug development; high throughput screening; in vivo/in silico screening; zebrafish

Highlights box

- 96% of clinical drug development fails. This low efficacy is especially important in emergency situations, such as the current pandemic or rare diseases.
- VS is one of the most powerful tools for the identification of therapeutically active molecules with a much lower investment in time and cost.
- Zebrafish larvae are the only vertebrate model suitable for *in vivo* high-throughput compound screening. The main biological pathway and chemical response are highly conserved between humans and zebrafish.
- A multidisciplinary team with experience in VS and *in vivo* zebrafish larvae screening can reduce the number of molecules that need to be tested next in mice models or in clinical trials , leading to time and money being saved and more efficient DD.
- The combination of VS and zebrafish larvae are so powerful in drug development that the only limiting factors are the knowledge of target and biological pathways together with the creativity of the researcher.
- DD should start from the computer, moving on to the aquarium and then finally to the patient's bedside.

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1. Introduction

'As an integral part of drug discovery (DD), drug screening (DS) involves screening new drugs or compounds with biological activity obtained from natural products or synthetic compounds. There are risks and time constraints associated with traditional drug discovery and development, including target identification and validation, lead compound discovery and optimization, as well as preclinical and clinical trials (1). The mean cost of developing a new drug is the subject of debate, with recent estimates ranging from $314 million to $2.8billion (1) and the attrition rate of drug candidates being as high as 96%, usually due to unfavorable absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties (2) contributing to excessive consumption of time, capital and human resources. It is therefore imperative to develop strategies which enhance the predictive value of current preclinical animal models and/or combine these with improved in silico and in vitro tools so that the best candidates can be narrowed down before entering costly preclinical and clinical phases

The discovery of innovative drugs requires collectively high throughput screening (HTS), low cost, low time consumption, high efficiency, automation, and physiological effect. As such, the in *silico* and *in vivo* screening combination is very attractive. Computer-aided drug discovery approaches help to mitigate the time scale and cost issues of candidate compounds. Then, the reduced number of chemical compounds needs to be evaluated experimentally either *in vitro or/and/in vivo*. *In vitro* models, while very useful, ultimately turn out to be artificial systems that do not reflect or mimic the biological desease complexity, and *in vivo* rodent studies are time-consuming and expensive, limiting the number of formulations that can be practically evaluated in any given study. As a promising, recent vertebrate model, zebrafish is unique in that it offers the opportunity of screening quickly and cost-effectively under in vivo conditions, bridging the gap between in vitro and rodent studies.

It is easy to visualize the combination of virtual screening (VS) and high/medium throughput zebrafish larval screening can save both money and time for rapid drug development, making it possible to advance compounds to clinical trials in a shorter time frame. After a survey of the literature, it is interesting to note that the number of scientific publications mentioning *VS* and *DD* has grown steadily (Figure 1A, 1B). In 2021, there were more than 86 published articles, approximately 8 times the number 15 years ago (Figure 1C). A similar trend can be observed for publications related to *zebrafish* and DD. Although the absolute number of scientific articles is lower than that of VS/DD, publications are 13 times higher than 15 years ago (Figure 1). Surprisingly, publications combining VS, zebrafish screening, and DD criteria are hard to find, although they are also increasing, although much more work is needed to exploit the combination of both methodologies.

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Figure 1

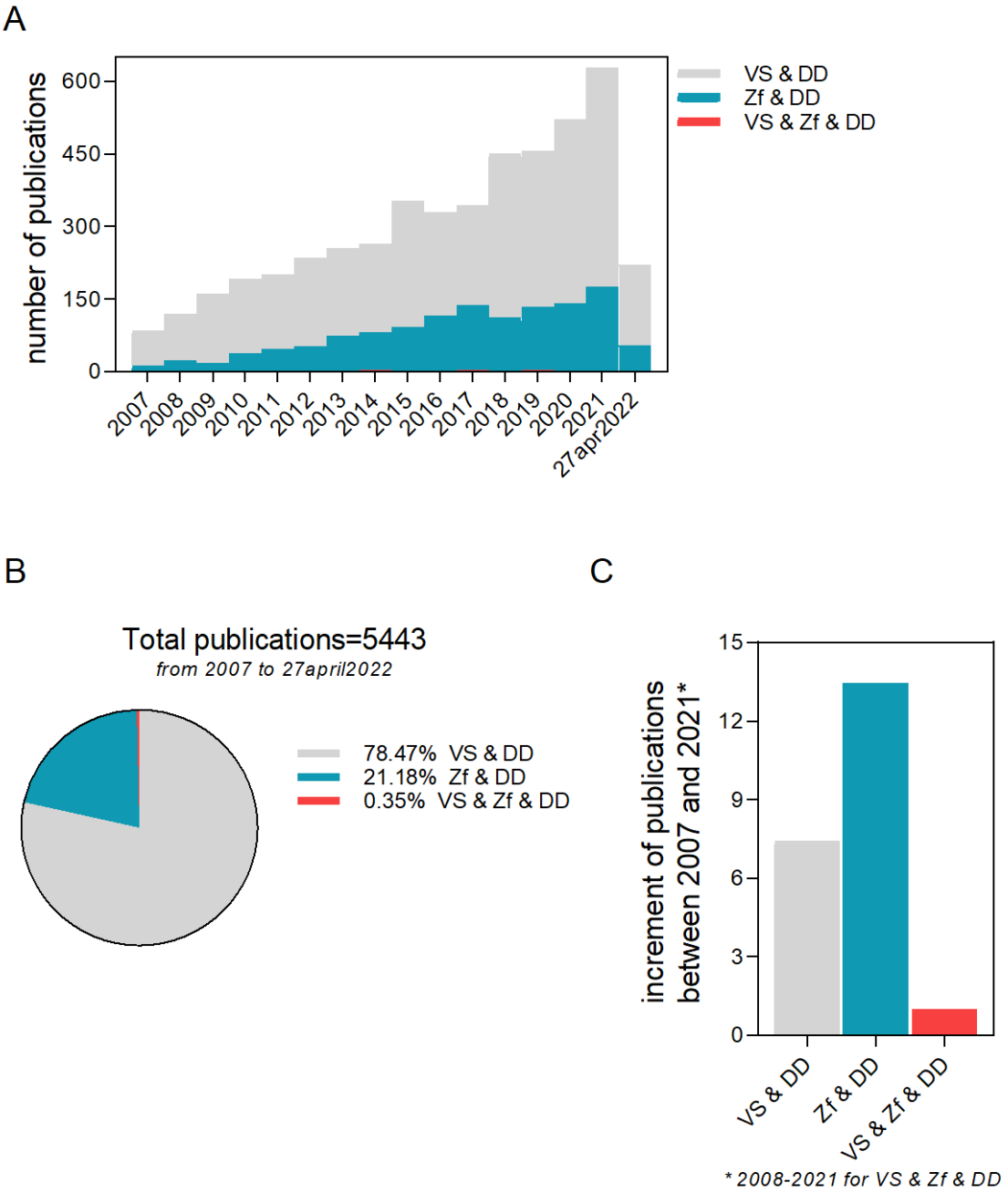

FIGURE 1. Evolution of publications in Virtual screening (VS) and in vivo zebrafish screening (Zf) and their combination with Drug Discovery (DD) from 2007 to April 2022. A) and B) Numbers of publications and proportions C) Comparison of the increase in publications in 2021/2022 compared to 2007.

This review will start by discussing the background, suitability, and advantages of virtual screening, *in vivo* whole animal approach screening, and the screening combinations, providing new hope for the discovery of safe, specific, and powerful new drugs in record time.

2. Virtual Screening (VS)

VS techniques were first used in the 1980s (3) but it was not until 1997 that the first publication on virtual screening appeared (3). This is a computational or in-silico technique, which allows cost-effective identification of new bioactive substances from large

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libraries of compounds (4) consisting of 1 dimension (D), 2D, and 3D chemical structures (5–8). In addition to bioactivity, VS can identify pharmacokinetics and/or pharmacodynamics properties (9,10) which can be divided into two main types: structure-based virtual screening (SBVS) and ligand-based virtual screening (LBVS) (Figure 2).

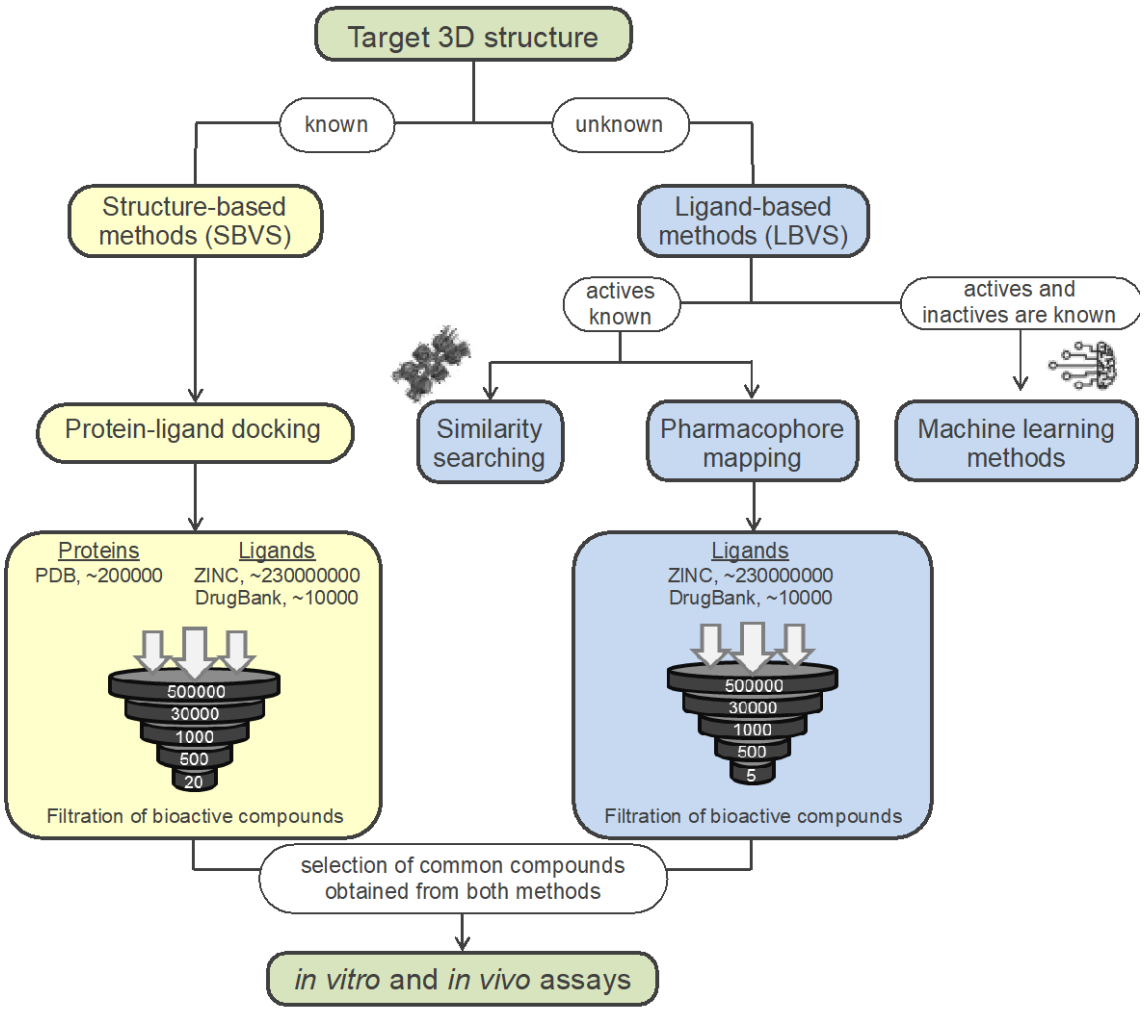

FIGURE 2: Schematic representation of the different virtual screening modalities.

2.1. Structure-Based Methods (SBVS)

SBVS methods predict the interaction between the ligand and the molecular target (ligand-receptor) (11). In this type of VS, compounds are ranked from a database by a scoring function that predicts ligand-receptor affinity and is implemented in the molecular docking process (12–14). SBVS shows the following advantages (12): i) It does not require expensive computational resources, ii) since each docking simulation typically only needs a few minutes. iii) when compared to full library wet-lab screening, fewer molecules are synthesized or purchased since only the top-ranked compounds are evaluated in the laboratory. Existence of available software tools (i.e., https://www.click2drug.org/) that help to perform SBVS.

Nevertheless, SBVS also have disadvantages that are detailed as follows (12) Other tools work better in specific cases (15) Low accuracy in predicting ligand-receptor binding affinity and subsequent classification of compounds; and finally, they can generate false positives as well as false negatives.

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Search algorithms are used to predict the orientations (16–18) and conformations that ligands can adopt at the binding site by the target molecule (4,16–21).

Accordingly, a good docking protocol will obtain more realistic ligand-receptor conformations and binding positions at the binding site. This algorithm performs a global search for most of the possible positions that can occur between the ligand and the receptor-binding site including rotational and translational degrees of freedom to the ligand (22).

2.2. Ligand-Based Methods (LBVS)

LBSV uses both the structural information and physicochemical properties of the structure of molecules, and if these molecules are active, it performs a VS under the principle of a previously defined similarity metric. Thus, from a set of known active molecules, new candidate compounds can be identified that should bind to a molecular target through a similar mechanism and hence exert a similar biological effect on the starting molecule (5,23).

2.2.1. Pharmacophore Methods

According to the International Union of Pure and Applied Chemistry (IUPAC) definition, a pharmacophore is "a set of steric and electronic characteristics necessary to ensure optimal supramolecular interaction with a specific biological target and to activate or block its biological response". This concept is based on the interactions observed in molecular recognition. Such interactions can be hydrogen bonds, positive or negative charges of molecules, and hydrophobic regions. This type of mapping has proved to be a very useful and successful tool in understanding the recognition process between the protein (receptor) and the ligand (24–26).

To generate a pharmacophore model, on top of knowing the interaction that occurs between the receptor protein and the ligand, the 3D structure of the receptor must be known. Currently, there is a platform where we can find 3D structures of proteins, expressly, the Brookhaven Protein Data Bank (PDB) (27,28), along with the ligand-binding site, where we can generate a 3D structural model of the most relevant ligand-based interactions (5).

The software LIGANDSCOUT (29) software allows the analysis and interpretation of structural information of ligands and posterior generation of pharmacophore models (29,30).

In contrast, when pharmacophore models are used for screening new ligands, the affinity estimation is based on the geometry of the atoms and the characteristics of the model (5). This type of screening yields many possible candidates, although it has a several other advantages including discrimination of non-binder compounds. Furthermore, the computational level has a lower cost when compared to other techniques such as high-throughput docking (5).

This VS method can be used if a group of ligands (compounds) is active and their 3D structure is known, meaning we can define their pharmacophores (31). Such a pharmacophore can serve as a model to perform VS, especially since this model is used when the 3D structure of the receptor is unknown and thus, SBVS cannot be performed (5).

With this pharmacophore approach, better performance is usually obtained than using just ligand similarity, since this method operates using a wider set of active molecules containing completely different chemical structures but showing the same pharmacophore features (5).

Having said that, Pharmacophore-based VS methods do show some disadvantages: i) both conformation and molecule overlap might be inaccurate or insufficient (32), ii) they might exhibit ambiguities in the pharmacophore model (this is related to the protonation and tautomer state of the compounds) (32), iii) they might show inadequate binding sites

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in the active center of the target molecule when ligand structures are not available, leading to incorrect ligand-receptor binding affinity (32).

2.3. Combination of SBVS and LBVS

Molecular modeling methods based on both ligands and structures that exist today have been very successful in VS by retrieving new compounds as potential candidates for the process of creating new drugs. These methods, as well as others, display some disadvantages such as i) the Structure-based (SB) pharmacophore method, ii) the selection of the main pharmacophore features not being trivial, and iii) having to take into account the potential ligand conformations.

These types of disadvantages must be considered when choosing the method to be used (33).

The pharmacophore model is very important when we do not know the active form of the ligand or the structure of the target protein (receptor), because in this case, it offers an advantage, aside from being able to identify a novel compounds, which is that a series of profiles can be designed to avoid the side effects that can occur when exerting a function foreign to the target protein (34). The SB pharmacophore model is provided by the structure of the target protein, after a prior investigation of all possible interaction zones in the binding cavity is performed (32).

Relevant interaction zones can be identified by energy- or geometry-based methods, resulting in a pharmacophore method. When all the necessary information about the ligand is available, such as 3D structure and pharmacophore characteristics, among others, is available, the structure-based pharmacophore model is used, where all possible interaction zones between the protein and the ligand are described.

The magnified advantage the SB pharmacophore method possesses is the higher probability for the identification of compounds that can be potentially active, leading to a higher computational cost which is considered to be a disadvantage (32,35). The combination of both techniques has delivered both good and bad results since at present there is an inability in the fusion strategies between the two methods to be able to consistently offer superior performance concerning techniques separately (32).

There are different drugs on the market that have been discovered with VS techniques, including saquinavir, ritonavir, and indinavir (antihypertensive drugs), saquinavir, ritonavir and indinavir (three drugs for the treatment of human immunodeficiency virus,) and others, that are in clinical phase III, such as nolatrexed, for the treatment of liver cancer (20,36–38). As previously, there are also different studies in which the combination of both VS techniques (SBVS and LBVS) is applied, where different drugs were found that are much more potent than that which currently exists, such as inhibitors of 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) (39,40). It is worth mentioning that the use of these techniques for the development, improvement, and repositioning of drugs is in general terms a much faster and less expensive way than using a traditional experimental method. A key example is the discovery of specific inhibitors of histone deacetylase 8 (HDAC8).

3. In vivo screening in zebrafish larvae

After the first step in the identification of therapeutically active molecules with a much lower time investment using computational techniques, preclinical studies should then be performed, in which the optimized molecules are tested in animal models to examine their pharmacokinetic properties and therapeutic potential. The final step is to carry out clinical trials.

As a result, a novel high-throughput *in vivo* evaluation system has recently been developed using zebrafish. The larvae of zebrafish show several advantageous properties over adult zebrafish or invertebrate models and even from rodent / human cells. First,

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husbandry costs are low, and larvae are readily available in large numbers and can develop externally from the mother. This allows for HTS set-ups under *in vivo* conditions. Second is the huge amount of transgenic zebrafish lines with a solid information network such as ZFIN (zfin.org). A third benefit is the optical transparency of larvae, which allows high-resolution viewing of specific biological events as they occur and across the entire living organism Finally, numerous molecular and biological tools are available to create new genetically modified zebrafish lines, which include morpholino oligonucleotides, CRISPR/Cas, mRNA, and transgenesis.

3.1. Zebrafish and humans

Around 70% of human genes have at least one clear zebrafish orthologue, based on comparison with the human reference genome. Interestingly, despite having no identifiable zebrafish orthologue for a few notable human genes (41). Zebrafish proteins with functionally similar activities may exist. In addition, in the Online Mendelian Inheritance in Man (OMIM) database, 3176 genes are described that are related to different diseases, of which 82% (2601 genes) have an orthologue in zebrafish (41).

Tools have recently emerged which are able to easily manipulate the zebrafish embryo's genome and this together with the knowledge of both the disease-leading genes in humans and the sequences of their zebrafish orthologues makes for a powerful methodology to create disease-specific models for the validation of novel drugs and the discovery of new therapeutics.

It is worth mentioning, human genes that are associated with many zebrafish genes (the 'one-human-to-many-zebrafish' class), with an average of 2.28 zebrafish genes for each human gene (41). This is because of an ancestor undergoing an additional round of whole-genome duplication. Gene redundancy, from an evolution point of view, helps an organism to survive when one copy of the homologs becomes non-functional or malfunctions or acquires a new function. However, it is undesirable for either the forward genetic approach to screen phenotypic mutants or reverse genetics to generate null alleles for target genes because the redundant genes might obscure the phenotypic drug screening or analysis.

Although the zebrafish is an aquatic animal, most zebrafish organs perform the same functions as their counterparts in humans, such as the pancreas (42), cardiovascular system (43) hematopoietic system (44), they also conserve critical parts of the innate and adaptive immune system. The inflammatory response has also been found to be well-conserved with human (45,46), The zebrafish brain and olfactory system share a significant degree of molecular an anatomical conservation with humans (47),

As above, processes such as aging or cancer are conserved (48,49). Interestingly, the human telomere length is more like that of zebrafish than of rodents, adding to a list of cases in which zebrafish physiology may be more relevant that its rodent counterpart as has been observed in their cardiac electrophysiology, vision dominated by cones, and diurnal behavior (50,51).

Several studies have shown that zebrafish respond biologically to chemicals, such as drugs and environmental toxicants, in a similar fashion to mammals, especially drugs that interact with the active region of the target protein. Indeed, drugs that modulate glucose homeostasis (52), hematopoiesis (53) cardiovascular pathways (54,55) inflammation (56) or teratogenicity (57) in humans have been proved o have identical effects on zebrafish.

3.2. Zebrafish drug screens

In addition to the advantages mentioned, it is added during the first days of development, zebrafish embryos can be kept in multiwell plates without the need for feeding. Chemical screening can be carried out by doing nothing more than diluting the compound of choice in the water in which embryos or larvae are raised. As such, a whole compound library can be tested quickly and efficiently in the direct context of a living vertebrate.

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The screening can be based on either phenotypic drug screening or target-based screening, in which drugs are identified based on the modification of a disease phenotype on whole organisms or in binding properties to specific molecular targets respectively. Both screenings allow simultaneous testing of the toxicity on other tissues. (Figure 3)

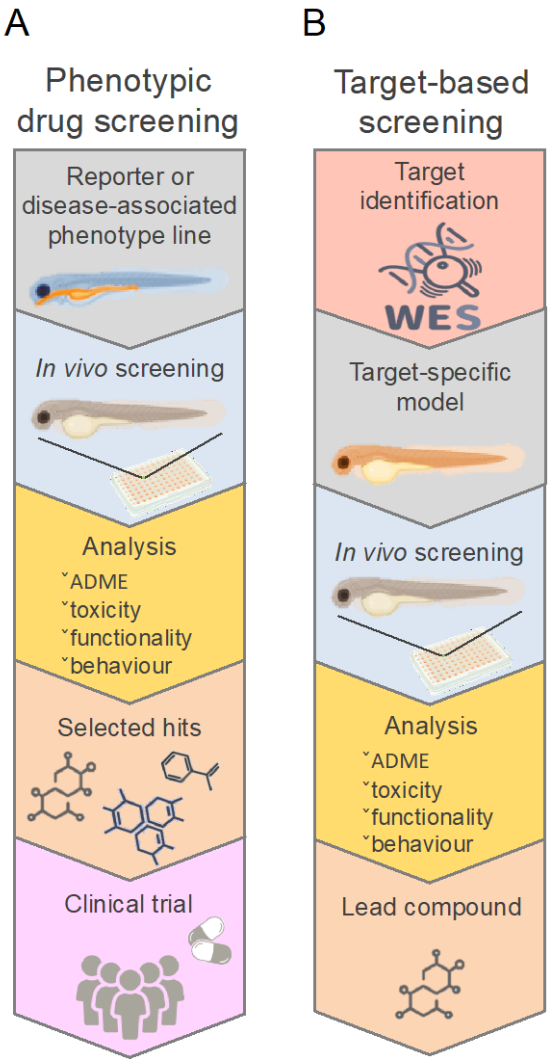

FIGURE 3: Schematic representation of the different zebrafish screening modalities. A) based on phenotypic drug screening or B) target-based screening.

3.2.1. A Phenotypic screening in which the DD is developed in a period of less than a decade driving new treatment for a rare disease (Dravet syndrome) is a good example from 'aquarium-to-bedside. The zebrafish deficient in scn1lab (homologous to SCN1A) recapitulate key clinical phenotypes in Dravet syndrome including spontaneous epileptic phenotypes at behavioral and electrophysiological levels (58). These unique features allow for high- throughput drug screening in the larvae of zebrafish (58,59). A phenotype-based screen of 320 compounds identified a US Food and Drug Administration (FDA) -approved compound (clemizole) able to inhibit convulsive behaviors and electrographic seizures. Additionally, clemizole (EPX-100) displayed a broad safety profile in recent phase I clinical studies and is now being researched as an 'add-on treatment' in a pivotal phase II clinical trial (60,61).

Structural analogs of clemizole and – as repurposed drugs – trazodone (Desyrel®) and lorcaserin (Belviq®) (62), were able to be effective in suppressing seizures. Lorcaserin

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and, to a lesser extent, have both shown efficacy against seizures in children suffering from DS. In a significant way, repurposed drug library allows a rapid clinical translation or compassionate-use trials.

3.2.2. A successful selected example of target-based screening is illustrated as follows. Generalized lymphatic anomaly (GLA) is a rare devastating disease spectrum of mostly unknown etiologies (63). The use of whole-exome sequencing (WES) allowed the identification of a recurrent gain-of-function ARAF mutation, which causes a conserved phosphorylation site to be lost, resulting in elevated ERK1/2 activity. The functional relevance of the mutation was also validated through the recreation of a lymphatic phenotype in a zebrafish model (tg(mrc1a :ARAFS214P, where the ARAF- expression targeted lymphatic vessels), with the anomalous phenotype being rescued by using a MEK inhibitor. Subsequent therapy of the lead proband with a MEK inhibitor brought about dramatic clinical improvement within 2 months of therapy, with the remodeling of the patient's lymphatic system, which showed resolution of the lymphatic edema and marked improvement in pulmonary function tests. Discontinuation of supplementary oxygen requirements together with near normalization of daily activities was observed after 12 months of therapy

4. Combination of VS and Zebrafish models

Surprisingly, despite the success in DD using VS and HTS in zebrafish, the combination of both tools has not been fully exploited. Using both models in tandem provides a way of offering great potential for both new drug discovery for different diseases and drug repurposing. Importantly, innovations in *silic*o target identification tools, mentioned above, allow faster and more accurate specific target determination, making target-based screening popular again. (Figure 4). Below, a selection of a wide range of different combined VS and *in vivo* screening strategies are illustrated.

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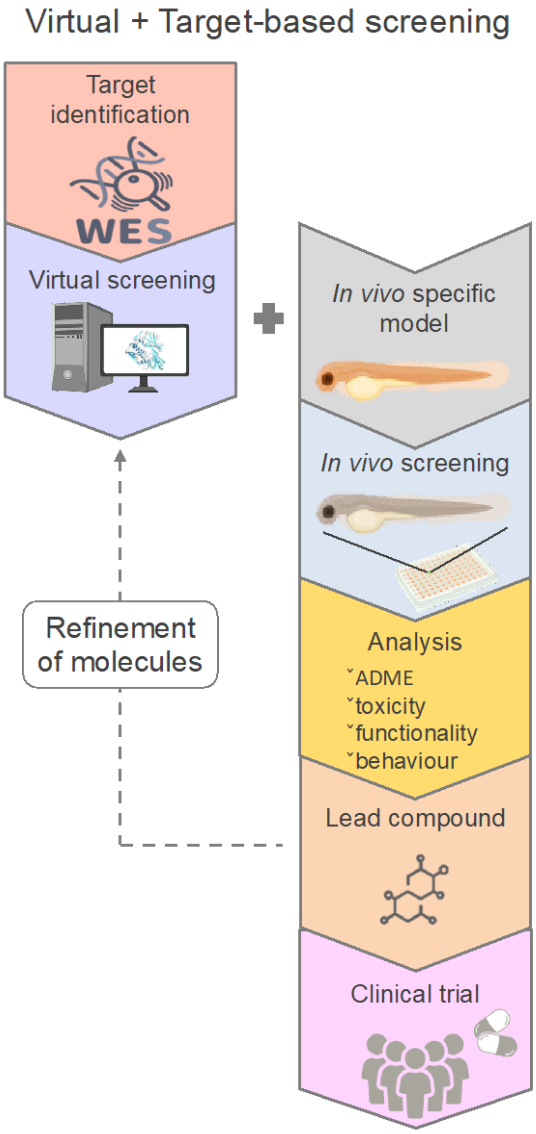

FIGURE 4. Combination of virtual screening and in vivo screening in zebrafish. A second round of screening is indicated in case refinement of molecules is necessary.

Xenograft. Fascin 1 is a vital actin-bundling protein involved in cancer invasion and metastasis whose expression goes hand in hand with poor prognosis. Therefore Fascin 1 is an excellent therapeutic target in cancer treatment. *In silico* screening calculations of 9591 compounds, including 2037 approved by the FDA, were performed, and analyzed by VS to identify a potential fascin1 blocker (64,65). Among the 20-30 top candidate compounds, imipramine (antidepressants) and raltegravir (anti-retroviral) were selected by different techniques such as thermofluor, fluorescence titration and in vitro characterization. Finally, both compounds were tested using the process of xenograft transplantation into larvae to evaluate their inhibitory activity in tumor growth, invasion, and metastasis. Imipramine is now an approved phase II clinical trial (64). The combination screenings allowed the repurposing to take place in less than 3 years and the number of compounds tested *in vitro/in vivo was* greatly reduced (99,5 % of the library were eliminated *in silico*)

Rapid development. Polo-like kinase 1 (PLK1), (66) one of the most important regulators of mitosis, is a target for cancer therapy given its abnormally heightened activity in several tumors. The first 10 mitoses of zebrafish embryonic cleavages take place

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every~30 minutes, providing a rapid assay to evaluate mitosis inhibitors, even those targeting Plk1. A computational virtual screen of~60,000 compounds against the human Plk1 3-D structure, which is highly conserved between zebrafish and humans, identified 370 candidates with the top free-energy scores. The candidates underwent a zebrafish assay and 3 were shown to inhibit cell division in the first 40 minutes after being added to the embryos. One of the 3, named I2, went on to demonstrate effective inhibition of' PC3 prostate cancer growth in the Xenograft mouse model *in vivo*. The IC_{50} values of I2 in these assays are compatible with those of ON-01910, a Plk1 inhibitor currently in Phase III clinical trials. Importantly, the authors compared results from a random screen using the ChemBridge DiverSet chemical library (from 5,376 compounds) with a computation-based pre-screen computation which was seen to increase the efficiency of identifying mitosis inhibitors by approximately 11-fold.

Green fluorescent protein* (*GFP) transgenic reporter. Zebrafish is a viable whole animal model for monitoring cell or biological processes (67). This is supported by the optical clarity of the embryo in combination with advancements in imaging technologies. 17 flavonoids as inhibitors or of angiogenesis were identified in almost 36,043 compounds from the Traditional Chinese Medicine (TCM) database (68). They were identified after computational screening against the crystal structure of human COX-2, human mPGES-1 and the 3D structure of CYP4A11 (Arachidonic acid metabolic enzymes), which all play an important role in glioma angiogenesis. Using a zebrafish transgenic line that expresses GFP in endothelia cells (Tg(*fli1a*:EGFP), isoliquiritigenin exhibited the most potent antiangiogenic activities**.** Therefore, in *silico* and zebrafish screening working in tandem is an efficient strategy for screening multiple-target inhibitors to block a biological process.

The homozygous double transgenic zebrafish, (Tg(*fli1a*: EGFP); Tg(*gata1a*:*ds*Red) which is expressed in endothelial cells and expresses the red fluorescent protein (*ds*Red) being controlled by *gata1* promoter in erythrocytes,) was used to study the inhibition of hemorrhages. The Rho/rho-kinase (ROCK) pathway is an important player in the pathogenesis of several cardiovascular diseases. To develop small-molecule inhibitors of ROCK1, molecular docking was employed to VS two chemical databases (which include 1.1 million structures). Chemical similarity clustering of the top 2000 compounds identified by Glide XP docking was carried out to maximize the chemical diversity of the selected compounds for biological assay. The top 200 compounds were then chosen from the individual clusters for experimental assay. The *in vitro* enzyme-based and cell-based assays showed that 12 compounds had good inhibitory activity against ROCK1 and the effects of 2 of its inhibitors were evaluated in an atorvastatin-induced cerebral hemorrhage in zebrafish larvae. Interestingly, one of the compounds exhibits more effective inhibition than fasudil (therapy for cerebral hemorrhage) in preventing the atorvastatin-induced zebrafish cerebral hemorrhage The structure of compound 24 is like that of nimodipine, the latter having been widely used to prevent a major complication associated with subarachnoid hemorrhage, namely vasospasm. Among these inhibitors, novel scaffolds by molecular dynamics simulations and free energy decomposition analysis were in evidence, thus forming the starting template for lead optimization and refinement.

Another zebrafish transgenic model has been used for the *in silico*/*in vivo* model combination. Such is the case of the zebrafish transgenic retinoic acid (RA) reporter line *Tg(12XRARE-elf1a: EGFP)*^{sk72} (69), which allowed agonists of nuclear receptors (Retinoic acid receptors (RARs), retinoid X receptors (RXRs) and peroxisome proliferator-activated receptors (PPARs)) to be identified. The stimulation of RARα, RXRα, and PPARα/γ may be beneficial in treating neurodegenerative diseases. Using a LBVS and in vivo transgenic reporters in zebrafish allowed the identification of a marine compound (Muqubilin A), which behaved as an agonist modulator of NR, paving the way for DD to treat neurological diseases and cancers . (70). As an alternative to fluorescent proteins, but based on imaging, a whole-mount staining strategy has been used in the assay of lipopolysaccharide (LPS)-induced leukocyte migration in zebrafish larvae. To accelerate the discov-

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ery of anti-inflammatory drugs, after virtual screening of an initial database of 1213 organic chemicals, 34 selected compounds were tested in zebrafish larvae and finally 6 of them were tested in the mouse model (71). More than 97% of the compounds were discarded by VS before the in vivo assay in zebrafish. It is important to note that 80% of the compounds tested in zebrafish were discarded before being tested in the mouse. Highlighted the importance of the combination of both screening methods that allowed the elimination of 99.5% of the compounds to be tested in mammalian models.(71).

Knockdown and humanized zebrafish model *the* development of new edition techniques, are powerful tools for the generation of mutant/Knock out, transgenic lines, including those that carry human gene mutated version (humanized zebrafish) for investigating and discovering treatment. This is especially interesting in the rare diseases (RD) as due to the low prevalence (from 1/2,000 to 1 /100,000) is not easy to conduct a clinical trial with a small number of patients.

Amyotrophic lateral sclerosis (ALS) is a devastating degenerative neuromuscular disease with an approximate incidence rate of 2/100,000 and [Cu-Zn] superoxide dismutase 1 (SOD1) was the first gene identified in ALS. The human SOD1 protein, when mutated, is more likely to induce other SOD1 wildtype proteins to misfold in a prion-like manner. A tryptophan residue at position 32 (W32) is predicted to participate in SOD1 misfolding and therefore in the toxic gain of function (72). The tryptophan at this residue is not well conserved, being serine in mice, and threonine in zebrafish. The transgenic embryos *mnx1*: GFP (where GFP expression is driven by *mnx1* promoter in motor neurons) were humanized by SOD1 wt and human SOD1 variant with W32 substituted for a serine (SOD1W32S). Substitution of tryptophan for serine prevented SOD1 toxicity meaning axonopathy and motor deficits were rescued.

A library of FDA-approved small molecules was ranked with VS based on predicted binding to W32, It then filtered analogs using a pharmacophore model based on molecular features of the uracil moiety of a small molecule as previous to interact with W32 (5'-fluorouridine or 5'-FUrd). Together with testing 5'-FUrd and uridine, a lead candidate from this list, telbivudine was selected as a result of its lower toxicity and improved blood-brain barrier penetrance, which significantly rescued SOD1 toxicity use as a potential drug to improve

An interesting strategy used for DD was the generation of a zebrafish model for Huntington's disease (huntingtin (HTT), -knockdown Zebrafish model) which allowed the identification of a compound, among 7 million molecules screened by VS, that mimics the HTT protein (73). The strategy was based on a search for small compounds or compounds that can perform the lost function of mutant protein (mimic) rescuing the disease.

Infection Model. Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is the second leading infectious disease-causing mortality worldwide. Topoisomerase I (Topo I), an essential mycobacterial enzyme, is heavily involved in the viability of the Mtb pathogen. Accordingly, the protein structure of Mtb Topo I 3D (74) was employed for VS of 5 million compound libraries in an identification process of various Mtb Topo I inhibitors. Hydroxycamptothecin was identified and structural derivatization of this compound yielded a set of fifteen compounds that were screened for in vivo anti-mycobacterial activity using the zebrafish infection model (75) and one of them was found to be more effective when compared to first-line anti-tubercular drugs, such as isoniazid and rifampicin.

There is an ongoing, albeit currently declining, global pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) This has posed a serious global public health emergency and with it, an unprecedented challenge to identify novel drugs for prevention and treatment. Although a concerted effort has been undertaken for DD using VS (76–78) nothing has yet been published regarding the combination with zebrafish. However, several laboratories have tried to analyze how useful zebrafish is as a model for COVID. These studies revealed that zebrafish showed an inflammatory reaction to SARS-CoV-2 rSpike protein (fragment N-terminal 16

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to 165), which led to organs (liver, kidney, ovaries, and brain) being damaged in a similar pattern to what happens in severe cases of COVID-19 in humans (79). In addition, it is known as Anosmia, and loss of smell is a prevalent symptom of SARS-CoV-2 infection. Interestingly, fragment of SARS-CoV-2 S1 protein, the RBD, caused olfactory pathology and loss of smell in adult zebrafish (80)

However, all this experiment has been performed in adult zebrafish, but only larvae are most suitable for high -throughput drug screening. Despite of low infectivity of SARS-CoV-2 in zebrafish larvae observed, it has been descripted a viral RNA stabilization after virus inoculation in the swim bladder, which is an aerial organ sharing similarities with the mammalian lung,(81). In this regard a humanized model has been generated by xenotransplantation with human lung epithelial cells (A549 cell line), into zebrafish swim bladders to study DD (80), test the safety of vaccines and immune response against SARS-CoV-2 and COVID -19. Future generation of transgenic zebrafish expressing human ACE2, will be needed to unlock the full potential of the zebrafish larva in the DD fight against COVID-19.

Drug toxicity testing. Zebrafish is fast becoming an *in vivo* platform to predict toxicity (with a particular focus on cardio-, neuro, hepato-, and nephrotoxicity) and teratogenicity of new compounds in the context of the whole animal, which could help to shift compound attrition to an early stage of drug development (82–85) As such, the developmental toxicity assay with zebrafish has become an interesting endgame for in-*silico* screening.

Such is the case of a structure-based VS of the Enamine database for 1.7 million (86) compounds that were applied in order to identify novel acetylcholinesterase (AChE) inhibitors. In this case, VS determined 29 compounds. Zebrafish were used as toxicity and safety *in vivo* models where in the end 3 compounds were chosen. Similar strategies have been followed for detecting inhibitor toxicity of O-GlcNAc transferase, NEDD8 activating enzyme, iNOS, which would be used in potential (85,87,88) anti-tumor, anti-inflammatory and anti-neurodegenerative applications.

All this proves once again that zebrafish assays coupled with computational screening significantly improve the efficiency of identifying specific regulators of biological targets and their toxicity, having been replicated in laboratories worldwide.

5. Conclusion

In the review, we wanted to show the importance of two very powerful tools in DD, such as VS (I Structure-Based Methods, Ligand-Based Methods, and Pharmacophore Methods) and the *in vivo* model of zebrafish larvae (phenotypic drug screening or target-based screening). In addition, this review highlights the importance of developing combined approaches that save time and money for rapid drug development and reach patients earlier which in this instance proved to be lifesaving.

This is especially important for RD and for pandemics such as the one we are currently experiencing, which require the development of rapid treatments and vaccines.

6. Expert opinion

The use of animal models is a fundamental part of biomedical research and crucial when developing new drugs. Mammalian models, such as rats and mice, have dealt with a huge variety of human diseases as preclinical models, but what cures the rat does not always cure the human (88). Many compounds have failed in clinical trials. The attrition rate of drug candidates is as high as 96%, usually due to unfavorable ADMET, properties, and others because the models were not well-characterized (2), leading to large consumption of time, capital, and human resources. This gives rise to a lack of improvement in' the health of patients. Progress is therefore needed in models (*in vitro* and *in vivo*) and *in silico* tools that improve predictions and accelerate DD.

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In this respect VS can be performed rapidly and at a low cost as several docking programs and other virtual screening tools are free for academic use (89). These screens are usually performed in a question of days using a recent mid-standard computer, but a high-performance computing cluster can do the same in less than an hour. Different screening strategies can be designed such as choosing a target or multiple targets in search of inhibitors that block a biological process, or even looking for a mimic compound, which functions as the protein that loses its function in the disease. The VS target and strategy depend on the knowledge and creativity of the researchers. In addition, VS may increase the efficiency of identifying drugs approximately 11-fold (66) when compared with a random screen using a chemical library. Although VS reduces the number of molecules analyzed from millions of compounds to hundreds or just tens of them, its procedures have yet to find correlation with more favorable *in vitro*/*in vivo* results.

The zebrafish animal model has become a prominent vertebrate model, allowing high- throughput *in vivo* screening for DD. It allows easy model generation, rapid generation of experimental replicas, the ability to incubate in multiwell plates and the ability to administer drugs in small amounts. Most drugs can be administered by simply adding them to the culture medium at a relatively low dose. Accordingly results are obtained in such a short period that the larvae do not even have to be fed. In addition, they are subject to fewer ethical regulations, and they comply with the 3Rs (Replacement, Reduction, and Refinement) to help reduce mammalian experiments.

As has been mentioned before, the zebrafish retains the main biological pathways (cancer, aging, hematopoiesis etc) and responds biologically to chemicals in the same way as mammals. There are some anatomical divergences but inherently several of these can be circumvented (72,75,90,91) The transparency of larvae and the use of transgenics zebrafish lines expressing fluorescent proteins in specific cells, favors the visualization of the effects of compounds on specific cells or organs. Furthermore, the easy genetic manipulation of embryos allows smart strategies. One of them is the developed humanized zebrafish model, in which either human cells are xenotransplanted into zebrafish or zebrafish native genes are exchanged for their human orthologs . Thus, allowing recapitulate the biological pathway but with an intact human target protein structure. These strategies would provide stronger evidence of drug-target interaction in DD (72,91).

The need is increasing for an efficient predictive tool of ADMET properties to reduce the risk of late-stage attrition. In this regard, deep learning techniques have often been used in ADME-Tox prediction. Zebrafish have been proposed as an excellent model for organ toxicity testing, but pharmacokinetics are not as well characterized in zebrafish as in mammals, so being able to understand pharmacokinetics better would help to optimize screening protocols. There is also a need for the development of automated techniques to facilitate throughout zebrafish screening.

Due to the field of knowledge of high-performance computing research being so far from that of zebrafish biological research, it would explain the low production in publications that include the VS and *in-vivo* ZF screening tandem. It is therefore necessary to encourage collaboration between the different professionals who know their models well enough to work synergistically and creatively in DD, thus allowing the process to be accelerated with extraordinary economic savings and more importantly, saving lives.

This review provides a representative demonstration of how the combination of VS and *in vivo* zebrafish screening offers a very powerful tool for rapid, inexpensive, and much more accurate DD (summarized in Figure 5) that, together with drug repositioning may constitute a new way to face future challenges, mainly in rare diseases and in current or future pandemics which need an urgent response.

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Figure 5

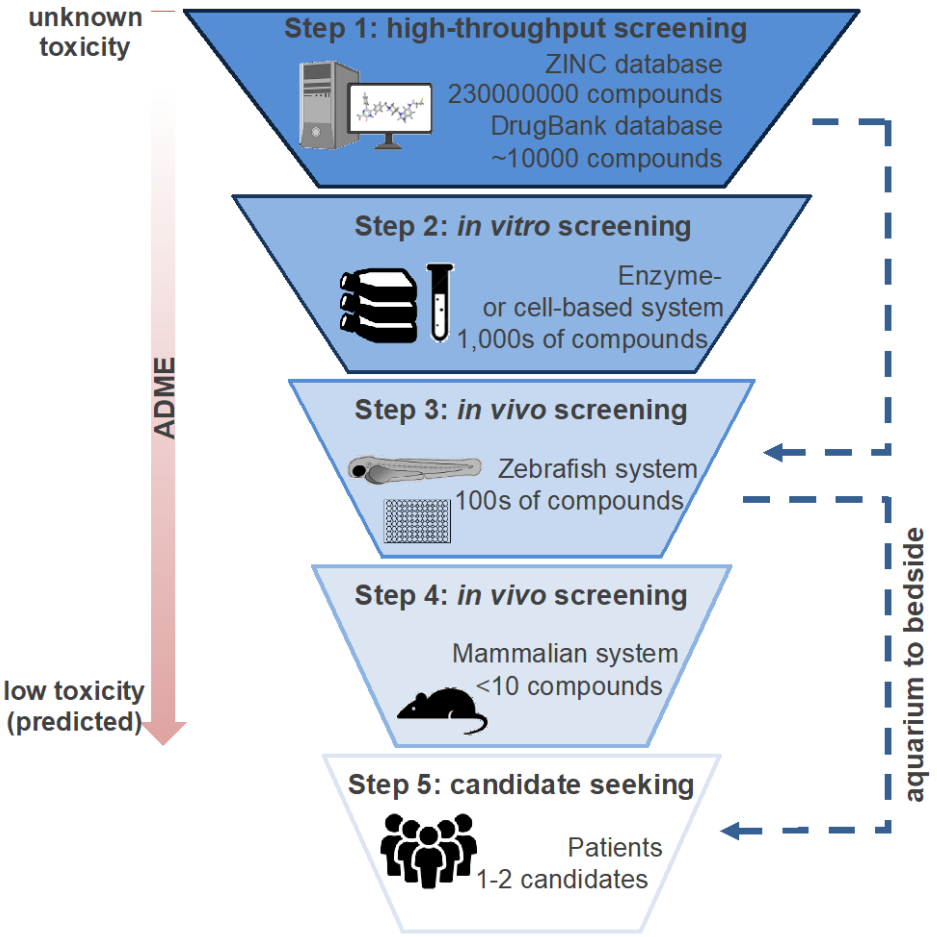

FIGURE 5. Integration of virtual screening and in vitro/in vivo modeling in clinical trials. An enormous reduction of compounds to be tested in vivo is achieved, which means time and cost savings. Simultaneous elimination of toxic compounds can be performed. Alternatives in the workflow are indicated with dashed lines, which allow further time savings and are especially useful in cases of rare diseases.

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Legends

1. FIGURE 1. Evolution of publications in Virtual screening (VS) and in vivo zebrafish screening (Zf) and their combination with Drug Discovery (DD) from 2007 to April 2022. A) and B) Numbers of publications and proportions C) Comparison of the increase in publications in 2021/2022 compared to 2007.
2. FIGURE 2: Schematic representation of the different virtual screening modalities.
3. FIGURE 3: Schematic representation of the different zebrafish screening modalities. A) based on phenotypic drug screening or B) target-based screening.
4. FIGURE 4. Combination of virtual screening and in vivo screening in zebrafish. A second round of screening is indicated in case refinement of molecules is necessary.
5. FIGURE 5. Integration of virtual screening and in vitro/in vivo modeling in clinical trials. An enormous reduction of compounds to be tested in vivo is achieved, which means time and cost savings. Simultaneous elimination of toxic compounds can be performed. Alternatives in the workflow are indicated with dashed lines, which allow further time savings and are especially useful in cases of rare diseases

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