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A countermeasure development pipeline

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Abstract

We have developed an integrated pipeline for countermeasure discovery that, under the auspices of the National Institutes of Health Countermeasures Against Chemical Threats network, is one of the few efforts within academia that by design spans the spectrum from discovery to phase I. The successful implementation of this approach for cyanide would enable efficient proof-of-concept studies that would lay the foundation for a generalizable strategy for parallel mechanistic studies and accelerated countermeasure development in the face of new and emerging chemical threats.

Keywords

cyanide; countermeasure; drug discovery; animal models; metabolomics

Cyanide is an archetypal chemical threat. This powerful natural toxin is encountered in many different settings, and potential exposures range from industrial accidents to major terrorist attacks. Cyanide has devastating consequences. Higher quantities lead rapidly to seizures, cardiovascular collapse, and death, while lower doses are associated with substantial long-term morbidity, including debilitating central nervous system injury. Currently available antidotes simply do not match the complexity or scale of the potential mass casualty threat scenarios, and there is an urgent need to develop novel countermeasures. To address these threats and to define an integrated approach that could be rapidly replicated for any new or emerging chemical threats, we developed a collaborative countermeasure discovery and development pipeline. In principle, the approach we describe is generalizable to virtually any therapeutic scenario that can be modeled in the zebrafish—extending beyond chemical warfare to disease models of many sorts.

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Conflicts of interest

The authors declare no conflicts of interest.

The major components of the drug discovery and development process we have put in place are (1) to identify novel classes of cyanide countermeasure using unbiased approaches in a validated zebrafish model system, (2) to explore *in vivo* the structure–activity relationships (SARs) of these novel countermeasure classes and use medicinal chemistry to generate a series of optimized lead compounds, and (3) to validate these optimized leads in established murine and rabbit models of cyanide toxicity. Together, these individual components are focused on the overarching goal of identification of novel cyanide countermeasures poised to move directly to formal preclinical testing and phase I clinical studies.

In order to accomplish this end, we integrated six discrete components: administrative, educational, and scientific cores and three research projects designed to accomplish the required steps from discovery to preclinical development. These include project 1—high-throughput *in vivo* discovery of cyanide antidotes; project 2—*in vivo* SAR and medicinal chemistry to generate optimized leads; project 3—validating leads in mouse and rabbit models of cyanide poisoning; core A—administration; core B—education; and core C—science (metabolomics).

Successful generation of validated countermeasures using this pipeline will not only lay the foundation for parallel work in preclinical development (through the Countermeasures Against Chemical Threats (CounterACT) Preclinical Development Facility (CPDF) as outlined below) and for subsequent clinical development of these compounds (in a logical extension of the current approach) but will also form a generalizable approach for the accelerated development of countermeasures to any existing or emerging chemical threat.

An outline of how each component relates and contributes to the others, including synergy between research projects and the scientific core, is given below and further elaborated in Figure 1.

Rationale

The need for novel cyanide countermeasures

The devastating consequences of large-scale chemical exposures remain among the great vulnerabilities of modern civilian populations. Whether the result of a terrorist attack or an industrial accident, we are poorly equipped to counteract the health consequences of such exposures.^{1–3} New, effective countermeasures are needed. The CounterACT initiative has recognized this need for “development of new or improved therapeutic medical countermeasures against chemical threats” and prioritized efforts around several specific chemical agents.^{4–7} Cyanide has been identified as among the highest-priority chemical threats.^{8–16} Milligram quantities of cyanide can cause convulsions, seizures, cardiovascular collapse, and death in minutes, and lower doses cause a spectrum of debilitating, long-lasting pathologies, including irreversible neuronal death in select brain areas.^{17,18} Unlike many other chemical threats, cyanide is readily accessible in large quantities, making it feasible for terrorists to acquire. In addition, cyanide is used widely in mining and other industrial processes, so the probability of mass exposure due to accidents is much higher than for other threat agents. Cyanide exposure can also occur as a consequence of smoke inhalation, consumption of cyanide-containing foods, and infusion of certain therapeutic

drugs.^{19–22} Therefore, development of improved cyanide countermeasures would have a positive impact on human health, even in the absence of a catastrophic mass exposure.

Existing cyanide countermeasures and their limitations

Several cyanide countermeasures are currently available in the United States: a two-component kit consisting of sodium nitrite and sodium thiosulfate⁴ and the single drug hydroxocobalamin. Nitrites are believed to function primarily by promoting formation of methemoglobin, which binds cyanide to form the relatively nontoxic cyanomethemoglobin.²³ Sodium thiosulfate is a substrate for the cyanide-detoxifying enzyme rhodanese. The combination of nitrites and sodium thiosulfate has many limitations. Importantly, the antidote is not easy to administer. Ideally, sodium nitrite and sodium thiosulfate are infused intravenously in sequence.²⁴ This requires significant time (sodium nitrite is typically administered over 10–20 min with continuous monitoring), relatively large infusion volumes (a typical dose of sodium thiosulfate is 12.5g in 50 mL, and hydroxocobalamin also requires substantial volume), and the involvement of experienced personnel. Thiosulfate has a distribution half-life of only 15 min after infusion²⁰ and exhibits limited intracellular distribution, while rhodanese is predominantly located in mitochondria, so the rate of rhodanese-mediated detoxification is low.^{17,25–27} Fourth, the methemoglobin formed by amyl/sodium nitrite can bind only about 10 mg of cyanide—larger quantities overwhelm this detoxification mechanism²⁰—though other unknown antidotal mechanisms allow better stoichiometry in practice. In addition, nitrites can cause dangerous decreases in blood pressure and systemic oxygen-carrying capacity.²⁸ Thus, this drug combination can benefit only small numbers of individuals and is inadequate for mass casualty scenarios. Hydroxocobalamin, a form of vitamin B₁₂, is a cyanide scavenger and is highly effective. It is safe at clinical doses and was licensed for use in the United States several years ago. However, like sodium nitrite and sodium thiosulfate, it requires intravenous or intraosseous infusion and so is of limited general utility as a countermeasure. Other antidotes, available outside the United States, exploit these same mechanisms, specifically methemoglobin formation (4-DMAP) or scavenging (dicobalt-EDTA).

Several cyanide antidotes are in development, including most notably cobinamide, a cobalamin analog, and sulfanegen, a substrate of the cyanide-detoxifying enzyme 3-mercaptopyruvate sulfur-transferase. Cobinamide and sulfanegen can be used singly or in combination to rescue animals from highly lethal doses of cyanide. Translation to clinical testing has been slowed by issues with solubility and stability, restriction in the total dose due to the volume required, and local toxicity with intramuscular delivery. Development of these two agents is being actively pursued, but there is room for new cyanide countermeasures that can be delivered rapidly using the intramuscular route in a mass casualty scenario. The discovery of such novel agents, enabling the neutralization of large cyanide exposures alone or in synergy with existing countermeasures, is the major focus of our countermeasure discovery platform and development pipeline.

Cyanide mechanisms

One of the major reasons why more effective mass casualty CN antidotes do not exist is the sheer complexity of the effects of cyanide. The cyanide anion has numerous toxic effects,

including inhibition of the mitochondrial cytochrome *c* oxidase complex blocking mitochondrial respiration, inhibition of adenosine triphosphate synthesis, and inhibition of the major antioxidant enzymes catalase and superoxide dismutase.^{3–6} Among the earliest effects of cyanide poisoning is the release of intracellular calcium and increased nitric oxide (NO) production through the induction of NO synthase.^{7,8} Cyanide also leads to the generation of reactive oxygen species, including hydroxyl radicals,^{9,10} and may itself be converted to the potent free radical of cyanide.¹¹ Exposure to a lethal dose of cyanide can cause death within minutes,¹² but there are also long-term consequences of sublethal exposure, particularly in the central nervous system, the precise mechanisms for which remain unclear. Short-term memory loss and a Parkinson disease–like syndrome are well reported, and there are also other less well-defined neurological problems in many subjects.⁶

Clearly, methemoglobin formation, rhodanese-catalyzed detoxification, and cyanide scavenging are not the only potential mechanisms by which a cyanide countermeasure might function. Several other candidate mechanisms have been proposed in the literature, each with varying degrees of experimental validation. Some of the potential countermeasure mechanisms focus on detoxification or elimination of cyanide itself, while others seek to reverse the biological sequelae of cyanide. However, the range of known toxic mechanisms, the broad spectrum of possible cyanide targets, and the potential for complex interactions among these targets suggest that a distinctive approach may be necessary. In the last decade or so, many previously intractable biologic problems have yielded to phenotype-driven screens, where a range of genetic or chemical manipulations are tested in an unbiased fashion to determine those pathways or compounds that result in the desired final phenotype.^{29–45} As more sophisticated phenotypic assays have been developed and automated, numerous examples have emerged implicating previously unsuspected pathways in specific processes or even in specific diseases.

Targeting multiple mechanisms in parallel

For many problems, including toxic exposures, it is important to model complex systems, such as the interactions between different tissues or organs. This has led to the concept of screening in intact organisms. Whereas target-based approaches lead to discovery of compounds that modulate an *in vitro* system but may not be effective *in vivo*, phenotype-based screens in intact organisms discover compounds that are by definition effective *in vivo*, without regard to the specific molecular target.^{46,47} This phenotype-based screening approach has been referred to as “chemical genetics” because it borrows from the logic of genetics, in which phenotype-based screening is used to identify novel genes that affect a biological process of interest. The development of many drugs in use today was guided by *in vivo* phenotypes,⁷ though not in any systematic manner. For conditions that affect the complex integrated physiology of the organism (such as cyanide intoxication), phenotype-based screens are a promising approach to discover novel therapies.

While systematic *in vivo* screens were initially undertaken in lower organisms, such as *Drosophila* or nematodes, in the last few years we have pioneered the use of the zebrafish for modeling vertebrate biology in health and disease at a scale compatible with screening libraries of tens of thousands of small molecules. The zebrafish has emerged as a powerful

tool for such phenotype-based screens.^{48–50} Its genome and body plan are similar to other vertebrates, but its optical transparency and external development make real-time observation of its internal organs simple. Numerous zebrafish disease models ranging from congenital heart defects to cancers have been developed,^{51–55} and the zebrafish is genetically and pharmacologically similar to humans.^{56,57} These attributes have already been exploited in multiple disease-suppressor screens, and the compounds identified have exhibited similar activities in mammalian models. Given the numerous potential mechanisms for reversing the effects of cyanide, we developed a large-scale, phenotype-based screen for cyanide countermeasures that we can then use to define diverse compounds with divergent mechanisms of action. Our prior work suggests that compounds that suppress chemical toxicity phenotypes in zebrafish may have direct utility as lead compounds for chemical agent countermeasures.^{58–75}

Validation in mammalian models

The zebrafish has emerged as the dominant vertebrate for *in vivo* high-throughput screening, but there are clearly significant differences between fish and human physiology. In the vast majority of instances to date, compounds with activity in the zebrafish model exhibit parallel activities in mammalian or even human systems. We have explored cardiovascular physiology at each scale from single channel to the integration of multiple ion channels and found remarkable homology between the larval zebrafish and adult humans. For all of the phenotypes studied, these homologies are at least as representative or even more so than rodent physiology. Nevertheless, we anticipate some differences in the way in which zebrafish and mammals respond to toxic exposures and to specific countermeasures. These include obvious differences, such as distinctive routes of entry (e.g., inhalational, intramuscular); distinctive metabolism, distribution, and toxicity of therapeutic compounds; and specific dosing schedules. However, there are also less well-understood issues, including the partitioning of duplicated genes into discrete expression domains in the zebrafish, the effects of poikilothermia on metabolic poisons, and distinctive lipids. To ensure that the countermeasures we develop have activity in mammalian cyanide poisoning, and to understand potential differences with zebrafish, we test each of our optimized leads in validated murine and rabbit models.

Center capabilities to enhance therapeutic development

Because of the need to improve our readiness as quickly as possible, efforts that will lead directly to the advancement of candidate therapeutics through the regulatory process are of highest priority. In our U54 Center, we have created an integrated pipeline using high-throughput *in vivo* zebrafish screening as a discovery engine to generate promising new potential antidotes for cyanide, further exploiting the fish for *in vivo* SAR to guide medicinal chemistry to allow the development of distinct lead compounds for rigorous validation in two mammalian species. The overarching goal for this effort is to identify lead compounds that are suitable for formal studies of pharmacokinetics and toxicology, which would then be positioned for phase I clinical studies under the U.S. Food and Drug Administration (FDA) Animal Rule.

The fundamental innovation underlying our pipeline is the integration of *in vivo* phenotype-driven screening in the zebrafish with robust mammalian models in what may be the only integrated discovery and development platform funded by the National Institutes of Health (NIH). Exploiting the comprehensive native context of the larval zebrafish and automated or semiautomated assays scaled for 96-well plates, it is possible to directly screen large chemical libraries for compounds that rescue end points relevant to human cyanide poisoning in this representative vertebrate. In addition, first-pass *in vivo* toxicology is also undertaken in the zebrafish allowing parallel evaluation for potential adverse effects during the screening phase. These capabilities for parallel counterscreening are further exploited through an *in vivo* approach to SAR that enables specific phenotypes to be correlated with structural features of the small molecule. When combined iteratively with streamlined efficacy and follow-up toxicity studies in mammalian models, these techniques offer a unique strategy for lead optimization. Notably, the murine models themselves incorporate features of the most likely threat scenarios, and the continuous optical and physiologic assessment available in the rabbit model allows the pathophysiology of cyanide toxicity and its rescue to be explored at a resolution that was not previously feasible. Along the pipeline, we have positioned metabolomics at each stage to link mechanisms in the different models and different tasks. This cutting-edge technology is very relevant to cyanide toxicity (and other chemical threats) and opens the possibility for novel insights into the mechanisms of action of cyanide and different countermeasures. Notably, we have previously validated the overall strategy with several agents in diverse therapeutic areas that are now in preclinical and clinical testing.

Implications for human health

Cyanide poisoning can result from accidental spills, ingestion, smoke inhalation, or as an iatrogenic outcome, such as from clinical sodium nitroprusside infusion.⁴ Novel, more effective cyanide countermeasures would provide measurable, life-saving benefits to the victims of these small-scale occurrences. However, safe and effective cyanide countermeasures would offer an even more dramatic impact in the face of a large-scale exposure, as might occur during an industrial accident or a terrorist attack, where no feasible options currently exist. In such an event, ready access to cyanide antidotes could transform a potentially devastating situation into a manageable one. Existing cyanide antidotes require administration by qualified personnel and have several associated toxicities that limit their utility.^{4,24,28,76} More importantly, they are limited in their mode of action. Existing antidotes focus on clearing cyanide from the system, typically by sequestration or conversion to a less toxic form. As such, they are not used prophylactically and do not mitigate the chronic effects of cyanide but are of utility when administered during the acute phase of cyanide exposure. The efforts in this proposal will lead to discovery of novel, potent, efficacious cyanide countermeasures. By identifying safe and effective cyanide countermeasures and expanding the range of mechanisms by which they function, our approach is designed to significantly enhance our ability to respond to chemical threats and protect human lives.

The integrated approach that we have designed screens ~25,000 novel compounds, optimizes four to five lead compounds, and validates one to two of these leads each year. Moreover, our approach has developed the infrastructure, scientific interaction, and

mechanistic understanding necessary to generalize this novel approach to countermeasure and drug development for other chemical threats and biomedical problems.

Specific components and their interactions

Phase I: high-throughput in vivo discovery of cyanide antidotes

This effort is focused on the discovery of novel cyanide countermeasures in phenotype-based chemical screens in the zebrafish.^{17,18} In prior work funded through the NIH CounterACT Program, we developed a zebrafish model of cyanide poisoning with stereotypical toxicities, including bradycardia, neuronal necrosis, and death. We have adapted this model for high-throughput screening and performed proof-of-concept studies in a screen for compounds that reproducibly rescue zebrafish from lethal cyanide exposure. In this screen (>100,000 compounds), we identified 26 small molecules that rescue cyanide toxicity at concentrations ≤ 1 μ M. We performed initial SAR with commercially available derivatives and identified two compounds with EC50s against cyanide of approximately 100 nM. These discovery efforts continue in several different ways. We are able to continue to prime the pipeline with discrete agents that emerge from new chemical libraries. We are able to screen in parallel zebrafish models of uniquely vulnerable or resistant populations, specifically early development, adolescence, and the aged. We are also prioritizing screen hits for development as optimized leads through the development of mode-of-activity profile assays for neurologic, cardiac, and systemic effects in the context of cyanide exposure and the testing of candidates in all potential pairwise combinations with all existing countermeasures and cobinamide and sulfanegen.

The hits generated are progressed for subsequent optimization through SAR and medicinal chemistry in project 2. Each of these hits must meet criteria for potency and satisfy initial screens for toxicity and mode-of-action profiling. Analog series generated in project 2 are also fed back to project 1 for efficacy and toxicity screens. Similarly, specific biological questions on the therapeutic or toxic effects of drug metabolites identified in project 3, as well as combination screens with existing agents, are undertaken in project 1. Project 1 uses the metabolomics platform for initial mode-of-action profiling and for characterization of the distinctive metabolic responses to cyanide across different age groups. These approaches exploit the compressed developmental timeline exhibited in the zebrafish and allow ongoing assessment of the interaction between cyanide and various countermeasures in both acute and chronic contexts.

Phase 2: optimizing novel cyanide countermeasures

This project builds on the hits with activity in our three cyanide toxicity–screening assays and uses SAR and medicinal chemistry to optimize the potency, toxicity, safety, and solubility of these novel classes to generate genuine leads that are then fed into project 3 for mammalian validation. Efforts furnish a series of optimized leads with potency, as well as absorption, distribution, metabolism, excretion, and toxicology characteristics suitable for further validation in mammalian models.

Interactions.—This project handles ~10 compounds per year, which undergo systematic SAR studies. Iterative rounds of compound design, compound acquisition (by purchase and synthesis), and compound testing deliver optimized compounds with high potency and efficacy. These optimized compounds are then profiled for physical and pharmacological properties, including pharmacokinetic stability and toxicology. Potent molecules with acceptable profiles are moved forward for further testing in mammalian models. Project 2 receives hits from project 1 and also returns optimized compounds to project 1 for high-resolution cardiotoxicity assessment. Project 2 passes optimized leads on to project 3 and also receives mouse and rabbit serum samples back from project 3 to assess compound pharmacokinetics. Project 2 exploits core C for absorption, distribution, metabolism, and excretion (ADME) studies of the optimized leads and for high-resolution mechanism of action studies.

Project 3: validating promising drug candidates in mammalian models of cyanide poisoning

Optimized lead compounds from project 2 are tested in two established mammalian models of cyanide poisoning, both singly and in limited combinations. In addition, range-finding toxicity studies are performed on the drugs in mice. Of the approximately 25 drug hits identified annually in project 1, we are able to optimize four to five per year for testing in mammals in project 3.

The mouse model is a cyanide gas inhalational model, which complements well with a potassium cyanide intravenous infusion model in rabbits. The drugs are administered by intramuscular injection to the mice and by intramuscular injection and inhalation in the rabbits, since the CounterACT program is primarily interested in finding a cyanide antidote that could be given rapidly in the field. The leads discovered in project 2 are also evaluated in a unique rabbit CN toxicity model monitored with noninvasive broadband optical technologies, including diffuse optical spectroscopy and continuous-wave infrared spectroscopy. This component enables the assessment of rate and extent of pathophysiologic events of CN poisoning as well as the therapeutic responses. It is also feasible to assess the effects of cyanide treatment agents in combined CN and smoke inhalation injury. All drugs are assessed for toxicity in the mice.

Interactions.—Project 3 tests each of the optimized leads emerging from project 2 for their efficacy and safety in mammals. This project uses core C for efficacy profiling as well as initial ADME studies in mammals. Drug metabolites identified in mammalian studies will be tested in project 1 and will also be used to inform additional rounds of medicinal chemistry in project 2. In addition, focused questions on mechanism of action or undetected drug toxicity will be explored further in projects 1 and 2. Ultimately, understanding the difference among the models employed in this proposal will inform future efforts to exploit this pipeline in the development of other countermeasures or other drugs and will contribute systems level information for the Animal Rule pathway.

The center is supported by three cores: an administrative core, an education core, and a metabolomics core. The administrative core is responsible for the fiduciary aspects of the

proposal. The education core focuses on the educational component of the U54 mission, executing it through the interdisciplinary training of a new generation of countermeasure scientists focusing on fostering interactions between countermeasure scientists and a range of emerging biologic disciplines. The metabolomics core offers scientific integration across the other disciplines within the program.^{75–86}

The metabolomics core

Recent advances in metabolic profiling technologies have significantly enhanced the feasibility of high throughput, phenotypic “snapshots” of a whole organism’s response to a given perturbation, though to date such technologies have principally been applied to studies in model organisms. The profiling of low-molecular-weight biochemicals, including lipids, sugars, and amino acids, that serve as substrates and products in metabolic pathways is particularly relevant to identifying diagnostic biomarkers that indicate exposure to toxic agents. In addition to serving as biomarkers, circulating metabolites may themselves participate as regulatory signals, such as in the control of blood pressure.

The core platform technology couples liquid chromatography with tandem mass spectrometry. A total of over 400 parent/daughter ion pairs (i.e., metabolites) are monitored through three selective reaction-monitoring experiments on each sample and quantitated based on internal standards. The core presently has the capability to analyze thousands of samples from humans or model organisms each year. The activities of the metabolomics core will directly benefit all three proposed projects. Metabolic profiling in zebrafish and mammalian models will contribute to the center in three significant ways: (1) it will lead to the discovery of novel diagnostic biomarkers of vulnerability to cyanide exposure, (2) it will help to establish similarities and differences between the various models of cyanide exposure, and (3) it will aid in the evaluation of countermeasure efficacy in each of the systems. The metabolomic platform is working actively with all three scientific projects at present, and we have tested the logistics of sample transfer between all the sites within the U54.

Long-term strategy

The work undertaken in this program will lead to the preclinical development of novel cyanide countermeasures, which will require formal phase I testing in humans under the Animal Rule pathway at the FDA, as cyanide cannot ethically be given to humans. This mechanism allows drugs to be approved through rigorous testing in animals in the absence of phase II and III clinical trials. If this platform proves as successful as we propose, we anticipate that three to four drugs will make it through all three projects, and we would plan in future projects to undertake rigorous preclinical testing via the unique mechanism of the CPDF at SRI. This resource within the CounterACT network facilitates the progression of bona fide lead compounds through formal preclinical testing toward phase I clinical testing. These studies bridge the gap between the initial efficacy and toxicity studies within our pipeline and the first-in-humans phase I studies that will be required for ultimate deployment as countermeasures. The specific studies required to progress toward phase I testing will vary with each compound but will likely include good manufacturing practices–compliant

studies of stability, manufacturing, and formulation in addition to formal ADME and toxicity studies.^{87–91}

Summary

The integrated pipeline for countermeasure discovery that we have developed under the auspices of the NIH CounterACT Network is one of the few efforts within academia that by design spans the spectrum from discovery to phase I. The successful implementation of this approach for cyanide would enable efficient proof-of-concept studies laying the foundation for a generalizable strategy to undertake parallel mechanistic studies and accelerated countermeasure development in the face of new and emerging chemical threats.

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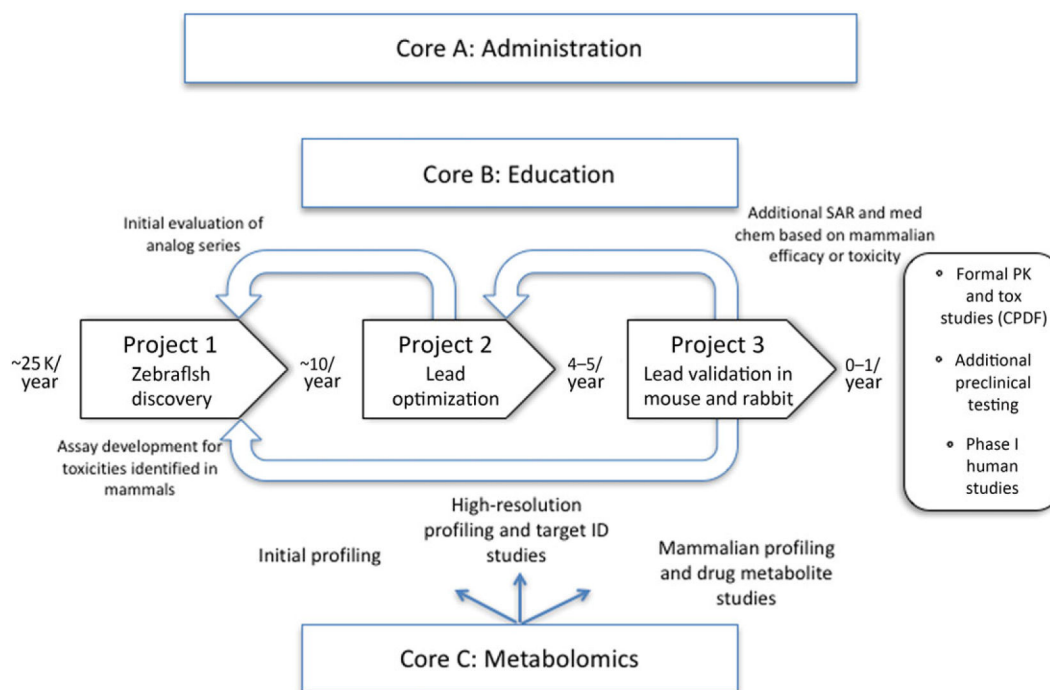


Figure 1. Schematic showing the relationship between the projects and cores. The approximate numbers of compounds moving forward at each stage are displayed in bold between each project.