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Evolutionary Biology and Drug Development

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1. Introduction

Evolution is the unifying framework in biology and scales to all living systems. It is the central organizing concept to explain seemingly disparate biological phenomena; from the very small (individual molecules) to the very large (ecosystems), from the rise and spread of molecular variants to the behavior and body shapes of elephants. In recent times, our appreciation for evolution in medicine has gained momentum. Individuals have championed the cause, dedicated journals have emerged, and new books on the subject are frequently published ("The Evolution and Medicine Review" is an excellent web-based resource providing updated information on the subject, http://evmedreview.com). This union between evolution and medicine has already advanced our understanding of pathological processes (Maccullum, 2007, Nesse & Stearns, 2008).

Drug development and therapeutic strategies are areas in which evolutionary principles may be particularly helpful. The avalanche of bioinformatic methods, genomic data and subsequent emergence of evolutionary genomics in the last few decades means that integrating these fields in drug design is now a possibility. Incorporating evolutionary information is not only helpful *a posteriori* when we may hope to understand why resistance to a particular compound emerged. It is also valuable *a priori*, to design more efficacious drugs, suggest potential resistance profiles and conceptualize novel treatment strategies. Many allopathic treatments, particularly those for chronic non-infectious diseases, relate to the manipulation of cellular functions within one individual's lifespan, for example, developing a drug aimed at a particular cardiac disorder. In these instances, evolutionary biology may explain why a particular disease arose, the evolutionary relationships between genes in the animal model and human or which pathological processes should be targeted. From an evolutionary perspective populations of reproducing individuals are the material on which evolution acts. Adaptive and non-adaptive changes occur over successive generations, and infectious organisms and cancer are therefore the premier examples to illustrate the role of evolution in drug development. In the current age it is almost unthinkable that evolutionary theory, the only scientific framework for studying ultimate causality in biology, doesn't already form the starting point for developing therapeutic interventions affecting evolving populations.

Here we wish to illustrate the role of evolution in allopathic medicine. A brief overview of the typical drug development pipeline is provided, followed by a discussion of relevant evolutionary questions. We discuss in greater detail the molecular evolutionary processes impacting on the emergence of drug resistance and offer suggestions to limit the problem.

Finally, we discuss the rapidly growing areas of evolvability and multilevel selection and how these inform our understanding of therapeutic strategies.

2. Drug discovery strategies

A drug discovery pipeline is a complex, costly and lengthy process involving several discrete stages (Fig. 1). The median time for the development of a new drug is estimated at ~13 years, with a potential cost upwards of ~1 billion US dollars (Paul et al., 2010). The funnel shape of the pipeline reflects the high failure rate between different stages and fewer than 1 in 50 projects deliver a drug to the market (Brown & Superti-Furga, 2003). In the last few years especially the number of new approved drugs has declined sharply despite an increase in research and development spending. Data from a survey of nine large pharmaceutical companies revealed that in 2010 only two new molecular entities from all these companies were approved by the FDA, a very poor return on their expenditure of approximately $60 billion dollars (Bunnage, 2011). Several strategies have recently been proposed to reduce the costs and improve the success rates, including closer cooperation between pharmaceutical companies and academia (Cressey, 2011, Frye et al., 2011); investigation of new uses for approved drugs (Littman, 2011); increased use of translational phenotypic assays (Swinney & Anthony, 2011); and improved target and lead selection (Brown & Superti-Furga, 2003, Bunnage, 2011).

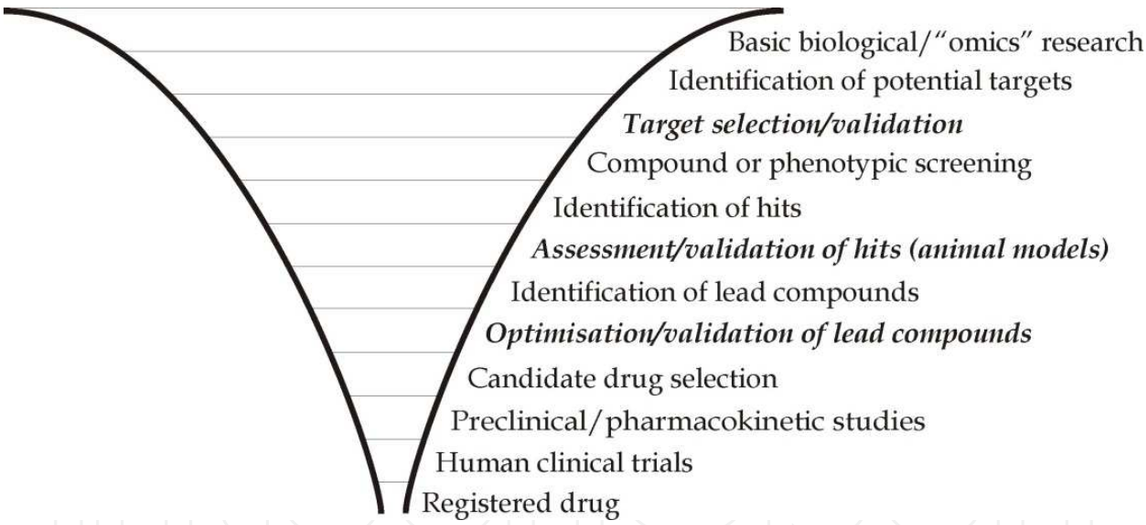


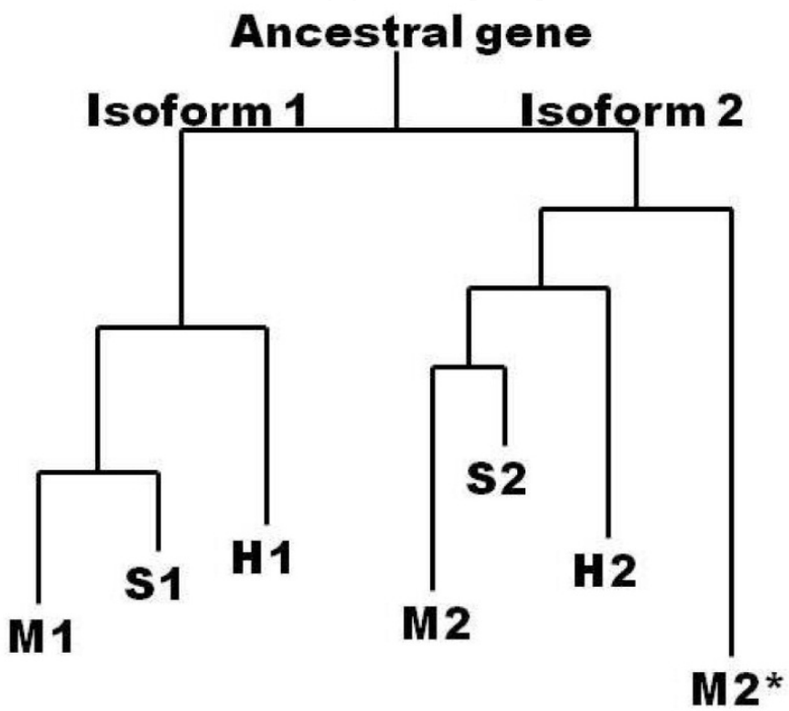
Fig. 1. The drug discovery funnel.

Evolutionary considerations are critical at steps in bold italics.

The availability of whole genome sequences, new discoveries regarding the molecular basis of disease, technological advances in target and lead validation, and high throughput screening strategies, provide exciting opportunities for drug discovery. However, translational research requires improved coordination and integration between different scientific disciplines to ensure a justified transition past key decision points in the drug development pipeline. In this regard, it is critical that evolutionary biologists participate in the process to ensure that fundamental evolutionary principles are taken into account, especially at the validation steps (Fig. 1), to reduce costs and attrition.

3. Evolutionary concepts relevant to drug design

To understand the evolutionary pressures on a potential drug target and the homologous relationships between target genes in the human and the proposed animal model, a few basic concepts should be addressed (Box 1). The reader is referred elsewhere for further discussion of general concepts of molecular evolution (Li, 2006).



Box 1. Orthology, paralogy and functional shifts. In the hypothetical phylogram an ancestral gene has been duplicated to give paralogous isoforms 1 and 2 in mouse (M), rat (R) and human (H). Speciation events gave rise to orthologues M1, R1 and H1 and orthologues M2, R2 and H2. In the mouse there has been a second duplication event giving rise to M2*. The finding that M2* is isolated on a long branch indicates a functional shift in this gene

3.1 Orthology and paralogy

Homologous genes share a common ancestry and depending on the events in their history are orthologous or paralogous (Box 1). Orthologues arise from speciation events; paralogues arise from gene duplication events and resolving these relationships is best done with phylogenetic reconstructions. A number of methods can be used to re-create phylogeny (Felsenstein, 2004) each with their own strengths and weaknesses, however, it should be borne in mind that phylogenetic reconstructions are not foolproof and may require significant interpretation and re-examination. Processes like concerted evolution, horizontal gene transfer and incongruent evolution cloud the picture (Felsenstein, 2004, Li, 2006). Nevertheless, establishing orthology and paralogy (as best one can) raises major questions and both are important for drug development and assessment of drug targets (Searls, 2003). Orthology informs one about the corresponding gene(s) in the animal model while paralogous relationships are often more important for identifying functional divergence.

3.2 Evolutionary rates

Related to the reconstruction of phylogenetic relationships is the determination of evolutionary rates and patterns. The simplest way of estimating the nature and intensity of the selective pressure is to quantify the ratio of non-synonymous to synonymous nucleotide substitutions in a coding sequence, corrected for opportunity, taking into account various features of sequence evolution such as transition/transversion ratios, base and codon biases, etc (Box 2) (some key references are Goldman & Yang, 1994, Hurst, 2002, Muse & Gaut, 1994, Nei & Gojobori, 1986, Yang, 2006, Yang & Nielsen, 2000). The ratio (*dN/dS* or ω) reflects fitness advantages or disadvantages resulting from changes in the amino acid sequence. A ratio $\omega>1$ indicates positive (diversifying or adaptive) selection; $\omega<1$ is negative (purifying or stabilizing) selection. In positive selection non-synonymous mutations are more prevalent in extant sequences presumably because they confer a fitness advantage. Negative selection indicates a fitness cost to non-synonymous substitutions. Furthermore, the lower the ω value, the stronger the stabilizing pressure as fewer and fewer non-synonymous substitutions are tolerated. If there is no difference between *dN* and *dS* substitution rates ($\omega=1$), the selective pressure is neither stabilizing nor diversifying and evolution is neutral. Examining the evolutionary pressures not only informs one about functional divergence; but guides the researcher in the selection of the target site. Briefly, sites that are fast evolving are typically poor drug targets, while structurally and functionally conserved sites are usually under purifying selection and make more suitable targets.

$$Q_{ij} = \begin{cases} 0 \\ \pi_j \\ \kappa\pi_j \\ \omega\kappa\pi_j \\ \omega\pi_j \\ \omega\kappa\pi_j \end{cases}$$

Box 2. A model for codon evolution (Goldman & Yang, 1994, Muse & Gaut, 1994).

Numerous methods are available to quantify evolutionary rates in nucleotide sequences. An extensively used approach is the maximum likelihood (ML) codon model for evolution. A simplified substitution rate matrix used by the ML method to estimate codon evolution is given (left). The matrix is used to statistically determine evolutionary pressures acting at individual codons: positive, negative or neutral evolution (see text for more discussion and references below). This model determines the probability that codon i mutates to j in a specified time interval and accounts for the transition/transversion rate ratio (κ); the equilibrium frequency of codon j (πj); and the non-synonymous/synonymous rate ratio (ω). Qij = 0 if i and j differ at more than 1 position.

4. Evolution and target selection

One of the major causes of attrition of a potential drug candidate is poor quality of the target. The critical steps of target selection and validation require greater emphasis and incorporation of additional evolutionary criteria to reduce subsequent failure.

4.1 Has the target undergone functional divergence?

Many genes of therapeutic interest have undergone expansion leading to functional redundancy. Targeting a specific protein isn't helpful if there are other family members that are immune to the drug and at the same time take over the function of the target.

To assess functional shifts, paralogy is a critical consideration. The reason is pleiotropy, which is often associated with paralogous genes. Pleiotropy occurs when a gene product has more than one function and can either precede gene duplications or result from duplication events where the duplicated gene is less constrained and free to evolve multiple functions. The impact of pleiotropy on drug discovery is apparent when one considers that in these situations, one must disentangle the compound's effect on multiple pathways. A good example of gene duplications leading to pleiotropy and functional divergence is that of the caspase family. The ancestral metazoan caspase has undergone numerous gene duplications over time resulting in at least 11 human and 10 murine true caspase genes (Nedelcu, 2009, Uren *et al.*, 2000, Wang & Gu, 2001). In humans, distinct clusters of caspases have been identified (Uren *et al.*, 2000, Wang & Gu, 2001), which may be involved in evolutionarily related but biochemically distinct pathways of inflammation or apoptosis. It also seems likely that some of the caspase family members are implicated in both processes. These proteins are primarily involved in one of the processes but are pleiotropically linked to the other. Disentangling the role of individual caspases in the two pathways would be important for developing drugs targeting either inflammation or apoptosis.

5. Evolution and hit validation

The assessment of hit compounds often requires *in vivo* testing in animal models and an inappropriate choice of model is one of the reasons why an apparently promising lead compound fails during human clinical trials. Understanding the phylogenetic relationships between genes in the two systems is therefore an important initial step.

5.1 What are the evolutionary relationships between genes in the model and target organisms?

The biochemical and pharmacological findings in an experimental model organism cannot be extrapolated to another organism without understanding the evolutionary relationships between the target genes. This is because evolutionary rates and functional divergence between homologous genes in related organisms may vary. It is important; therefore, to establish at the very least the homologous relationships between the gene used in the experimental system and the proposed target gene in the human.

Even a slightly improved understanding of orthologous gene differences between model and target species can have an impact on the progression of a compound with major implications for scientific and financial resources. However, while orthology tells us about the evolutionary relationships between genes in related organisms and *suggests* similar function, this is not guaranteed. A phylogram may reveal that a particular gene in the model

organism is orthologous to the gene in the target organism. Despite this orthologous relationship isolation of the gene on its own long branch indicates sequence divergence and a functional shift. This should alert the researcher to be wary of using that particular model as a basis for studying the biochemistry of the protein in the human target. In the example given in Box 1 a duplication event has led to genes M2 and M2* in the mouse. This duplication occurred after the speciation events giving rise to humans and rats and both M2 and M2* are therefore orthologues of R2 and H2. However, the isolation of M2* on its own long branch strongly suggests functional divergence and it should not be used as a model for developing a drug compound targeted at H2.

Molecular evolution of homologous proteins may vary along different lineages, which means that a protein may appear highly stable and conserved in one branch leading to humans, but under a different selective pressure in the experimental model animal. If this is not taken into account inappropriate models may be selected. A good example of this is the finding that leptin is associated with obesity in mice (Chen *et al.*, 1996). The discovery was greeted with tremendous excitement since it implied that rodents could be used as a model organism for studying the pathogenesis of obesity in humans. However, it subsequently emerged that there is evidence for positive selection in leptin in primates (including humans) but not in rodents (Benner *et al.*, 2000). This indicates adaptive evolution and a functional shift in leptin after the divergence of primates from rodents. Using the mouse therefore, as a model for understanding the biochemistry of the protein and its potential use as a drug target in humans is problematic.

6. Harnessing evolution to minimize the emergence of resistance

Drug resistance is an ever present threat that curtails the effective lifespan of a drug and has enormous financial implications for pharmaceutical companies. Resistance can develop very rapidly, for example, resistance to the anti-malaria drugs pyrimethamine and proguanil (Hyde, 2005) developed within a year of introducing the drug to the market. Similarly, chronic myeloid leukaemia (CML) cells have become refractory to treatment with tyrosine kinase inhibitors targeting the bcr-abl oncogenic protein, necessitating the development of second and third generation inhibitors (Kantarjian *et al.*, 2008). It is therefore vital that evolutionary principles are applied to the key decision points in the drug development process relating to the validation of targets, hits and lead compounds to minimize the emergence of resistance.

6.1 What is the possible evolutionary response to drug pressure?

Evolution occurs by non-adaptive and adaptive (Darwinian) means. Non-adaptive evolution includes pleiotropic phenomena and genetic drift, or may appear non-adaptive at one level of selection and adaptive at another. Adaptive evolution occurs by natural selection and is more closely associated with the concept of fitness. For pharmacological interventions against infections and cancer to be effective, they are aimed at killing or at least inhibiting growth of infective organisms or cancer cells. This means they generally act on adaptive traits so that fitness is compromised. Targeting a non-adaptive trait such as the pleiotropic effects of paralogous genes discussed above may have a minimal effect on fitness, limiting the drug's usefulness. Targeting fitness-related traits is important for a drug to be effective, but doing so induces a Darwinian response if there are any survivors following the treatment. Furthermore, the greater the fitness cost resulting from the drug

pressure the stronger the evolutionary response (Read *et al.*, 2011), and it is usually a question of *when*, rather than *if*, resistant mutants will emerge.

There is effectively a therapeutic trade-off. From the perspective of treatment efficacy, the trade-off is between maximizing the fitness cost to the target (infectious agent or cancer cell) and minimizing the undesirable evolutionary escape response. Of course, if the fitness cost is absolute and all individuals in the population of infective organisms or cancer cells are killed, there is no trade-off. This is the ideal situation; but often not the case. Unless there is complete cure, there will be a therapeutic trade-off. Optimizing this trade-off is seldom given any consideration and therapy results in a temporary hiatus in the disease. Eventually the most resistant mutants take over and, as indicated, the most aggressive therapies elicit the strongest escape response (Read *et al.*, 2011). In addition, not only does therapy select the most virulent individuals, but the group level dynamic is disrupted, further intensifying the escape response. Studies in mice infected with the malaria parasite *Plasmodium chabaudi* found that more virulent clones are controlled by less virulent ones (Wargo *et al.*, 2007). Treatment that failed to eradicate all clones allowed the more virulent ones to thrive leading to a more serious secondary relapse. Humans living in malaria endemic areas can be infected with over 15 genotypically distinct clones of *P. falciparum* (Juliano *et al.*, 2010). If the mouse model study is extrapolated to humans, then aggressive chemotherapy may actually be harmful in the long-term. Instead, to optimize the therapeutic trade-off, it is suggested that the guiding principle should be to impose no more selection than is absolutely necessary (Read *et al.*, 2011). This holds particular relevance for infections or malignancies where cure is unlikely and therapy is aimed more at disease management, for example, chronic leukaemias and infections like HIV.

An understanding of the evolutionary constraints acting at the molecular level is not only helpful when predicting the intensity of the evolutionary response, but it is also important for identifying the appropriate target sites of a protein.

6.2 Can we identify target sites with minimal risk for resistance?

There are various computational approaches (see Yang, 1997 and later versions) to determine the selective pressures acting on whole genes, specific codons or on lineages in a phylogenetic tree. Whole genes are seldom under positive selection; however, those that are, rapidly escape the fitness cost associated with the drug therapy. Non-synonymous substitutions already confer a fitness advantage in sequences demonstrating positive selection and the added drug pressure rapidly leads to resistance. To obtain a more informative view of a gene's evolutionary rate, it is helpful to examine substitution rates at individual codons in the coding sequence. Maximum likelihood estimates of ω at individual codons will usually reveal variation across the coding sequence. Highly conserved or functionally important amino acids are likely to be under purifying selection, while others in the sequence may be evolving neutrally or be under positive selection. Targeting the positively selected or neutral sites will drive the emergence of resistance mutations and should be avoided, while sites under intense purifying selective pressures are far less likely to produce viable mutations and make suitable targets.

Drug treatments add to the naturally occurring selective pressures and codon sites that code for resistant mutations are frequently evolving more rapidly than others. A good example of this is a study of serially sampled reverse transcriptase coding sequences isolated from a group of HIV-1 subtype C–infected women before and after single-dose nevirapine (Seoighe *et al.*, 2007). Nevirapine is a standard therapy for preventing mother-to-child transmission. A

directional selection evolutionary model differentiated codons under positive selection from those subject to purifying selection and the differences in evolutionary rates would reliably have predicted *a priori* the sites of amino acid change leading to nevirapine resistance. This study provided proof of concept that quantifying the evolutionary pressures acting at individual codon sites can predict the likelihood of resistance emerging if the drug-protein binding site is known. Even before this study, others developed iterative approaches for use in development pipelines to guide experimentalists to biologically relevant sites based on sequence conservation. The first approach known as Evolutionary Tracing (ET) (Lichtarge *et al.*, 1996, Lichtarge & Sowa, 2002) and another, Evolutionary Patterning (EP) (Durand *et al.*, 2008), which directly quantifies the evolutionary rate, provide useful examples for further illustration.

6.2.1 Evolutionary tracing

ET generates a trace sequence from multiple sequence alignments of functional classes of a protein family. Clusters of invariant amino acids are identified and incorporated into 3D structures to identify the most suitable target sites in terms of their conservation, functional and structural importance and access. ET is particularly helpful for modeling functional specificity and architecture-defining residues. ET predictions have been verified experimentally. The most complete demonstration that ET anticipates mutational and crystallographic analyses was performed on the regulator of G protein signaling proteins that act to increase G_α GTP hydrolysis rates (Sowa *et al.*, 2000). Based on the ET data specific amino acids were mutated causing profound effects on enzyme activity and led to the prediction of an allosteric binding site (Sowa *et al.*, 2001), which was subsequently confirmed by crystallography (Slep *et al.*, 2001).

6.2.2 Evolutionary patterning

In ET, one of the premises for identifying target sites is that structurally and functionally essential amino acids are conserved in a trace sequence. However, while conservation suggests purifying selection it does not necessarily equate to it. To more accurately quantify the selective pressure acting at a particular amino acid residue, it is important to study the substitution rates at individual codons across a coding sequence. This is the approach adopted by EP, which makes use of a maximum likelihood substitution matrix to estimate the ratio of non-synonymous / synonymous substitutions at individual codons in a coding sequence (Box 2). The Bayes Empirical Bayes posterior probability of the MLE (maximum likelihood estimate) of ω falling into a particular category (for example, positive selection $\omega>1$ or extreme purifying selection $\omega<0.1$) is computed using PAML software (Yang, 2007). The distribution of these probabilities across a potential target protein can be examined and mapped to the predicted 3D structure, guiding the researcher as to which residues to target and which to avoid. It is argued that codons subject to extreme purifying selection are evolutionarily constrained, perhaps because the amino acid is essential for protein structure or function. The data from extant sequences indicate that non-synonymous mutations at these sites are not tolerated and make ideal drug targets if the encoded amino acids are accessible to interactions with lead compounds. In contrast, residues that are subject to positive or neutral selection, or only weakly conserved should be avoided. Non-synonymous mutations at these sites have arisen naturally during the evolutionary history of the protein indicating that amino acid

changes do not significantly compromise protein fitness. These sites should not be targeted therapeutically as any mutants that arise are likely to be selected for by the drug pressure. Mapping the amino acids under extreme purifying selection to a structural model is important so that the accessibility and interaction between target sites and lead compounds can be assessed *in silico*. As with ET, this is an iterative process. Docking studies uncover drug-protein interactions and the strength of chemical bonds assessed; interactions with undesirable amino acids are revealed and the lead compound may be modified so that contacts with target sites are optimized. The process can then be repeated as often as necessary to maximize favourable interactions.

The application of EP to a potential drug target, *P. falciparum* glycerol kinase may be used as an illustration (Figures 2 and 3) (Durand *et al.*, 2008). Six separate target sites comprising stretches of contiguous amino acids subject to extreme purifying selection were identified. The targets were mapped to a 3D model generated using the *E. coli* homologue as a template, which revealed that four were accessible to potential lead compounds. These sites were also found to overlap with functional domains and were suggested as therapeutic targets. The EP approach was validated by examining resistance mutations in the *P. falciparum* dihydrofolate reductase-thymidylate synthase protein, which is targeted by the anti-malarial drug pyrimethamine. EP predicted that none of the five known mutations conferring pyrimethamine resistance would have been subject to extreme purifying selection - a factor which would have facilitated the evolution of resistance. This was indeed the case, confirming that the likelihood of an evolutionary escape response was greater if the codon was under more relaxed evolutionary constraints.

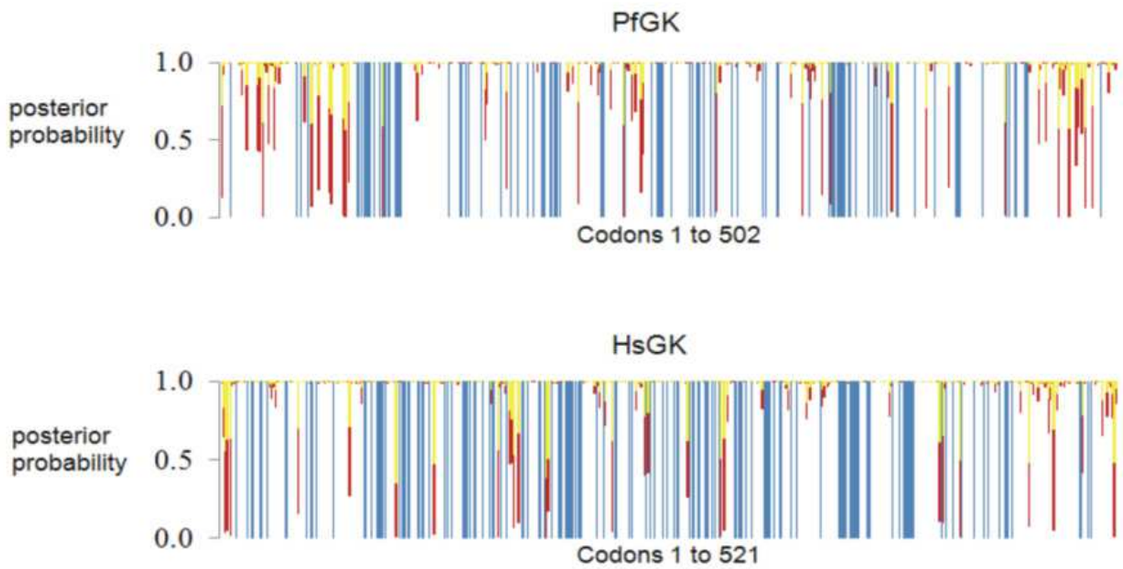


Fig. 2. Posterior probabilities for four categories of ω across GK coding sequences (from Durand *et al.*, 2008). Bayes Empirical Bayes posterior probability estimates for each category of ω across *P. falciparum* (PfGK) and human (HsGK) GK coding sequences are shown. Residues under extreme purifying selection (ω≤0.1) are potential drug target sites and were mapped to a 3D model to assess drug accessibility (Fig. 3). Categories for ω are indicated with coloured bars: yellow (ω>1.0), red (ω=1.0), white (0.1<ω<1.0), and blue (ω≤0.1).

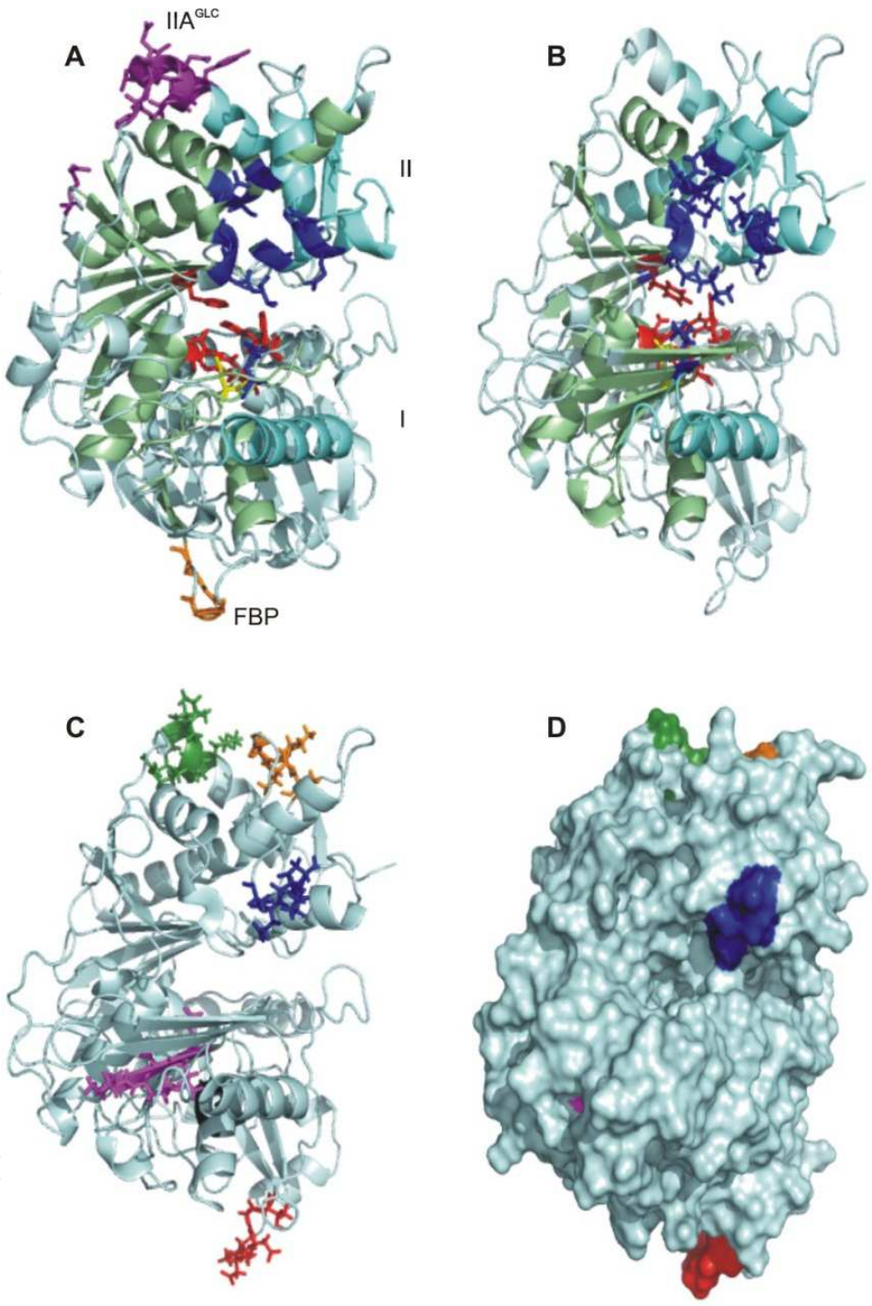


Fig. 3. *E. coli* and *P. falciparum* glycerol kinase 3D models (from Durand *et al.*, 2008). Ribbon models (with functional residues as sticks) of E. coli glycerol kinase (EcGK) (A) and P. falciparum glycerol kinase (PfGK) (B) are displayed. In EcGK, coloured residues are involved in binding to ADP (blue), Mg2+ (yellow), glycerol (red), FBP (fructose-bis-phosphate) (orange) and IIAGLC (purple). Alpha helices and ß sheets in the ATPase site (light green) and subunit interactions (aquamarine) are shown. The PfGK model is based on EcGK. Five regions were identified in Fig. 2 as good target sites and mapped to ribbon (C) and surface (D) models. One of the regions (black) is partly obscured and is in the core of the molecule, indicating the region would not be accessible to drug compounds.

ET and EP provide guidance for selecting the most suitable drug target sites and thus assist in designing more effective drugs, which may limit the emergence of resistance for long periods. However, any drug pressure will still invoke an evolutionary response, so at some point escape mutants are still likely to arise. A truly fundamental shift in the approach to allopathic therapies would involve strategies aimed at having evolution work in our favour rather than against us. Doing so is a major conceptual challenge.

7. Evolvability and multilevel selection: Future avenues for drug research

Basic science research into the fundamental nature of evolution has resulted in what some biologists think is tantamount to a paradigm shift. For a review of these advances see discussions around an "extended theory of evolution" (Danchin *et al.*, 2011). Two areas in which evolutionary thinking has rapidly progressed are the concepts of evolvability and multilevel selection. Both have relevance for future drug development strategies.

7.1 Evolvability

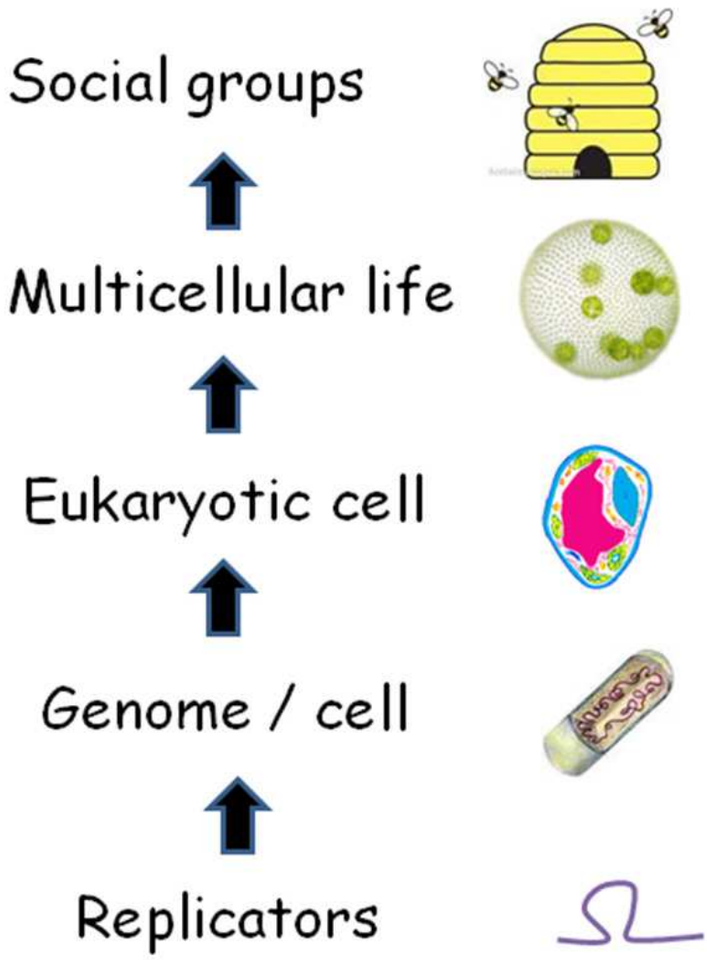
Advances in evolution hold promise for exploiting under-appreciated biological phenomena in drug design. The eloquent statement "Not only has life evolved, but life has evolved to evolve" (Earl & Deem, 2004) implies that the genetically encoded propensity to adapt to environmental pressures (known as evolvability) is a selectable phenotype. The variation in response to changing environments confers heritable variation in fitness. It is argued therefore, that evolvability can be acted on by natural selection leading to populations of organisms that are more or less likely to adapt to environmental pressures. Experimental evolution studies using model organisms like *Escherichia* (Leroi *et al.*, 1994) and *Chlamydomonas* (Bell & Reboud, 1997) date back nearly two decades and seem to support these assertions although whether evolvability itself is always adaptive (as opposed to being non-adaptive) is not always clear (Creavin, 2004). Evolvability also appears to play a role in pathogen virulence. For example, the HIV reverse transcriptase (RT) is notoriously error-prone leading to the evolution of populations of quasispecies that evade host immunity and escape drug pressures (Bebenek *et al.*, 1993). However, as indicated above, whether the error prone nature of HIV RT evolved as an adaptation or whether it is the result of other adaptive or non-adaptive effects is uncertain. Nevertheless, what is clear is that the error rate of HIV RT confers a fitness advantage. Targeting the pathogen's evolvability rather than phenotypic traits that are easily overcome by the propensity to evolve is therefore likely to have a greater impact in the long term. It can be argued that where evolvability forms part of a pathogen's life history strategy, this consideration should be included in drug design efforts. For further discussion on plasticity and evolvability in parasitic infections such as malaria with relevance to chemotherapeutic strategies the reader is referred elsewhere (for example Reece *et al.*, 2009).

7.2 Multilevel selection theory

7.2.1 Multilevel selection, sociobiology and the conceptualization of novel drug strategies

Our understanding of the living world has been transformed by the finding that natural selection acts at multiple levels of biological organization (Box 3). Multilevel selection theory (MLST), which includes group selection and for which there is now a significant body of

evidence, describes the living world in terms of hierarchically structured levels where the tenets of selection are applicable to evolutionary units across these levels (Keller, 1999, Lewontin, 1970, Okasha, 2006, Wilson, 1975). Evolutionary transitions gave rise to increasing complexity including groups of genes, which form genomes, which form cells, which form multicellular organisms, which may form social groups and so on (Maynard Smith & Száthmary, 1995). The fact that the units of evolution span levels of biological organization raises the question of whether it may be better to target other levels of organization such as groups rather than individual cancer cells or infectious organisms.



Box 3. The multilevel selection paradigm. The living world is characterized by quantum leaps in organization and complexity. Early replicators cooperated to form genomes and cells, which formed a eukaryotic cell, multicellular life, social groups and so on. Evolution by natural selection acts on any system where there is a group of reproducing individuals, so long as there is heritable variation in fitness. All these levels are therefore subject to natural selection. The related concept of group selection fits into this framework of MLST and describes competition between rather than within groups. While there is ongoing debate regarding the mechanisms, terminology and extent of group selection most researchers accept the fundamentals.

For drug development strategists, there are some key aspects to MLST that must be appreciated. Group level traits and adaptations arise because of selection and dynamics between groups rather than individuals within a group. These traits arise in various ways. They can be aggregates of properties within the group or arise as irreducible 'emergent properties' only existing at the group level (Thompson, 2000). Selection pressures at different levels of organization can vary; a particular trait can be beneficial at one level and harmful at another (see programmed cell death later) or the trait may have differential fitness benefits at two or more levels. Unpacking the relative selection pressures at different levels requires an understanding of Fisher's fundamental theorem of natural selection, which states that "t*he rate of increase in fitness of any organism at any time is equal to its genetic variance in fitness at that time*" (Fisher, 1930). Fisher's theorem is applicable at any level of organization, whether it is a population of groups or a population of cells. Drugs targeting more than one level of selection will therefore induce differential responses and the intensity of the evolutionary escape response will depend on fitness variances at the different levels. Targeting the group level, rather than individuals as is the conventional approach has distinct advantages for therapies against infections (Pepper, 2008) and cancer (Pepper *et al.*, 2009). The advantages typically concern the phenomenon of cooperation and the group fitness variance.

The phenomena of cooperation and its more extreme form altruism are commonly found in groups of pathogens or cancer cells. Mechanistically, cooperation can take the form of "public goods" (Wessler *et al.*, 2007), molecules that are produced by individuals but have a group level action. The quantity or quality of molecule produced by one of the individuals is of such a nature that it may have little or no direct benefit for the producer, but in combination with the molecular products from others results in a group level fitness-enhancing trait. For example, in bacterial biofilms quorum-sensing molecules regulate cell division of individuals so that the group responds to challenges as a collective (Wessler *et al.*, 2007). Similarly, in solid tumours angiogenic factors are produced by individual cells but only when sufficient quantities are produced by the group does neo-vascularization occur (Kerbel, 1991). Targeting public goods makes good evolutionary sense.

Consider a situation where individuals (cancer cells or pathogens) secrete molecules that only have a group level benefit. There is initially no benefit for a mutant individual because the concentration of its molecular product is either too low or on its own cannot modify the group phenotype and increase group fitness. More likely, its mutant nature means that its role in the group network is compromised and group fitness is diminished. Unlike conventional therapies which actively select for resistance, there is no immediate fitness benefit to mutants and resistant individuals die along with others in the group or they are selected against. Of course, a larger clone of resistant cells may survive and reproduce, but from the outset and all else being equal, mutants have the same or lower fitness than susceptible cells and generally do not take over the population. This scenario is more than just conceptual. It is supported empirically. One of the most detailed illustrations comes from the 15 year old study of tumour neo-vascularization and drug resistance referred to above (Boehm *et al.*, 1997, Kerbel, 1991).

In solid tumours cancer cells eventually outgrow their nutrient supply. Angiogenesis factors are produced by the tumour leading to neo-vascularization and subsequent survival of the group. Cytotoxic cancer drugs create a powerful selective pressure and in the heterogeneous population of cancer cells resistance rapidly emerges. However, targeting the group level benefit with "anti-angiogenic therapy does not induce drug resistance" (Boehm *et al.*, 1997).

This is because while the angiogenesis blocker is applied, resistant mutants do not produce sufficient angiogenesis factors for neo-vascularization to occur and they die along with others before reaching a critical mass.

Similar strategies have been advocated or used for a number of infectious diseases with some success, including the escalating challenge of methicillin-resistant *S. aureus* (MRSA) (see Pepper, 2008 and references therein). *S. aureus* produces numerous virulence molecules that act at the group level and are required for establishing and maintaining infections. The prototypical public good example in *S aureus* is α–toxin, without which infections in animal models are unsustainable (Bhakdi & Tranum-Jensen, 1991). A literature survey suggests that an appropriate and current opportunity for using MLST in drug development is tuberculosis (TB). TB is one of the major global health challenges and with the emergence of multidrug resistant (MDR) and extreme drug resistant (XDR) strains the need for novel strategies has never been more urgent. Laboratory studies of the resuscitation promoting factors (rpfs) in *Mycobacterium* species indicate that these factors may be prime targets for group level chemotherapy (for a review of rpfs see Kana & Mizrahi, 2010). Knockout experiments suggest rpfs have a negligible role in individual cell fitness; however, at the group level they are important as virulence factors and for the resuscitation of latent infections.

With regards to fitness variance and the rate of evolution, Fisher's theorem bodes well for drug strategies targeting cooperation in groups. The fitness variance of the phenotype decreases as the cooperative behaviour increases and is shared equally within the group (for a detailed discussion see Price, 1972). When fitness variance is zero, the implication is that either all the individuals in the group receive the benefit of the public good or none at all. The evolutionary rate of the group level phenotype is therefore exceedingly slow, as is the likelihood of resistance developing. This is in contrast to the evolutionary rates when the unit of selection is the individual in the group. In these instances fitness variance is usually greater and resistance evolves more rapidly. The fundamental properties of evolutionary rates as they relate to fitness variance coupled with cooperation and group level traits opens a whole new avenue for drug development strategies.

7.2.2 Multilevel selection and the intriguing case of programmed cell death

The phenomenon of programmed cell death (PCD) in unicellular eukaryotes brings together many aspects discussed in this chapter. It provides a useful context for integrating homology, adaptations, evolutionary rates, evolvability and MLST as they relate to infections, cancer and drug development. Our discussion of PCD below is based on a few key papers and requires far more investigation, but as an example it illustrates how evolutionary thinking could lead to a radical shift in drug design.

Programmed cell death (PCD), previously considered a hallmark of multicellularity, has been reported in all major lineages in unicellular eukaryotes and prokaryotes (see Table 1 in Nedelcu *et al.*, 2011). From an evolutionary perspective (with implications for drug design in infections and cancer) the burning question has been: why would an organism actively kill itself? For an individual unicellular organism PCD has no fitness benefit and adaptive evolution cannot explain the phenomenon. Likely explanations are that it is either maladaptive pleiotropy or adaptive in a MLS context (i.e. at a group level). While strong arguments can be made for both scenarios (Nedelcu *et al.*, 2011), laboratory evidence from two model organisms favour the hypothesis that PCD in unicells is adaptive for the group. In *S. cerevisiae* PCD-related aging assists re-growth in a related mutant subpopulation

(Fabrizio *et al*., 2004, Herker *et al*., 2004). A direct fitness-related experiment in *C. reinhardtii* demonstrated that molecules released by cells dying by PCD provide fitness benefits to others (Durand *et al*., 2011). Genomic studies have also revealed that many of the homologues for key protein domains involved in PCD are conserved across a wide range of organisms (for example Nedelcu, 2009), although there has been expansion of many of the gene families, particularly in vertebrates and plants as organism complexity evolved. As discussed in sections 3 and 4, an understanding of the evolutionary rates and relationships between homologues in model and target organisms will be helpful if expanded gene families in the PCD pathway are to be targeted therapeutically.

S. cerevisiae and *C. reinhardtii* are already used as model organisms for a range of diseases including cancer (for example Fang & Umen, 2008). The genomic and empirical data for PCD as an adaptation in these organisms shed new light on PCD in human parasitic infections and cancer and can help explain some puzzling phenomena. With regards to parasitic disease, numerous organisms demonstrate PCD, including apicomplexa, stramenopiles, trichomonads, diplomonads, kinetoplastids and trypanosomatids (see Table 1 in Nedelcu *et al*., 2011 and references therein). The group effect of PCD in most of these organisms has not been studied; however, in *Leishmania* (a kinetoplastid) infections, PCD as a group level adaptation explains the counterintuitive finding that virulence is associated with PCD (Van Zandbergen *et al*., 2006). If the infective inoculum contains a proportion of apoptotic (PCD) cells, the population has greater virulence and fitness. Removing the apoptotic forms diminishes disease severity. The interpretation is that apoptotic forms enhance group fitness, which is in keeping with the *C. reinhardtii* findings. Similar experiments have not been performed with cancer cells, but the role of apoptosis is not always clear. Tumour suppressor genes are usually mutated in cancer; however, the apoptosis pathway is malignant cells is frequently activated through the FAS ligand receptor. Curiously, the FAS ligand pathway can also promote tumour growth (Chen *et al*., 2010). Whether this is due to crosstalk between this pathway and another anti-apoptosis pathway is unclear. However, in light of the *C. reinhardtii* experiments (Durand *et al*., 2011) and that the essential pathology of cancer is atavism (regression to the ancestral unicellular state) (Davies & Lineweaver), is it possible that apoptosis in some cancers also provides benefits to other cells in the population? In a bizarre twist, can chemotherapy exacerbate a cancer or infection by inducing PCD in some cells, which then provide fitness benefits to others?

8. Concluding remarks

The potential role for evolutionary biology in drug design is vast and can be applied at various stages in the development process. The aim here is to provide the reader with an overview of evolutionary medicine, with specific reference to drug design and the emergence of resistance in infections and cancer. Some key concepts such as phylogenetic relationships and evolutionary rates are introduced to illustrate how evolutionary studies can predict the most suitable drug target sites in a protein and limit resistance. Perhaps the most exciting union between evolution and drug development is the future use of evolvability and multilevel selection, heralding a new era for therapeutic strategies.

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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.

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