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The impact of synthetic biology on drug discovery

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The emergence of synthetic biology is holding great hopes for providing solutions to the unmet needs of humankind. This review article describes how synthetic biology can deliver on this promise in the field of drug discovery by providing novel opportunities throughout the entire drug discovery process.

Synthetic biology tools enable disease mechanisms and target identification to be elucidated and also provide avenues to discover small chemotherapeutic molecules or design novel biopharmaceuticals. Furthermore, synthetic biologists can design cost-effective microbial production processes for complex natural products, which could help overcome global drug shortages. These impressive advances have been achieved in only a few years, and are an indicator for the potential of synthetic biology.

Introduction

It took almost a century after synthetic biology was first introduced in 1912 [1] until research in biology had sufficiently characterized fundamental biologic processes for them to be used for designing and constructing new biological parts, devices, and systems and redesigning existing natural biological systems (http://syntheticbiology.org/). Since 2000, pioneering work in synthetic biology has led to the construction of living systems displaying complex dynamic behavior, such as synthetic epigenetic imprinting [2,3], time-delay functions [4], oscillating expression patterns [5–8] as well as synthetic ecosystems emulating the coexistence of different species in the same habitat [9,10] (for reviews, see [11–13]). Along with this ‘fundamental synthetic biology,’ whose aim is to elucidate and establish design principles for biologic parts, devices and systems, ‘translational synthetic biology’ [11] may provide solutions to pertinent challenges in biotechnology [14], agriculture [15] or environmental sciences [16,17].

Detailed analysis of enzymes, transcription factors and receptors provides a collection of functional and dynamic information on these individual ‘biobricks’ [18,19], which, when processed by a mathematical model, can offer precise clues for rapid and successful assembly of complex synthetic biological devices in living systems [20–22]. The speed for rational design and implementation of synthetic devices has significantly increased in recent years because of a combination of advances in (i) gene synthesis technology providing large custom-made DNA sequences at moderate cost and within shorter timelines [23,24], (ii) viral and nonviral gene-transfer technologies, enabling straightforward installation of transgenes in different target cells [25,26] and (iii) model-based evaluation of genetic assemblies, identifying crucial design parameters and simulating device dynamics before experimental implementation. Such progress holds the promise of streamlining and intensifying the drug discovery process chain, as exemplified by studies highlighted in this review article. Target identification can be facilitated by the synthetic reconstruction of signaling pathways, which enables identification of disease-causing elements. Once a target is identified, whole cell screening assays can be designed, using synthetic biology strategies, to allow for a streamlined and integrated drug discovery process. Beyond the discovery of small-molecule-based therapeutics, synthetic biology provides novel opportunities for the design of biologics, for example by constructing bacteriophages with superior anti-infective properties. Synthetic biology can further be used to produce complex plant-derived natural compounds in a microbial host, thereby providing an economic manufacturing processes.
**Synthetic biology and the understanding of the molecular basis of disease**

Whereas classical hypothesis-driven basic research disciplines are taking native biological systems apart to understand their function, synthetic biology focuses on the assembly of artificial devices from well-established natural or engineered components to unravel biological phenomena; a science which is based on the statement by Richard Feynman ‘what I cannot create, I do not understand’.

In an exemplary study, B cell antigen receptor (BCR) signaling processes were synthetically reconstructed in *Drosophila* S2 Schneider cells where they could be studied in an interference-free cellular environment because of the evolutionary distance between insects and mammals [27]. S2 cells were easily transfectable, with many different vectors enabling the reconstruction of multistep signaling pathways. The isolated investigation of the signaling components permitted novel insights into the signaling network topology, revealing a positive feedback loop resulting in strong amplification of the BCR signal. The allosteric tyrosine kinase Syk binds to and is activated by the phosphorylated BCR immunoreceptor tyrosine-based activation motif (ITAM). Activated Syk phosphorylates neighboring ITAM, resulting in the binding and activation of more Syk in a positive feedback loop, which thereby triggers the rapid amplification of the BCR signal [27]. In a follow-up study [28], the same group applied the synthetic reconstruction of BCR signaling in S2 cells to elucidate the molecular basis of a rare form of agammaglobulinemia, a primary immunodeficiency characterized by the early blocking of B cell development. Inducible expression of the IgM BCR complex constituents in S2 cells revealed that a nonsense mutation in Igα prevented the correct association with Igμ, thereby inhibiting functional BCR formation [28].

The studies described above highlight how the synthetic reconstruction of natural signaling events in an orthogonal host can elucidate physiologic and pathologic network topologies for understanding the genetic underpinning of inherited diseases. Recent studies have synthetically reconstructed Severe Acquired Respiratory Syndrome (SARS) coronaviruses to reveal how zoonotic viruses can acquire human tropism [29,30]. Bioinformatics analysis suggests that the SARS epidemic at the beginning of the 21st century originated from a bat coronavirus having crossed the barrier from zoonotic to human tropism [29]. Understanding the evolutionary strategies for jumping the species barrier would provide clues as to what future epidemic SARS viruses might look like and lay the basis for designing preventive or therapeutic vaccines.

The investigation of the natural zoonotic virus reservoir in bats was hampered by the fact that it was not possible to propagate bat coronaviruses in cell culture or laboratory animals [29]. Therefore, Becker et al. [29] exchanged the ectodomain of the Spike type I membrane glycoprotein from the bat coronavirus for a synthetic construct harboring mutations found in SARS coronaviruses. The synthetic viruses replicated in murine and primate cells as well as in infected mice, demonstrating that the insertion of the synthetic Spike protein efficiently promoted the cross-species shift in tropism. Moreover, they demonstrated that the chimeric viruses were able to bind to the human angiotensin-converting enzyme 2 (hACE2) as receptor required for cell entry. This synthetic infectious sequence might help predict and directly test pathways that could assist in rapidly recovering and testing newly identified pathogens, hence adapt intervention strategies for future epidemics [29]. Similar to the approach of reconstructing SARS coronaviruses, the sequencing of the 1918 Spanish influenza pandemic virus enabled the synthetic reconstruction of all eight gene segments of the pandemic virus which were fused to the noncoding gene sequences of a closely related H1N1 virus. In contrast to contemporary influenza viruses, the reconstructed virus was able to replicate in the absence of trypsin, was lethal to mice and embryonated chicken eggs and showed a high growth phenotype in human bronchial epithelial cells [31]. Correlating these deadly properties with the mutations observed in the 1918 influenza gene sequence might help to understand the evolution of the virus [32] as well as to characterize its virulence factors to ultimately design preventive vaccinations.

A possible strategy to design vaccines from reconstructed viruses has recently been implemented using poxvirus variants engineered for differential codon usage [33]. It was shown that unfavorable codons resulted in a virus with attenuated virulence so that it could be used to immunize mice and provide protective immunity after challenge. Given the high number of unfavorably altered codons, the attenuated viruses are very unlikely to revert into the natural virulent strain and might therefore represent a safe approach for the design of human vaccines.

**Synthetic biology in the discovery of new drugs**

Availability of well-characterized and standardized biologic parts such as repressor–DNA interactions, transactivation domains, minimal promoters and reporter systems enables the assembly of designer circuits which mimic pathways essential for human pathogens or involved in major pathologies and could be used to screen for interfering small-molecule drugs using transgenic host cells.

**Discovery of antituberculosis compounds**

In a recent synthetic biology study using engineered mammalian cells, small molecules were discovered that switched off the resistance of mycobacteria to the antibiotic ethionamide [34] (Fig. 1). *Mycobacterium tuberculosis* is inherently resistant to ethionamide, which must first be activated by *M. tuberculosis*’s Baeyer-Villiger monooxygenase EthA. However, activation tends to be inefficient, because EthA production is repressed at the transcriptional level by the TetR/CamR-type repressor EthR [35]. Therefore, inactivation of EthR was expected to be correlated with increased EthA expression and higher sensitivity of this pathogen to ethionamide [36]. In an attempt to screen for compounds that inactivate EthR but that are not toxic to mammalian cells and can penetrate the mammalian cell membrane to reach the intracellular pathogens, an EthR-based synthetic genetic circuit was designed and engineered into human embryonic kidney cells [34] (Fig. 1). A chimeric transcription factor was constructed, comprising EthR fused to the VP16 transactivator, which binds the EthR-specific operator O_{ethR} and triggers activation of the downstream minimal promoter, resulting in the expression of the reporter gene SEAP (human placental secreted alkaline phosphatase). This configuration was used to screen a chemical library, and revealed several compounds which inhibit EthR, most of which were not followed up on as viability of
the screening cell line was compromised. The small-molecule ester 2-phenylethyl-butyrate met all the above criteria and activated EthA expression in *M. tuberculosis* and to render this pathogen more sensitive to the antibiotic ethionamide, thereby providing novel therapeutic options for the treatment of tuberculosis [34].

**Screening for anti-infective molecules**

A variant of this screening technology was described by Aubel et al. [37], where the *Streptomyces pristinaespiralis*-derived streptogramin-responsive repressor PIP [38] was engineered into mammalian cells to repress a PIP-responsive promoter that controlled transcription of a reporter gene. Addition of the streptogramin antibiotic pristinamycin I released PIP from its promoter, thereby de-repressing reporter activation. This system was used for screening a metabolic library of *Streptomyces* and identified bacterial strains producing compounds active against clinical pathogens, able to penetrate into mammalian cells and that do not show toxic side effects on mammalian cell physiology. Similar approaches might be feasible using other antibiotic-responsive repressor proteins functionally transferred into mammalian cells, such as macrolide-specific MphR(A) [39] or tetracycline-specific TetR [40].

**Screening for anticancer drugs**

A key characteristic of anticancer drugs is that they selectively kill dividing cells but do not affect mitotically inactive cells. We recently described a synthetic gene network that emulated the emergence of proliferating cancer cells in a nondividing G1-phase cell population [41]. Chinese hamster ovary cells were engineered with a tetracycline-triggered gene network controlling the expression of the cycline-dependent kinase inhibitor p27\(^{kip1}\). In the presence of tetracycline, the tetracycline-dependent transactivator tTA is inactive and p27\(^{kip1}\) expression is silent. However, upon tetracycline withdrawal, tTA binds its cognate promoter P\(_{tet}\) and drives the transcription of the cycline-dependent kinase inhibitor, triggering a G1-phase-specific growth arrest. However, during conditions of sustained proliferation control individual cells emerged at a precise frequency which had escaped p27\(^{kip1}\)-mediated G1-phase arrest and resumed growth. Such a situation in which proliferation-competent cells appear within a growth-arrested terminally differentiated tissue population is reminiscent of the development of cancer. The mixed population of growing and growth-arrested cells was subjected to different clinically licensed cancer therapeutics, followed by scoring the number of growing and growth-arrested cells. It could be shown that cancer therapeutics, including 5-fluorouracil, doxorubicin and etoposide, selectively killed growing cells but did not affect the G1-arrested population. This validates the growth-arrested engineered cells as a cancer model suitable for identifying novel compounds that can selectively kill neoplastic cells [41,42].

**Rational design of antimicrobial peptides**

In a recent study, Stephanopoulos and coworkers analyzed the sequences of natural antimicrobial peptides and treated their amino acid sequences as formal language whose set of regular grammars was then used to design new unnatural antimicrobial peptides showing bacteriostatic activity against several species of bacteria including *Staphylococcus aureus* and *Bacillus anthracis* [43]. These new antimicrobial peptides conform to the formal syntax of natural ones but populate a previously unexplored region of sequence space. Such antimicrobial peptides have a potential use in combating bacterial infections as they are suggested to be less susceptible to bacterial resistance compared to traditional antibiotic agents [44].
Synthetic biology-based therapy and disease prevention
Besides serving as targets for the discovery of small-molecule drugs designer circuits and synthetic devices could directly be used as therapeutic compounds tailored to treat a particular disease.

Designed phages for combating pathogenic bacteria
In two pioneering studies, Jim Collins’s group recently used synthetic biology approaches for engineering bacteriophages to achieve superior antibacterial activity [45,46]. They first addressed the eradication of biofilms crucial in the pathogenesis of clinically important infections. Biofilms are difficult to combat because they resist antibiotic treatment or removal by the host immune system [45]. To dissolve biofilms, T7-derived bacteriophages were engineered to express Actinobacillus actinomycetemcomitans-derived dispersin B (DspB), which hydrolyzes β-1,6-N-acetyl-d-glucosamine, a crucial extracellular polymeric substance (EPS) needed for biofilm formation and integrity in Staphylococcus and Escherichia coli. Administration of the engineered bacteriophage to E. coli biofilm resulted in the initial infection of few cells, intracellular progeny phage and DspB production, and subsequent release of both compounds by cell lysis. The released DspB loosens the biofilm structure by degrading EPS and the released phages attack neighboring cells, thereby re-initiating the antibacterial cycle. It was shown that the application of DspB-engineered phage reduced bacterial biofilm cell count by 4.5 orders of magnitude, about 2 orders of magnitude higher than that of the wild-type control phage. This indicates that engineered phages might become a viable option in biofilm control in environmental, industrial and clinical settings [45].

Aside from biofilm formation, the increased resistance to antibiotics is a major concern in combating bacterial infections. While previous efforts focused on eliminating drug resistance by using small molecules that inhibited the determinants of resistance [34,47], Lu and Collins pioneered the engineering of bacteriophages to target antibiotic resistance mechanisms in E. coli [46]. They constructed an M13-derived phage engineered for the production of LexA3, a repressor of the SOS response system. Upon LexA3-mediated SOS inhibition, bacteria were more susceptible to antibiotics and less prone to develop resistance to antibiotics [48]. Co-administration of the lexA3-engineered phage with the antibiotic ofloxacin resulted in a reduction of viable bacteria by 2.7 orders of magnitude compared with the unmodified phage. It was further demonstrated that lexA3 expression acted in synergy with β-lactam and aminoglycoside antibiotics to kill bacteria and increase the survival of E. coli-infected mice [46].

The approach of using engineered phages to genetically suppress antibiotic resistance opens the avenue to simultaneously target multiple antibacterial targets that are not easily addressed by using traditional drug compounds. Infection of E. coli by a phage simultaneously expressing csrA (a global regulator of energy metabolism and repressor of biofilm formation) and ompF (a porin used by quinolones to enter bacteria) resulted in a synergistic killing of bacteria when co-administered with the quinolone ofloxacin [46]. The enhanced bacterial killing of synthetic biology-based phages could potentially be combined with the findings of previous studies that prolonged the half-life of phages in mammals and avoided their clearance by the immune system [49,50], which would represent a further step toward the eradication of drug-resistant bacterial infections.

Environmentally controlled invasion of cancer cells by engineered bacteria
In a similar approach to the engineered phages described above, Chris Voigt’s group has pioneered the design of environmentally controlled bacteria to invade cancer cells to deliver a cytotoxic agent [51]. E. coli were engineered to target human cancer cell lines derived from cervical (HeLa) or hepatocellular (Hep G2) carcinomas. For a selective invasion of solid tumors, the authors constructed a hypoxia-inducible expression vector because in general, solid tumors display a low-oxygen environment. The authors used the invasin gene from Yersinia pseudotuberculosis, which was cloned under the control of the hypoxia-regulated formate-dehydrogenase promoter (Pfdh). E. coli harboring the constructs selectively invaded cells under low-oxygen conditions. This result paves the way for future anticancer studies in animals [51].

The selectivity in targeting tumors and sparing healthy tissue could potentially be further improved using a synthetic network that was recently developed to guide bacteria to desired locations by gradients of small molecules [52]. In E. coli, the phosphatase CheZ triggers dephosphorylation of CheY, which in turn makes E. coli run and tumble while phosphorylated CheY induces tumbling only. To control chemotaxis, the cheZ gene was fused to a theophylline-responsive aptamer, resulting in theophylline-induced cheZ expression. It could be shown that engineered E. coli follow a theophylline gradient, which opens up the possibility of engineering bacteria to follow gradients of pathologic messengers (e.g. tumor markers) to invade and kill the malignant cells [52].

Preventing infection by eliminating the pathogen habitat
Treating a disease is good, preventing it from happening is even better. In recent studies, Luke Alphey’s group has described strategies for controlling insects [53] that serve as hosts for human pathogens like Plasmodium falciparum, the causative agent of malaria [54]. With the aim of eradicating specific insect populations, the group developed synthetic gene switches to confer inducible, female-specific, dominant lethality in the Mediterranean fruit fly (Ceratitis capitata), an agricultural pest [15]. In this approach, male flies harboring the engineered transgenes would be released into the environment where they would mate with wild-type females, thereby competing with wild-type males for wild-type females. Female progeny is expected to die, compromising reproduction of this insect pest [55]. To enable efficient breeding of the engineered flies, female-specific lethality must be suppressed in the breeding phase. Therefore, the authors [15] disrupted an insect codon-usage adapted variant of the tetracycline-responsive repressor (tTAV) with an intron, exclusively spliced out in female organisms, to yield in translation-competent tTAV (Fig. 2). The intron-encoding tTAV was placed under the control of a tTAV-inducible promoter, resulting in a positive feedback loop that triggered high levels of tTAV only in females. This was toxic in the developmental phase probably because of transcriptional squelching effects of the VP16 transactivation domain [56] contained in tTAV which resulted in the killing of all female progeny. In the presence of tetracycline in the insect food, the tTAV-amplifying positive feedback loop was interrupted, yielding
female progeny for breeding purposes. Owing to the conservation of the sex-dependent splice mechanism [15], the authors suggest this procedure might be of more general use in controlling mosquito-transmitted malaria [54]. In fact, the United States Department of Agriculture has decided on May 12, 2009 to include the use of genetically engineered insects in the agency’s future plant pest control programs as this approach is considered to minimize potential impacts on human health, nontarget species and environmental quality compared to traditional chemical-based insect control strategies.

**Synthetic biology for improved drug production**

While the discovery of a drug provides the basis for treating a disease, manufacturing the drug substance in an economic, large-scale manner affects whether the drug will be available for people outside rich industrialized countries. While most small molecules can be addressed by classic synthetic chemistry, one-third of all marketed small-molecule therapeutics are drugs derived from natural products [57] and isolated from the natural host. While production of therapeutics in *Streptomyces* or fungi (e.g. antibiotics) can be done cost-effectively, large-scale production of plant secondary metabolites poses enormous economic challenges. For example, the antimalaria drug artemisinin, a sesquiterpene lactone endoperoxide extracted from the sweet wormwood (*Artemisia annua* L), is in short supply and unaffordable for most malaria patients [58]. Another example is taxol, a highly decorated diterpenoid derived from the pacific yew tree (*Taxus brevifolia* Nutt.) with a high chemotherapeutic value against lung, ovarian and breast cancer [59]. Taxol precursors are currently produced from plant cell culture and transformed into taxol by chemical synthesis, which is a costly process given the low yields from plant cell culture.

To design cost-effective manufacturing processes for therapeutic compounds that are difficult to produce, synthetic biologists have worked at assembling complete biosynthesis pathways in *E. coli* and *Saccharomyces cerevisiae*. This is a complex endeavor as the expression levels of all biosynthetic enzymes must be orchestrated to result in a streamlined biosynthesis and avoid the (toxic) accumulation or depletion of intermediate substances [60].

In a seminal effort, Jay Keasling’s group engineered *E. coli* to produce artemisinic acid, a substance readily convertible to artemisinin by organic chemistry [60]. The production of artemisinic acid required 11 enzymatic reactions, starting from acetyl-CoA with mevalonate, farnesyl pyrophosphate and amorphadiene as key intermediates (Fig. 3).

The following work illustrates general engineering strategies to optimize designer biosynthesis pathways for increased yield:

(i) Splitting the overall biosynthesis operon in suboperons (e.g. one catalyzing acetyl-CoA to mevalonate and one transforming mevalonate to farnesyl pyrophosphate) enables the independent construction, validation and optimization of both operons [61].

(ii) It was shown that similar pathways from different hosts differ in efficacy. In this example it was shown that the yeast mevalonate pathway transferred to *E. coli* resulted in substantially higher amorphadiene concentrations than using the native deoxyxylulose-5-phosphate (DXP) pathway from *E. coli* [60,61].

(iii) To avoid the accumulation of intermediate metabolites to toxic concentrations (e.g. the direct mevalonate precursor 3-hydroxy-3-methylglutaryl-CoA, HMG-CoA), additional copies of the next downstream enzyme can be incorporated (e.g. HMG-CoA reductase) thereby increasing the overall yield [62].

(iv) To optimally balance the expression levels of the three biosynthetic genes determining the conversion of acetyl-CoA to mevalonate, a library of tunable intergenic regions (TIGR) was constructed containing control elements that
included mRNA secondary structures, RNase cleavage sites or RBS sequestering sequences. The TIGR elements were cloned in the intergenic regions of the mevalonate pathway and screened for high mevalonate production, resulting in the production increased up to sevenfold as compared to the nonoptimized operon [63].

(v) Heterologous enzymes are sometimes difficult to express in *E. coli*, potentially limiting the overall biosynthetic capacity of the microbial systems. For example, the high-level expression of the cytochrome P450-based amorphadiene monooxygenase required codon optimization, the insertion of heterologous transmembrane domains as well as the coexpression of redox partners [64].

In a four-year concentrated effort, combining the above-mentioned strategies resulted in 10⁶-fold increased production levels of the intermediate substance amorphadiene and in final production yields of 300 mg/L artemisinic acid. These can readily be converted into active artemisinin by organic chemistry [60].

In a similar approach, Jay Keasling’s group engineered the artemisinic acid biosynthesis pathway in *S. cerevisiae* [65]. This resulted in the production of 2.5 g/L artemisinic acid in an optimized bioprocess [66]. Such work on the optimization of artemisinin production is the most advanced demonstration of how synthetic biology makes it possible to produce difficult natural compounds and overcome shortages in the supply of natural therapeutics [58].

**Conclusion**

The emergence of synthetic biology was characterized by the design of synthetic gene networks showing complex dynamic behavior, as well as providing tools for the rational construction of cells with desired features. Such designer networks are currently being considered for the next-generation of gene- and cell-based therapies as well as for all levels of the drug discovery process. This development is known as the second wave of synthetic biology [67].

Production of artemisinin is the paradigm of synthetic biology’s impact on drug development. Manufactured by a synthetic biology-based production process, this antimalarial compound is expected to enter the market in 2010 supported by an alliance of the Institute of OneWorld Health, Amyris Biotechnologies and Sanofi Aventis. Also, the use of genetically engineered insects has recently been validated in open field trials and has now obtained positive evaluation by the United States Department
of Agriculture. The clinical validation of synthetic biology-based biopharmaceuticals such as engineered phages or cancer-invading bacteria is imminent and will probably be successful because wild-type phages have already been used to treat bacterial infections [68] and the treatment of cancer patients with attenuated Salmonella was promising [69].

The rapid development of synthetic biology as exemplified by the identification, definition and characterization of biologic parts [18,19], the elaboration of computer-based modeling and design principles [21,22] as well as advances in genome synthesis technology [23,24,70] will revolutionize the entire drug discovery process. Assembly of designer networks and reconstruction of synthetic signaling pathways will lead to the identification of novel targets and the development of tailored drugs. However, synthetic biology will impact clinical applications beyond drug discovery because components of synthetic designer circuits [71] were recently adapted for the construction of stimuli-sensing biomaterials that release an embedded therapeutic growth factor in response to a small-molecule drug [72].

The recent achievements of medically related translational synthetic biology described above provide an inkling of how synthetic biology is poised to become a ground-breaking tool in the drug design and discovery process. It will help facilitate the discovery and production of novel therapeutic and preventive strategies to address unmet medical needs.

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