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Discovery of a new method for potent drug development using power function of stoichiometry of homomeric biocomplexes or biological nanomotors

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Abstract

Introduction—Multidrug resistance and the appearance of incurable diseases inspire the quest for potent therapeutics.

Areas Covered—We review a new methodology in designing potent drugs by targeting multi-subunit homomeric biological motors, machines, or complexes with $Z > 1$ and $K = 1$, where Z is the stoichiometry of the target, and K is the number of drugged subunits required to block the function of the complex. The condition is similar to a series, electrical circuit of Christmas decorations; failure of one light bulb causes the entire lighting system to lose power. In most multisubunit, homomeric biological systems, a sequential coordination or cooperative action mechanism is utilized, thus K equals 1. Drug inhibition depends on the ratio of drugged to nondrugged complexes. When $K = 1$, and $Z > 1$, the inhibition effect follows a power law with respect to Z , leading to enhanced drug potency. The hypothesis that the potency of drug inhibition depends on the stoichiometry of the targeted biological complexes was recently quantified by Yang-Hui's Triangle (or binomial distribution), and proved using a highly sensitive *in vitro* phi29 viral DNA packaging system. Examples of targeting homomeric bio-complexes with high stoichiometry for potent drug discovery are discussed.

Expert Opinion—Biomotors with multiple subunits are widespread in viruses, bacteria, and cells, making this approach generally applicable in the development of inhibition drugs with high efficiency.

Keywords

Drug development; drug target; antibacterial; antiviral; cancer drug development; phi29 dsDNA packaging motor; viral assembly; hexameric ATPase; binomial distribution

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1. Introduction

The continuous escalation of drug resistance has been threatening human health and life, i.e., many microorganisms including bacteria, viruses, and even cancer cells are developing resistance to current chemotherapies. Drug resistance in cancer has partially contributed to ~600,000 deaths in the USA in 2012[1]. To combat the on-rising drug resistance, different approaches for developing new drugs have been explored. One method is to develop drugs that target novel mechanisms. Components highly important for cancer cell growth have been explored as drug targets for the treatment of multidrug resistant cancer[2, 3]. The first FDA-approved drug to treat multidrug-resistant tuberculosis, bedaquiline, follows a novel mechanism of inhibiting the bacterial ATP synthase of *M. tuberculosis* and other mycobacterial species[4]. Another approach is to use nano-drug carriers to enhance the binding efficiency of drugs to cancer cells[5-8]. A third approach is to develop new combinational drugs acting on multiple targets to enhance its efficacy [9, 10], including cocktail therapy[11]. This involves identifying multiple targets that when treated simultaneously lead to a synergetic therapeutic effect and optimizing the design of multi-target ligands[12]. Still, there is unmet need for treating multi-drug resistant disease. Thus, new approaches for drug development are needed to combat drug resistance.

A new hypothesis that potent drugs can be developed by targeting proteins or RNA complexes with high subunit stoichiometry was reported recently[13]. The major challenge for testing this hypothesis is to evaluate the significance of the target stoichiometry and the binding affinity of the drug molecule with respect to its efficacy. In order to quantitatively correlate the drug inhibitory efficacy to the stoichiometry of the target biocomplexes, a well-studied multicomponent system is required, which allows an empirical comparison of functional inhibition efficiency of individual components with different numbers of subunits.

The DNA packaging motor of bacteriophage phi29 was an ideal model for testing this theory. The morphology and stoichiometry of the individual components in the phi29 DNA packaging motor have been well studied. The Phi29 biomotor (Fig.1A) is composed of three essential, co-axially stacked rings[14-17]: a dodecameric connector ring located at the vertex of the viral procapsid; a hexameric packaging RNA (pRNA) ring bound to the N-terminus of the connector[16, 18], and a hexameric ring of ATPase gp16 attached to the helical region of pRNA[19-21]. The stoichiometry of pRNA was first determined using Yang Hui's Triangle (or binomial distribution) in 1997[22], and similar mathematical methods were applied to determine the stoichiometries of the protein subunits [14]. Furthermore, dsDNA packaging utilizes a revolution mechanism without rotation to translocate its genomic DNA powered through the hydrolysis of ATP[20, 21, 23-28]. The copy number of ATP molecules required to package one full Phi29 genomic dsDNA has been predicted to be 10,000[20, 21, 23-27, 29]. Phi29 DNA packaging, thus, offers an ideal platform to test the novel concept described above: the dependence of the inhibitory drug efficiency on the stoichiometry of its targeted biocomplex.

Although the theory of targeting multisubunit complexes for developing potent drugs was reported and validated recently[13], real cases of targeting multisubunit complex for new

drug development have been practiced[30-32]. Since multicomponent biomotors are widely spread in nature[26, 27, 33, 34], the approach of targeting multisubunit complexes for potent drug development discussed here is generally applicable, especially in developing new generations of drugs for combating the rising acquired drug resistance in viruses, bacteria, and cancers [35-37].

2. Rationale for selection of multi-subunit biocomplexes as efficient drug targets

Inhibitory drugs are typically designed to bind selectively to a target site, thereby blocking the site from interaction with other biomolecules leads to the loss of essential activity of the biological target. This target can be a single element, composed of only one subunit, or a complex consisted of multiple subunits, such as the biomotors of the hexameric ASCE (Additional Strand Catalytic E) superfamily[20, 38]. Conventional drugs are designed to inhibit pathogenesis through targeting of a single subunit molecule, such as an enzyme or a structural protein of a virus. As discussed below, the key in designing potent drugs lies in targeting multisubunit biological motors, machines, or complexes as drug targets that follow a sequential coordination or cooperative mechanism. The stoichiometry of the complex, Z , is larger than 1 and the number of drugged subunits that are required to block the activity of the target complex, K , equals 1 ($Z > 1$ and $K = 1$). Similar to in-series connected decorative Christmas lights, where one broken light bulb will turn off the entire chain, one drugged subunit will inhibit the entire complex and therefore biological activity. Sequential action or cooperativity in multisubunit complexes has been widely reported in biological systems[39-43]; inhibiting any subunit leads to inhibition of the entire complex, or in other words K equals 1.

For a conventional drug that inhibits its single subunit target ($Z=1$) with efficiency p , the fraction of undrugged target molecules q will be $1-p$; and those undrugged target molecules will remain active to maintain their biological function. In this situation, the inhibition efficiency is proportional to the substrate targeting efficiency p [39-41]. When targeting a dimeric complex ($Z=2$), for example, inactivating any subunit results in inhibition of the whole complex. For a drug targeting a dimeric complex with substrate targeting efficiency $p=0.9$ (90%), only 10% of the first subunit and 10% of the second subunit remain active after drug targeting. Thus, the fraction of undrugged complexes will be effectively reduced to 0.01, leaving 1% of complexes active. Since drug inhibition depends on the ratio of drugged to undrugged complexes, the efficiency of the inhibition is proportional to the product of the inhibition of the individual subunits, in other words, it follows a power law with respect to Z .

Consequently, a complex composed of Z subunits with the smallest number of blocked subunits (K) to inhibit activity of the complex is 1, when p percent of subunits are interacting with the drugs, the fraction of uninhibited biocomplexes will be q^Z and the proportion of inhibition equals $1 - q^Z$.

2.1 Drug inhibition efficiency predicted by binomial distribution model

The scenario outlined above follows a binomial distribution which can hence be used to outline the relation between drug inhibition efficiency and target stoichiometry in general. When the target element is a monomer, the inhibition efficiency can be calculated using Equation 1, where p and q are the fractions of drugged (substrate targeting efficiency) and undrugged (normal active elements) subunits, respectively ($p + q = 1$).

$$X = (p + q)^1 \quad (1)$$

However, when the target element contains multiple subunits, a higher order binomial distribution (Equation 2) is required to calculate the ratio of active complexes, where Z represents the total number of subunits (the stoichiometry) and M the number of drugged subunits in one biocomplex.

$$X = (p + q)^Z = \sum_{M=0}^Z \binom{Z}{M} p^{Z-M} q^M = \sum_{M=0}^Z \left(\frac{Z!}{M! (Z-M)!} \right) p^{Z-M} q^M \quad (2)$$

The probability of drugged subunits (M) and undrugged subunits (N ; $M + N = Z$) in any given biocomplex can be determined by the expansion of Equation 2. When $Z = 3$, The expanded form of Equation 2, $(p + q)^3 = p^3 + 3p^2q + 3pq^2 + q^3 = 1$, displays the probabilities of all possible combinations of drugged and undrugged subunits of a homoternary complex composed of three (p^3), two (p^2q), one (pq^2), or no (q^3) drugged subunits; the sum equals 1. Assuming that 70% ($p=0.7$) of subunits are inactivated by bound drugs leaving 30% ($q=0.3$) unaffected, then the percentage of complexes possessing at least two copies of normal subunits would be the sum of those possessing one copy of drugged and two copies of undrugged *wild-type* subunits, $3pq^2$, and those possessing three copies of native subunits is q^3 , i.e., $3pq^2 + q^3 = 3(0.7)(0.3)^2 + (0.3)^3 = 0.216$. In another example, if a complex contains 6 subunits, and biological activity requires 5 out of the 6 subunits to remain uninhibited, the fraction of active complexes in the total population equals the sum of probabilities of obtaining: 1) 5 and 2) 6 undrugged subunits.

Using the binomial distribution, the probabilities that a population contains any combination of undrugged *versus* drugged subunits can be predicted. The effect of the targeting efficiency p on the probability of obtaining a given complex with M drugged and N undrugged subunits is displayed in Table 1. The probabilities are calculated using equation 2, $\frac{Z!}{M!N!} p^M q^N$, with the coefficients $\frac{Z!}{M!N!}$ obtained from Yang Hui's Triangle, which is also called Pascal's Triangle, or binomial distribution (Fig. 1B)[44]. The use of Yang Hui's Triangle and binomial distribution to determine the stoichiometry of biological motor was published in Guo Lab in 1997[22, 45] for RNA component and restated in 2014 for protein component[14] in phi29 DNA packaging motor.

2.2 Cooperativity in multisubunit biocomplexes leads to high inhibition efficiency

The cooperativity of multisubunit biocomplexes is the key to high drug inhibition efficiency. Cooperativity means that multiple subunits work sequentially or processively to accomplish one essential biological reaction [23, 40-42, 46-50]. Blocking any subunit of the complex

inhibits the activity of the whole complex. Many reactions involving multiple subunits work cooperatively, e.g. assembly pathways in viral assembly systems [39, 51]. An analogy to such a biological reaction mechanism is given by the difference between parallel and series circuits. When a chain of light bulbs is arranged in a parallel circuit, burning out one light bulb will not affect others, while in a series circuit, breaking any one light bulb turns off the entire lighting system. The K value, the smallest number of subunits that needs to be inhibited in order to inhibit function of the light chain is therefore, $K=1$. Thus, the K value is a key factor in estimating the probability of obtaining inactive nanomachines or biocomplexes by combination and permutation of all subunits.

$K = 1$ is critical for obtaining ultrahigh inhibition. The foundation of the approach in this report is the difference in inhibition probability for biocomplexes with the same ratio of drugged target subunits but different K values. Biological systems display complicated reactions that involve several steps and multiple components interacting in series or parallel. Based on the binomial math model and cooperative nature of biological reactions, we suggest that targeting of multi-subunit biocomplexes can serve as a tool to develop highly potent drugs. In a conventional six-component system, when one drug is designed to target only the component #3 to stop the entire system, such a condition resembles the model in equation 2 with $Z=1$ and $K=1$. Thus, the inhibition efficiency is linear to the substrate targeting efficiency (p) of the drug. However, in a homohexameric component system, the entire complex is blocked when a drug targets any subunit of the hexamer, which resembles $Z=6$ and $K=1$. Thus, the probability of active target complexes equals q^6 ($q=1-p$). In other words, the drug inhibition efficiency is equal to $1-q^6$, which scales with the 6th power of q compared to linearly with q as for conventional mono-subunit approaches (see Table 1).

Targeting a biological complex that exhibits a higher stoichiometry substantially reduces the fraction of non-inhibited complexes. $K=1$ implies that drug binding to one subunit inactivates the subunit, in which one drugged subunit is sufficient to inhibit the function of the entire complex. As an example, a probability calculation for $Z=6$ and $K=1$ is given below. As all 6 ($Z=6$) copies of the subunits are required for function, while one drugged subunit ($K=1$) is sufficient to block the activity, all elements possessing 1 through 5 copies of drugged subunits are non-functional (Fig. 1C). Only those complexes possessing 6 copies of undrugged subunits are functional. The probability that a complex contains 6 copies of unaffected subunits is q^6 and therefore the inhibition efficiency is $1-q^6$ [12, 23, 39, 46, 51, 52].

Consequently, for a drug with binding efficiency p , a larger stoichiometry of the target complex substantially increases the inhibition efficiency. To illustrate, we compare the fraction of non-inhibited complexes for $Z=6$ and $Z=1$, while keeping $q=0.4$ and $K=1$ fixed for both target systems. The fraction of non-inhibited complexes for $Z=1$ amounts to $q^Z=0.4^1=0.4$, resulting in $1-0.4=60\%$ of inhibited complexes. In contrast, for $Z=6$, the fraction of non-inhibited complexes is $q^Z=0.4^6=0.0041$ and therefore $1-0.0041=99.59\%$ of complexes are inhibited. The ratio of the remaining non-inhibited complexes ($0.4/0.0041=98$) shows a 98-fold decrease in non-inhibited complexes for $Z=6$ compared to $Z=1$. At a targeting efficiency of $p=0.9$, the inhibition efficiency for $Z=6$ is $1-q^Z=1-0.1^6=0.999999$ resulting in a 10,000-fold increased inhibition efficiency compared to

$Z=1$ ($0.1/0.1^6=10^5$, see Table 1). The binomial distribution indicates that the inhibitory effect follows a power law with respect to the stoichiometry of the target. Thus, for $K=1$, the fraction of uninhibited biocomplexes equals q^Z ; the larger Z , the smaller q^Z , (as $0<q<1$). That is to say when developing drugs with the same binding affinity to their targets, the higher the stoichiometry of its multimeric target, the fewer uninhibited targets will remain and the more efficient the drug will be.

2.3 IC₅₀ decreases as the stoichiometry of target complexes increases

The half maximal inhibitory concentration (IC₅₀) is one parameter used to evaluate drug efficacy. It quantitatively indicates how much of a particular drug is required to reduce the activity of a given biological process by half. It is universally used as a measurement of drug potency in pharmacological research. The median lethal dose LD₅₀, also known as 50% of lethal concentration, is an important parameter to evaluate the safety profile, i.e., acute toxicity of a drug. Most importantly, a larger ratio of LD₅₀ to IC₅₀, results in a safer drug. By increasing the inhibition efficiency through targeting components with high stoichiometry, the effective drug dosage is greatly decreased, thus decreasing the IC₅₀. As a result, the ratio of LD₅₀ to IC₅₀ increases, resulting in an enlarged therapeutic window of the drug.

If we denote P_{IC50} as the percentage of drugged subunits needed to reach 50% inhibition, then $1 - (1 - p_{IC50})^Z = 50\%$. Solving this equation, $p_{IC50} = 1 - 0.5^{1/Z}$. Figure 2 shows the relationship between stoichiometry (Z) and drug targeting level p to reach the inhibition effect (IC), where p is a combined result of drug binding efficacy and drug concentration (dosage). When the stoichiometry Z of the multimeric drug target increases, the dosage of drug to reach IC₅₀, IC₂₀, or IC₈₀ decreases, presented by the percentage of drugged subunits. This clearly shows that as Z increases, p_{IC50} decreases, and hence the drug is more potent.

Focusing on the stoichiometry of the target complex for drug development differs from conventional approaches. Conventional drug molecules are sought to have a high binding affinity to the target, which means we expect more drug molecules to bind to one target molecule. Here stoichiometry refers to the copy number of subunits within a biocomplex or nanomachine that serves as the drug target. This idea agrees with a newer model for predicting clinical drug efficacy, the receptor occupancy. Receptor occupancy acts as a predictor for human pharmacodynamics and antihistamine potency and takes into account both the affinity of the drug for its receptor and its free plasma concentration [53].

3. Inhibition efficiency as a power function of target stoichiometry proved by Phi29 viral assembly system

The hypothesis that drug inhibition efficiency follows a power function with respect to the target stoichiometry has been proved using the Phi29 viral assembly system [54]. This well-defined *in vitro* assembly system is composed of four components, each of which is comprised of different subunits that can act as the nano-machine target. Inhibition of viral assembly is achieved using mutant components that represent drugged target components. The inhibition efficiencies were analyzed with Yang Hui's triangle for targeting each of the

Phi29 DNA packaging motor components. Binomial distribution analysis of these viral assembly competition assays confirmed the concept that drug targeting biological complexes with higher stoichiometry results in a higher efficiency than drugs acting on a single subunit target.

The highly sensitive *in vitro* Phi29 assembly system was used to determine the inhibition efficiency of drugs targeting multi-subunit complexes [22, 39, 45, 55], thus validating a new method for developing potent drugs. The bacteriophage Phi29 DNA packaging motor contains one copy of genomic dsDNA, 6 copies of packaging RNA, 6 copies of ATPase protein gp16, and consumes more than 10,000 copies of ATP during genome packaging. The hexameric stoichiometry of Phi29 pRNA has been extensively shown using single-molecule techniques[54], AFM imaging[56, 57], pRNA crystal structure determination[58], and statistical evaluations[22]. The hexameric stoichiometry of Phi29 gp16 has been proved by native gel binding, capillary electrophoresis assays, Hill constant determination, and by titration of mutant subunits using binomial distribution[20, 23]. The copy number of ATP molecules was calculated based on the fact that 6 ATP molecules are required to package one pitch of dsDNA containing 10.5 base pairs [59], thus one ATP is used to package 1.7 base pairs of dsDNA. The entire Phi29 genome is composed of 19,400 base pairs, thus, it is expected that more than 10,000 ATP molecules are required to package an entire Phi29 genome. The availability of a motor system with multiple well-defined and characterized components makes an ideal disease model for the analysis of drug inhibition efficiency versus the subunit stoichiometry of individual subcomponents within the same assay.

Inhibition efficiencies were determined for ATP, pRNA, ATPase gp16, and DNA as drug targets with stoichiometries of 10,000, 6, 6, and 1, respectively. Among these components, targeting of ATP showed the strongest inhibition, while drugged mutant pRNA and mutant gp16 still showed stronger inhibitory effects than mutant DNA (Fig. 3). For example, adding 20% mutant DNA caused 20% inhibition of viral assembly, while 20% of drugged mutant pRNA exerted 74% of inhibition on viral assembly and 20% of γ -S-ATP almost completely inhibited the viral assembly, indicating that higher stoichiometry results in stronger inhibition efficacy.

The target with ten-thousand-subunits showed higher inhibition than those with six subunits, which in turn showed higher inhibition than the single subunit target. In conclusion, these results show that inhibition efficiency displays a power function with respect to the stoichiometry of the target biocomplexes. Drug inhibition potency depends on the stoichiometry of the targeted components of the biocomplex or nano-machine. Since biomotors share certain common structural and operational mechanisms across viruses, bacteria, and other cells, this approach has general application in drug development.

4. Wide-spread distribution of biomotors with multiple subunits or high order stoichiometry

Biological systems contain a wide variety of nanomachines with highly ordered stoichiometry that are essential for DNA replication, DNA repair[60], homologous recombination, cell mitosis, bacterial binary fission, Holliday junction resolution[61], viral

genome packaging[62], RNA transcription, nuclear pore transport, as well as motion, trafficking, and exportation of cellular components. Here we use biological motors as an example to elucidate the rationale of $Z>1$ and $K=1$. These biological motors can generally be classified into three categories according to their DNA transportation mechanism: linear motors, rotation motors and the newly discovered revolution motors[23, 27, 34]. High order stoichiometries are widely observed among biomotors, especially in rotation and revolution motors. Thus, biomotors are feasible targets for the development of potent inhibitory drugs that exploit the power law behavior of the subunit stoichiometry.

4.1 Rotation nanomachines

FoF1 ATP synthase and helicases are representatives of rotary motors [63, 64]. FoF1 ATP synthase is a ubiquitous membrane enzyme that plays a key role in biological energy metabolism [65, 66]. It consists of two linked rotary motors, F1 and Fo, which are distinct in structure and function. F1 ATPase, forming the catalytic core, shows strong ATP hydrolysis activity. It is composed of 5 subunits ($\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$), with three α and three β subunits forming a hexameric ring with part of a long coiled coil γ subunit. Fo is the proton pore that is embedded in the membrane, it consists of at least 3 subunits ($a_1b_1c_{8-15}$) whereby subunit c differs among species.

Helicase DnaB is a hexameric nanomachine (Fig. 4A) that unwinds dsDNA in front of the replication fork during DNA replication[67, 68]. Recently, a hand-over-hand translocation mechanism was proposed for DnaB based on the crystal structure of the DnaB hexamer complexed with ssDNA and GDP-AIF4 [69]. In this mechanism, the 5'-3' translocation of the subunits at a stepsize of two nucleotides is coupled with the sequential hydrolysis of NTP [70]. The sequential hand-by-hand migration of the individual subunits results in DNA translocation.

RecA, a family of ATP-dependent recombinases, plays an important role in dsDNA repair and genetic recombination in Archaea, Bacteria, and Eukaryota. It can interact with ssDNA forming right-handed helical filaments as a complex with approximately six monomers of RecA per turn (Fig.4B)[71, 72]. Electron microscopy studies have demonstrated that ATP binding induces a re-orientation between the RecA ATPase domains, resulting in the relative rotation of the protein on DNA substrate during DNA translocation powered by ATP hydrolysis.

4.2 Revolution nanomachines

All the dsDNA viruses known to date utilize similar mechanisms to transport their genome into preformed protein shells during replication. For example, Bacteriophage phi29, HK97, SPP1, P22, and T7 all share a common revolution mechanism for dsDNA packaging that employ a hexameric ATPase and predominantly dodecameric connector channels for packaging dsDNA. The phi29 DNA packaging motor is composed of three coaxial rings: a dodecameric channel ring and an ASCE hexameric ATPase linked by a hexameric ring of pRNA (Fig. 4C)[20, 54, 58]. During genome packaging, more than 10,000 ATP molecules are consumed by the hexameric ATPase as energy source to drive the translocation of one copy of the dsDNA genome[59].

The ASCE superfamily, including FtsK-HerA superfamilies and the AAA+ (ATPases associated with diverse cellular Activities), is a clade of nanomachines that display a hexameric arrangement[73-76] of subunits. Their biological function is to convert chemical energy from ATP into mechanical motion[20, 29, 77, 78], typically associated with conformational changes of the ATPase enzyme [20, 79, 80].

FtsK belongs to the ASCE superfamily. It is a multi-domain protein composed of a C-terminal ATPase domain FtsK(C) containing α , β and γ sub-domains, an N-terminal membrane-spanning domain FtsK(N) and a 600-amino acid linker[81-83]. It is responsible for conjugation between bacterial cells and dsDNA bidirectional translocation[84, 85]. It has been proposed that FtsK subunits acts in a sequential manner employing a revolution mechanism to translocate dsDNA[86, 87]. The crystal structure and electron microscopy of FtsK(C) demonstrates formation of a ring-like hexamer with DNA passing through the hexameric ring (Fig.4D)[87, 88].

5. Targeting biocomplexes for developing potent drugs

As illustrated above, drug efficiency follows a power function of the stoichiometry of the subunits of the multimeric target biocomplex. Targeting biocomplexes with higher stoichiometry therefore can lead to the development of more potent drugs. Experimentally, approaches targeting receptor dimers, hetero- and homo-oligomers for drug screening open exciting possibilities for drug discovery and development[89].

5.1 Targeting homomeric channel proteins for drug development

In the history of drug development, one important property of most channel protein receptors has been overlooked, their stoichiometry. As a matter of fact, many channel proteins are expressed as dimers or oligomers on cell membrane, including most G-Protein-Coupled Receptors (GPCR) proteins [89]. Targeting of GPCR hetero- and homo- oligomers is generally starting to be considered for drug development. Therefore, new models for multisubunit protein binding are being developed[89]. Cooperative binding affinity between ligand and multisubunit targets has been reported and cooperativity factors were calculated by fitting to the Hill equation[23, 89].

The ATP-sensitive homotrimeric P2X7 receptor (P2X7R) acts as a ligand-gated ion channel. It forms a chalice-like channel with three ATP binding sites localized at the interface of the three subunits. Occupancy of at least two of the three sites is necessary for activation of the receptors which results in opening of the channel pore allowing passage of small cations (Na^+ , Ca^{2+} , and K^+). P2X7R has received particular attention as a potential drug target for its widespread involvement in inflammatory diseases and pivotal roles in central nervous system (CNS) pathology [30]. These concepts will broaden the therapeutic potential of drugs that target multi-subunit channel proteins, including receptor heteromer-selective drugs with a lower incidence of side effects. They will also help to identify novel pharmacological profiles using cell models that express heteromeric receptors.

5.2 Targeting homomeric enzyme for antibiotics development

Targeting of key enzymes in essential biosynthesis pathways is an important approach for antibiotics development. Many key proteins in the fatty acid synthesis pathway and nucleotide synthesis pathway are found to be multivalent. The highly ordered oligomeric enzymes in biosynthesis pathways could be promising targets for developing more potent antibacterial drugs. Some examples of developing potent drugs by targeting multisubunit biocomplexes are discussed below.

Fatty acid synthesis is an essential lipogenesis process in both Gram-positive and Gram-negative bacteria. A key enzyme in the fatty acid biosynthesis pathway is fatty acid biosynthesis 1 (FabI), which is a homotetramer complex acting as the major enoyl-ACP reductase present in *Burkholderia pseudomallei* (Bpm). A recent X-ray structure study revealed the binding mode of the inhibitor PT155 with the homo-tetrameric BpmFabI [31] (Fig. 5A). The substrate BpmFabI is a homo-tetramer, one PT155 molecule bound to each monomeric subunit has shown significant promise for antibacterial drug development[31]. Another example of targeting multisubunit biocomplex as drug target is found in the guanine nucleotide biosynthesis pathway to control parasitic infection. Inosine 5'-monophosphate dehydrogenase (IMPDH) is a homo-tetramer enzyme[32](Fig. 5B), which plays an important role by catalyzing the oxidation of IMP to XMP in guanosine monophosphate(GMP) biosynthesis[32]. Structural characterization of IMPDH with chemical inhibitor drugs indicates that binding to the repeating units shows a more potent inhibition effect[90].

These examples of successfully targeted homotetramer enzymes for potent drug development further proved the importance of the stoichiometry of target homo-meric complexes. When applying this method to search enzymes as drug targets, it is critical to test whether the stoichiometry of the complexes (Z) is > 1 , and the number of subunits needed to inhibit to block biological function (K) equals 1.

5.3 Targeting homomeric drug transporters for drug development

The mechanism of drug transporter, very similar to that of the revolution motor, involves entropy induced transitions by ATP. High stoichiometry of the target complex is a key consideration in drug efficiency. Targeting multidrug efflux transporters with high stoichiometry has a better chance to develop drugs for treating multi-drug resistant disease. The structure of bacterial multidrug efflux transporter AcrB is composed of three α -helix subunits, that connect to form a funnel around a central cavity(Fig. 5C)[91]. The multidrug exporter MexB from *Pseudomonas aeruginosa* also forms a homotrimer (Fig.5D) [92]. Pyridopyrimidine derivatives have been reported to be promising drugs to treat multidrug resistant pathogens by specific inhibition of the homotrimeric AcrB and MexB transporters[117]. The structural architecture of ABC transporters consists minimally of two TMDs and two NBDs. These four individual polypeptide chains combine to form a full transporter such as in the *E. coli* BtuCD[93]. Although the stoichiometry of the heterodimer is not very high, the stoichiometry of ATP per transporter is high. It is involved in the uptake of vitamin B12. The TMDs of ModBC-A and MalFGK2-E have six helices per subunit. These unique structural features can be used in target considerations.

6. Conclusion and Future Perspective

Targeting functional biological units with higher stoichiometries allows for higher inhibition efficiencies. The inhibition efficacy follows a power law with respect to the subunit copy number when targeting multimeric biocomplex, compared to a linear effect of the drug-target binding affinity when targeting a single-subunit substrate. This new concept outlined herein suggests that potent drugs can be developed by targeting biocomplexes with high stoichiometries with the potential of complete inhibition of the targets activity. Possibly, this method can further be applied to guide development of dominant negative proteins for potent gene therapy, which can be incorporated into a multimeric protein nanomachine and results in a change of its activity [94]. Since bio-motors share certain common structural and operational mechanisms across viruses, bacteria, and cells, this approach has general applicability in drug development.

Living systems contain many elegant arrays, motors and nanomachines that are composed of multiple identical subunits. As reported here, these homomeric biocomplexes can serve as potent drug targets. For example, most members of the ASCE family are hexamers[20, 95-99]. As these machines are common among living systems, specificity and toxicity need to be considered. In the development of anti-bacterial and anti-viral drugs, specificity and toxicity is not problematic since the target biocomplexes differ from those found in human cells and thus all targets are intended to be killed nonexclusively. In the development of anti-cancer drugs, mutations in multiple-subunit biocomplexes of cancer cell will present ideal targets for potent drug development.

7. Expert Opinion

Drug discovery is a multidisciplinary science including the fields of medicine, biotechnology and pharmacology. Aiming to find a method for developing drugs with ultra-high potency, much effort has been placed in the screening of new drug compounds, uncovering of new drug targets, and illumination of functional pathways, but little attention has been paid to the exploration of new methods for the design and development of more efficient drugs. Here we propose that the inhibition efficiency of a given drug depends on the stoichiometry of the biocomplex or bio-machine that serves as drug target. Here the notion of “stoichiometry” differs from the conventional concept in drug development. Conventionally, stoichiometry refers to the number of drug molecules bound to each substrate or cell membrane. In the current study stoichiometry refers to the number of identical subunits that the target biocomplex is composed of. Phi29 viral components with a series of variable but known stoichiometries were evaluated as mock drug targets to test the hypothesis. Both *in vitro* and *in vivo* virion assembly assays were employed to compare inhibition efficiencies for targets with differing subunit stoichiometries. Viral inhibition efficiency was analyzed with Yang Hui's (Pascal's) Triangle (also known as binomial distribution) (Fig. 1), as shown in equation 2.

It was observed that inhibition efficiency of virus replication correlates with the stoichiometry of the drug target. The inhibition efficacy follows a power law behavior where the percentage of uninhibited biocomplexes equals q^Z (see equation 2). For a system with

fixed q and K values, the inhibition efficiency thus depends on Z , the number of subunits within the target biocomplex or bio-machine. This hypothesis is supported by empirical data that a target with ten-thousand-subunits shows higher inhibition effect than a target with six subunits, which in turn shows higher inhibition than a single-subunit target (Fig. 3). The unconventional hypothesis described in this article for the development of potent drugs with power function behavior with respect to the target stoichiometry can be foreign or even outlandish to the main force of the pharmaceutical field. The approach of developing highly potent drugs through targeting of protein, RNA or other macromolecule complexes with high stoichiometry has never been reported due to challenges to prove the concept.

Traditionally, it is almost impossible to prove this concept by comparing efficacies of two drugs where one of them targets a biocomplex with multiple subunits. When reporting the efficiency of this new approach, it is very difficult to distinguish essentiality of the two targets in biological function, it is also very challenging to compare the binding affinity of two different drugs to two different targets. For instance, if two drugs target two stoichiometrically different complexes, it becomes extremely difficult to prove whether the difference in drug efficiency is due to differences in their target binding affinity or essential level of the target in the biological organism.

The mechanism of drug inhibition mainly relies on blocking an essential biological target element from functioning. The target elements can be monomers or a complex of multiple homosubunits; such as the biomotors of the hexameric ASCE superfamily[20, 38]. Conventional drugs are designed to target a single subunit molecule to inhibit pathogenesis, such as an enzyme or a structural protein of a virus. The key in designing potent drugs is to target multi-subunit biological motors, machines, or complexes with $Z>1$ and $K=1$, where Z is the stoichiometry of the complex and K is the number of drugged subunit that are required in order to block the function of the entire complex. Similarly, in a series circuit Christmas decorative light chain, one broken light bulb will turn off the entire lighting system.

In most, if not all, multi-subunit biological systems, sequential coordination or cooperative action mechanisms are utilized, thus, K equals 1. Drug inhibition depends on the ratio of drugged to the non-drugged complex. For $K=1$, and $Z>1$, inhibition efficacy follows a power function with respect to Z , leading to an increased potency of the drug since inhibition of any subunit results in complete inhibition of activity. For a drug designed to target a single-subunit molecule at targeting efficiency p , the fraction of undrugged target molecules q that will remain active is $1-p$. In this situation, the inhibition efficiency is proportional to the substrate targeting efficiency p and the inhibition efficacy is of the first order of p .

Sequential action or cooperativity in multi-subunit complexes has widely been reported in biological systems[39-41]. Drugs targeting a complex with multiple subunits can inhibit the complex activity if any homosubunit of the target is inactivated. Thus, if the copy number of this cooperative complex is $Z>1$, and the least number of blocked subunit to inhibit complex activity (K) is 1, the fraction of uninhibited biocomplexes is q^Z , and the inhibition efficiency is $1 - q^Z$, where $1-q$ is the portion of drugged subunits.

The binomial distribution analysis allows prediction of the inhibition efficiencies. For example, in targeting a six-subunit biocomplex with $K=1$, the inhibition efficiency is

determined by drug binding to any one of the six homosubunits. Therefore, the probability of inhibiting any subunit at random position is $\frac{1-q^6}{1-q}$ times higher than inhibiting a monomer substrate. With this new elucidation and understanding of the concepts behind targeting of cooperative multi-homosubunit complexes, a new generation of potent drugs may emerge in the near future.

Our discovery is an approach, not a drug. This approach will have general impact in the development of drugs for many diseases such as cancer, viral or bacterial infections. In living systems, biological machines or complexes with high stoichiometry and operated by sequential cooperative action or coordination with $Z > 1$ and $K = 1$ are ubiquitous. This class of biological machines is involved in many aspects of crucial cellular processes to the survival of viruses, bacteria, and eukaryotic cells. For example, multi-subunit biomotors are involved in chaperon, ATPase, ATP synthase, cell mitosis, bacterial binary fission, DNA replication, DNA repair, homologous recombination, Holliday junction resolution, nuclear pore transportation, RNA transcription, drug transporters, muscle contraction, viral genome packaging, as well as motion, trafficking, and exportation of cellular components. These systems use a sequential mechanism similar to the serial circuit of the Christmas decoration lighting chain. Thus, our approach will have broad application in drug development in many biological systems. Drugs targeting to these motors will be highly efficient.

Biomotors belonging to the multi-subunit ATPase are widely spread in organisms, including bacteria, viruses and cancer cells. Successful implementation of this new methodology will lead to the development of the next generation of potent drugs. In fact the first drug approved to treat multidrug-resistant tuberculosis, bedaquiline [4], is acting on the ATP synthase which is a multisubunit biomotor [100-111]. Treating multidrug-resistant tuberculosis had been very challenging previously. Although this drug's inventors were not aware of the concept of targeting multisubunit complexes for potent drug development, the success in this drug conquering the tough *Mycobacterium tuberculosis* organism supports the concept of using the multisubunit complex as a potent drug target. Cancer or bacterial mutant multi-subunit ATPase can be used as target. The drug developers can simply check the published literature and identify a multi-subunit machine as the drug target. For cancer treatment, it is to find a multi-subunit machine with mutation.

The concept of $K = 1$ for high efficiency inhibition may be impactful in gene or protein therapy. By introducing the dominant negative protein[94] or inactive mutant protein into the cell, either by intracellular expression or direct introduction of proteins, which resembles the above illustrated approach and mechanism used for phi29 DNA packaging motor systems [13, 14, 45, 112](Figure 3). This involves the incorporation of mutant proteins, either intracellularly expressed or directly introduced, into a highly multimeric complex that is identified as the target unit. For purposes of serving as a small molecule drug target, a multimeric complex might be identified, such that binding of one drug molecule to any one binding site on the complex will inactivate the whole complex. The fact that the complex composed of Z subunits holding one drugged subunit will only come into play as the drug concentration is at the high end. However, if the strategy was to express a dominant negative protein, as has been done in recent cardiac gene therapy with dominant negative

phospholamban[94], a high inhibition efficacy will achieve. The greater the value of Z the more the effect of the dominant negative protein subunit or mutant subunit will be achieved.

Another possibility is the use of homomeric drug transporters [113, 114] as drug targets (see section 5.3). The mechanism of drug transporters is very similar to that of the revolution motor featuring an entropy transition induced by ATP. High stoichiometry of target complex is a key consideration for achieving high drug efficiency. Targeting multidrug efflux transporters with high stoichiometry has a better chance to develop drugs for treating multi-drug resistant disease.

While the hypothesis behind this method might theoretically seem challenging, elucidation of the mechanism should greatly facilitate application of this approach. Two factors are essential for drugs development: efficiency and specificity. The strategy described herein focuses on drug efficiency, while specificity is similar to the general consideration in the development of chemicals and drugs. Nevertheless, design of potent drugs to common machines or general targets is still possible. For example, if an oncogenic mutant hexameric ATPase is found in one specific type of cancer cells, drugs targeting to this mutation of the altered ATPase will not only be highly efficient but also specific.

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Article Highlight Box

- Using multisubunit homomeric complexes such as AAA+ family ATPase, biomotors or drug transporters with high stoichiometry could lead to development of highly potent drugs.
- Most multisubunit complexes work in a sequential and cooperative manner, that is $K=1$, which is the key for these complexes to be used as target for potent drug development.
- Bacterial virus Phi29 DNA packing motor contains many multisubunit components with different stoichiometries; it is a good model to elucidate the concept of stoichiometry in drug development.
- Multisubunit nanocomplexes such as biomotors or ATPase are widely spread in nature. Thus, the method should have broad application in the development of new drugs. For example, the use of ATP synthase as drug target has led to the development of a new drug for treating multidrug resistant tuberculosis.

Figure 1.

The morphology and stoichiometry of Phi29 DNA packaging motor. (A) Illustration of Phi29 DNA packaging motor composed of 1 copy of genomic DNA through a channel composed of three coaxial rings, a 12 subunit connector, 6 subunit pRNA, 6 subunit ATPase gp16. (B) Binomial distribution equation with its coefficient displayed by Yang Hui Triangle. (C) Illustration of Z=6 and K=1, drug targeting any subunit of a hexameric complex would block its function. Reproduced from [13] with permission of Future Medicine.

Figure 2.
The relationship between the stoichiometry of homomeric target complex (Z) and target complex inhibition effect (IC). Reproduced from [13] with permission of Future Medicine.

Figure 3.

Comparing Phi29 viral assembly inhibition efficiency by targeting components of the system with different stoichiometries(left pannel), DNA with stoichiometry of 1, ATPase gp16 with stoichiometry of 6(right upper pannel), pRNA with stoichiometry of 6(right lower pannel), and ATP with stoichiometry of 10,000. Reproduced from [13] with permission of Future Medicine.

Figure 4.

Widespread biomotors or nanomachines are composed of multisubunit complex. (A) Rotation nanomachine DnaB helicase is a hexamer [69] (PDB ID: 4ESV), (B) rotation nanomachine RecA motor protein is a hexamer [72] (PDB ID: 1N03), (C) revolution Phi29 DNA packaging motor contains a hexameric pRNA [58], (D) revolution DNA motor protein FtsK is a hexamer [87] (PDB ID: 2IUU).

Figure 5.

Examples of homomeric multisubunit complex as drug target for developing potent drugs.

(A) Tetrameric bpFabI is a key enzyme in fatty acid synthesis in bacterial, inhibitor PT155 forms a tetrameric complex with BpmFabI [31] (PDB ID: 4BKU). (B) Inosine monophosphate dehydrogenase (IMPDH) [32] (PDB ID: 1AK5) is a key enzyme in guanine nucleotide biosynthesis pathway, inhibitors have been developed targeting the tetrameric IMPDH. (C) Bacterial multidrug efflux transporter AcrB forms a homotrimer[91](PDB ID: 1IWG). (D) Multidrug exporter MexB from *Pseudomonas aeruginosa* forms a homotrimer[92](PDB ID: 2V50).

Table 1

Probability of obtaining complexes containing M copies of drugged and N copies of undrugged subunits

Inhibited Subunits (<i>p</i>)	Z=1		Z=6						
	M=0, N=1	M=1, N=0	M=0, N=6	M=1, N=5	M=2, N=4	M=3, N=3	M=4, N=2	M=5, N=1	M=6, N=0
0%	100%	0%	100%	0%	0%	0%	0%	0%	0%
10%	90%	9%	53%	35%	10%	1%	0%	0%	0%
20%	80%	16%	26%	39%	25%	8%	2%	0%	0%
30%	70%	21%	12%	30%	32%	19%	6%	1%	0%
40%	60%	24%	5%	19%	31%	28%	14%	4%	0%
50%	50%	25%	2%	9%	23%	31%	23%	9%	2%
60%	40%	24%	0%	4%	14%	28%	31%	19%	5%
70%	30%	21%	0%	1%	6%	19%	32%	30%	12%
80%	20%	16%	0%	0%	2%	8%	25%	39%	26%
90%	10%	9%	0%	0%	0%	1%	10%	35%	53%
100%	0%	0%	0%	0%	0%	0%	0%	0%	100%