Emerging Themes in Drug Resistance

Gunjan Arora, Ankur Kulshreshtha, Kriti Arora, Puneet Talwar, Rishi Raj, Gurpreet Grewal, Andaleeb Sajid, and Ritushree Kukreti

Abstract In the last century, advances in biomedical sciences led to improvements in quality of human life mainly by decreasing the disease burden and providing various healthcare innovations. Effective medications were developed for most infectious diseases, lifestyle disorders, and other diseases. For once, we all believed that we can create a disease-free world; however, the excessive use of medications generated drug-resistant human pathogens leading to untreatable forms of many diseases. In the last few decades, the focus has been to understand the evolution of drug resistance, tackle the current drug-resistant disease agents, and develop new drugs that are potent and reliable for long-term usage. In this chapter, we will discuss the emerging themes in drug resistance research. As biologists are gaining deeper understanding of cellular complexity and disease agents, numerous modern themes have been pursued. The focus is to identify novel drug targets and develop specific drug molecules detrimental for the disease causing pathogens, and to harness host immunity. For the scope of this chapter, we will primarily discuss

G. Arora (✉)
National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville 20852, MD, USA
e-mail: arorag1983@gmail.com

A. Kulshreshtha
CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India

International Centre for Genetic Engineering and Biotechnology (ICGEB), Aruna Asaf Ali Marg, New Delhi, Delhi 110067, India

Jawaharlal Nehru University, New Delhi, Delhi 110067, India

K. Arora • A. Sajid
Tuberculosis Research Section, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda 20892, MD, USA

P. Talwar • G. Grewal • R. Kukreti (✉)
CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India
e-mail: ritus@igib.res.in

R. Raj
Molecular Biophysics Unit, Indian Institute of Science, Bangalore, Karnataka 560012, India

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pharmacogenomics, therapeutic antibodies, cell-to-cell communication, exosomes, structuromics, and posttranslational modifications.

1 Pharmacogenomics

1.1 Introduction

The completion of human genome project in 2003 followed by emergence of high-throughput sequencing and genotyping technologies has led to the initiation of a new era in the research field with central focus on genomics. This has changed the way of looking at conventional study approaches leading to merging of different domains, which resulted in the opening of new research frontiers such as pharmacogenomics, network medicine, and systems pharmacology to name a few. In this section, we aim to provide a broad overview of the pharmacogenomics, including major developments that have occurred in this field and finally highlighting the significant achievements and future perspective.

Pharmacogenomics (PGx) and pharmacogenetics (PGt) involve individual's genetic signatures to study drug response and/or drug toxicity behavior often referred to as phenotype. PGx differs from PGt in terms of the involvement of entire genome instead of few selected genes with no prior hypothesis. Genetic signature refers to the markers such as single-nucleotide polymorphisms (SNPs) and copy number variations (CNVs). Drug response phenotype implies to individual who responds well to the treatment (the good responder) and to one who doesn't (the poor responder) based on drug metabolism profile categorized into extensive metabolizer, intermediate metabolizer, and poor metabolizer. Individuals with extensive-metabolizer phenotype excrete the drug from the body completely earlier than expected, failing to achieve its desired therapeutic effect. These individuals require higher doses than the normal individuals which are basically intermediate metabolizers. Poor metabolizers accumulate the drug in the body and may experience adverse drug reactions (ADR). Some people also show drug toxicity due to hypersensitivity reactions that may also be due to the individual specific genetic signatures such as genes in the human leukocyte antigen (HLA) system. So, these individuals may require a lower dose or switch to another drug.

Several factors affect the outcome of a PGx/PGt study including ethnicity, disease (brain disorders-serum drug levels are not useful), genetics (host susceptibility genes) and environment (diet, smoking, alcohol); and therefore these are considered an important part of study design based on the study hypothesis. The steps involved in a PGx/PGt study are depicted in Fig. 1.

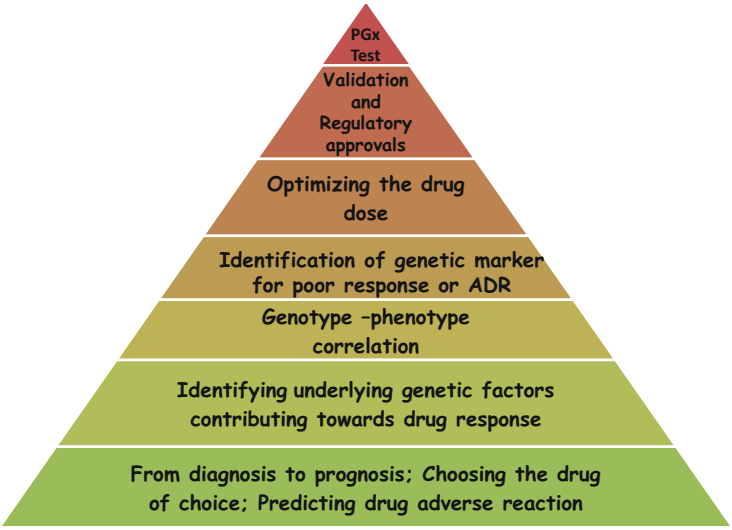

Fig. 1 Steps toward personalized medicine: To date around 136 FDA-approved drugs are available with pharmacogenetics information in their labeling

1.2 Role of Pharmacogenomics in Drug Resistance

The ultimate goal of pharmacogenomics is the development of optimized drug therapy based on the genetic makeup of an individual with maximum efficacy. The drug effects are determined by number of drug-metabolizing genes categorized into three groups—phase I (functionalization by cytochrome P450 superfamily), phase II (conjugation by conjugating enzymes such as sulfotransferases and UDP-glucuronosyltransferases), and phase III (excretion by drug transporters). The genetic variation in these genes may lead to differential response to drug treatment which can have important clinical implications. Drug transporters (phase III) have pivotal role in regulating the absorption, distribution, and excretion of many medications. There are number of studies where inherited variation in these transporters has been associated with differential response in different diseases and development of drug resistance in many diseases. Few such studies are discussed below.

1.2.1 Tuberculosis

Rifampicin is a first-line drug used to treat tuberculosis. Weiner et al. conducted a pharmacokinetics study in pulmonary tuberculosis patients from Africa, North America, and Spain compared with North American healthy controls to investigate the reasons for the interindividual variations in rifampicin levels. The study found that polymorphisms in the *SLCO1B1* gene (encodes drug transporter OATP1B1—

organic anion transporter peptide) had a significant influence on rifampicin exposure. Approximately 36 % lower exposure of rifampicin was found in patients with *SLCO1B1* 463 CA genotypes than CC genotypes (Weiner et al. 2010).

1.2.2 Epilepsy

There are number of studies in epilepsy where poor or no response to antiepileptic drugs (AEDs) is associated with variation in drug transporters. Siddiqui et al. investigated the role of genetic variation in *ABCB1* (ATP-binding cassette subfamily, B1), encoding P-glycoprotein (P-gp) for multidrug resistance in epilepsy (Siddiqui et al. 2003). *ABCB1* can efflux AEDs out of the cells. They studied *ABCB1* C3435T variant (rs1045642) in 200 patients with drug-resistant epilepsy, 115 patients with drug-responsive epilepsy, and 200 controls, all of European ancestry. It was found that multidrug-resistant epilepsy patients were more likely to have the CC genotype than the TT genotype (Siddiqui et al. 2003).

1.2.3 HIV

The pharmacokinetics profile of antiretroviral drugs used in HIV therapy is also dependent on genetic differences in drug transporters. Anderson et al. conducted a study to understand the relationship of antiretroviral drug pharmacokinetics and pharmacodynamics with polymorphisms in drug-metabolizing genes. They observed that "indinavir" oral clearance was 24 % faster in individuals having multidrug resistance-associated protein 2 (MRP2)-24C/T variant which can interfere with drug efficacy as indinavir is a substrate of MRP2 and thus can limit drug absorption and accelerate drug clearance (Anderson et al. 2006).

1.3 Role of Pharmacogenomics in Drug Discovery

The information from PGx can help in drug discovery and development. The example of Herceptin (trastuzumab), a humanized monoclonal antibody against ErbB2 receptor used in breast cancer treatment (Vogel and Franco 2003; Noble et al. 2004), explains how well PGt tests can help in progressing drug discovery and development. It has been observed that more positive response was observed in patients with tumors overexpressing ErbB2. With PGt testing, individuals having overexpression of ErB2 and appropriate for Herceptin treatment can be identified (McCarthy et al. 2005). Similar approach can be used with drug transporters. One approach can be the use of specific inhibitors against transporters to stop efflux of drugs to overcome drug resistance in subgroup where overexpression of specific transporter is observed. The other approach can be identifying the molecular players like nuclear receptors regulating expression of transporters as their genetic

variability is responsible for nonresponsiveness to drugs and drugs can be designed against those molecular players to achieve therapeutic efficacy. More research in PGx and development of specific technologies can really help in progress of medicinal sciences.

2 Therapeutic Antibodies for Bacterial Diseases

2.1 The Growing Problem of Antimicrobial Resistance

Antibiotics are the most commonly prescribed class of therapeutic agents both prophylactically and therapeutically. However, the use of antibiotics is not just limited to clinical settings. In an attempt to make food safer to eat, antibiotics have been introduced in poultry and dairy farms. In addition, several antibiotics are also being exploited in water sources for irrigation to prevent crop from diseases. This indiscriminate use of antibiotics has contributed heavily to the spread of antimicrobial resistance. Resistance to most antibiotics has been observed within 4–7 years of their introduction (Clatworthy et al. 2007). This has led to reemergence of infectious diseases, which had previously been effectively controlled by chemotherapy. This has in turn prompted surveillance programs by the World Health Organization (WHO) and Centers of Disease Control and Prevention (CDC) and European Center for Disease Prevention and Control (ECDC) to monitor the rise and spread of antibiotic resistance (http://www.who.int/drugresistance/global_action_plan/en/, http://www.cdc.gov/drugresistance/cdc_role.html, http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/database.aspx).

According to current estimates by the CDC, there were two million cases of infections with 23,000 deaths from antibiotic-resistant organisms every year in the United States alone (2013 Threat Report, CDC http://www.cdc.gov/drugresistance/threat-report-2013/index.html). Several bacterial and fungal pathogens have made a comeback and have once again become an unmet clinical need of infectious disease (Fauci and Morens 2012). The ESKAPE pathogens, namely, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *and Enterobacter* species, are posing a serious challenge in the nosocomial environment and are rapidly becoming pan resistant to all known classes of antibiotics used against them (Rice 2008).

2.2 The Need for New Therapeutic Interventions

Owing to the growing problem of antimicrobial resistance, there is an urgent need for development of new therapeutic agents against infectious pathogens. Antimicrobial resistance has surfaced against all known classes of drugs including colistin

which has been considered the last-resort antibiotic against several multidrug-resistant pathogens (Liu et al. 2016). With the exhaustion of the clinically useful antibiotic repertoire, attention is now being focused on other biologics that can serve to incapacitate pathogens. The post-antibiotic era has begun, and with that drug discovery programs are shifting focus toward large-molecule therapies which are based on an understanding of the host's own immune defenses. Antibodies are one such class of agents that have historic precedence for use in successful treatment of certain infections. In the pre-antibiotic era, as early as the 1890s, antibodies were being used for the treatment of select infections, but with the discovery of antibiotics, antibody therapies took a backseat. As a result, their potential was neither fully realized nor exploited.

2.3 The Foundations of Antibody Therapy

The discovery of passive antibody immunotherapy for infectious diseases is credited to Emil von Behring and Shibasaburo Kitasato. They were the first to show that serum from guinea pigs infected with a sublethal dose of *Corynebacterium diphtheriae* could be used to protect healthy guinea pigs from a lethal challenge of the organism. Similar protection was also shown by them against another bacterial pathogen, *Clostridium tetani* (Von Behring and Kitasato 1965, 1991). For this discovery in 1901, von Behring secured the first physiology/medicine Nobel Prize. As is evident, the protection was afforded by antibodies directed against the diphtheria or tetanus toxins. At the same time, Paul Ehrlich made his contribution to the field by standardizing the therapeutic dose by titrating the antiserum in infected animals and establishing methods for generation of high-titer antiserum using large animals such as horses (Bosch and Rosich 2008). This was an important advancement in dose determination for immune-based therapies for which he was awarded the Nobel Prize in 1908 (along with Élie Metchnikoff for his discovery of phagocytosis). In the United States, the mass production of diphtheria antiserum for therapeutic use began in 1895 by the H. K. Mulford Company in Glenolden, Philadelphia (later Merck Sharp & Dohme Corp. in 1929). Subsequently, antiserum was successfully used in the treatment of some bacterial diseases such as diphtheria, tetanus, pneumococcal pneumonia, and meningitis (Casadevall 1996). These early advances not only helped lay the foundations of vaccinology but also established the therapeutic potential of antibodies. However, despite these early achievements, interest in serum-based therapies rapidly declined in the 1930s. The reasons for this decline included the problem of serum sickness (due to the presence of foreign animal proteins), unpredictable therapeutic outcomes (which was due to instability of preparations, differences in batches, and a lack of understanding of disease stage at the time of administration), and most importantly perhaps the rise of antibiotics. While antibodies continued to be exploited for vaccine discovery, their application to treatment of infections quickly decreased. After the discovery of penicillin by Alexander Fleming in 1928,

antibiotics quickly became the magic bullets for treatment of life-threatening and nonlife-threatening microbial infections.

2.4 Antibody Structure

The typical immunoglobulin G (IgG) molecule is a Y-shaped molecule which has two longer heavy chains each of which is in turn linked to two shorter light chains by disulfide bonds. The heavy chains are also linked to each other by disulfide bonds. The light chains and their associated heavy-chain regions which form the fork of the "Y," known as the Fab fragment, are involved in antigen binding. The stem of the "Y" which is composed of the two heavy chains is known as the Fc portion and is involved in binding to receptors on the surface of phagocytic cells. The Fab and Fc fragments are separated from each other by a hinge region which allows flexibility of movement to the Fab fragment. Further, the heavy and light chains are divided into distinct domains known as the variable and constant regions. Each heavy chain has three constant regions (C_H1, C_H2, and C_H3) and one variable (V_H) region. Each light chain has one constant (C_L) and one variable (V_L) region. Each Fab fragment is therefore composed of the V_H and V_L regions (which form the V region) and the C_H1 and C_L regions (which form the C region). The amino acid residues in the variable region define the specificity of the Ab. The V region of the Fab fragment which makes contact with the antigen is known as the paratope, and it is complementary to the residues on the antigen which form the antigen's epitope (Putnam et al. 1979; Litman et al. 1993; Mattu et al. 1998; Maverakis et al. 2015).

2.5 Types of Therapeutic Antibody Molecules

Therapeutic antibodies come in many flavors (Chames et al. 2009). From the conventional polyclonal sera to antibodies with multiple specificities and unconventional structure, all forms are currently being explored in therapy (Fig. 2).

1. While polyclonal antibodies are not as common in development anymore, *monoclonal* antibodies are still at the forefront of antibody therapies.
2. *Chimeric* antibodies combine the portions of mouse and human antibodies where the human portion makes up to 70 %, allowing better specificity of the Fc region and reduced toxicity.
3. Further advancement of technology has made possible the generation of *humanized* antibodies which are 85–50 % human, and these therefore have even better safety profiles.
4. Antibody fragments comprising solely of the VL and VH domains, termed *scFv* (single-chain variable fragment), are used to provide specificity in the absence of Fc receptor-mediated opsonophagocytosis.

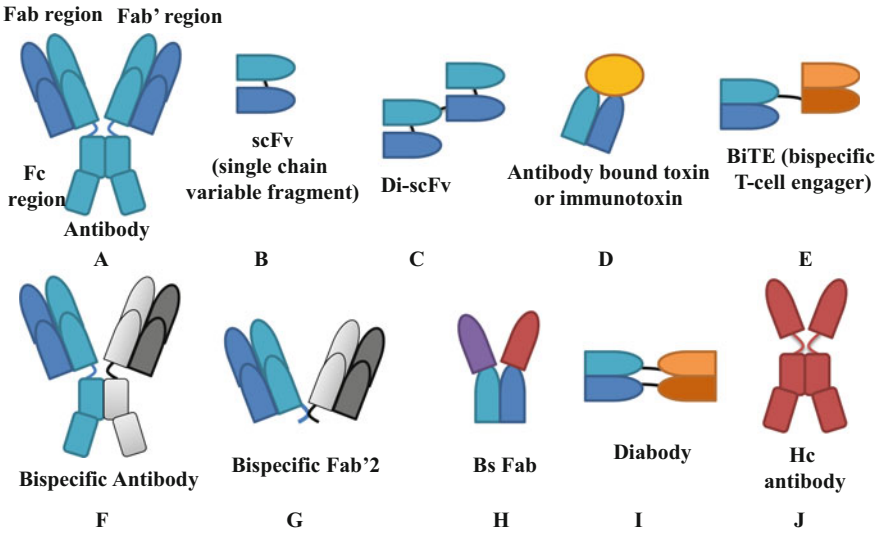

Fig. 2 Therapeutic antibody structural representation: (**a**) basic antibody structure, (**b**) single-chain variable fragment, (**c**) dimer of single-chain variable fragment, (**d**) toxin/therapeutic compound bound to single-chain variable fragment, (**e**) bispecific T-cell engager, (**f**) bispecific antibody, (**g**) bispecific F(ab)2 region, (**h**) bispecific Fab region, (**i**) diabody, and (**j**) heavy-chain only antibody

5. Two scFv linked together with a very short linker make a structure known as the *diabody*.

2.6 Clinical Pipeline

The first monoclonal antibody to be approved for clinical use against an infectious agent was Palivizumab/Synagis for respiratory syncytial virus (RSV) in 1998. Palivizumab has been developed by MedImmune against the F protein of RSV (Johnson et al. 1997), for treatment of severe respiratory infection in high-risk infants. In recent years, antibody therapies have been in development against many infectious pathogens. Antibody therapies are being developed to enable pathogen clearance by diverse approaches.

2.6.1 Neutralization of Viruses and Toxins

Direct neutralization by formation of antigen-antibody complexes which can be cleared by the immune system.

2.6.2 Opsonophagocytic Killing

Antibodies specific for proteins on the pathogen's surface. The Fc region is in turn able to bind the Fc receptors on surface of phagocytic cells. This allows the phagocyte to directly engulf and mediate killing of the pathogen.

2.6.3 Complement Activation

Binding of antibodies can also lead to complement activation which in turn can lead to pathogen lysis by formation of the membrane attack complex (particularly in Gram-negative bacteria and fungi).

2.6.4 Antibody-Drug Conjugates

Antibody-drug conjugate is a novel class of biologics where an antibody provides a means of delivery of a small-molecule chemotherapeutic agent.

There are several candidates currently in clinical development that are based on one or more of the abovementioned approaches (ter Meulen 2007, 2011; Bebbington and Yarranton 2008; Nagy et al. 2008; Saylor et al. 2009). These therapies are frequently directed against surface structures of the pathogens which are often critical for attachment, toxin injection, and immune evasion. Also, many candidates in development are directed against bacterial toxins which continue to cause injury to the host even after elimination of the pathogen. Some of them are discussed below:

Bacillus One such target is the protective antigen (PA) of *Bacillus anthracis*. PA is a pore-forming protein of *B. anthracis* which heptamerizes, forming a pore in the endosomal membrane through which it delivers lethal factor (LF) and edema factor (EF) into the cell cytosol of host. All the antibodies in development against *B. anthracis* PA act by blocking the binding of PA to its receptor. Raxibacumab (Human Genome Sciences, NCT02016963), AVP-21D9 (Emergent BioSolutions, NCT01202695), Anthim (Elusys Therapeutics, NCT01453907), and Valortim (PharmAthene, NCT00964561) are antibodies in development for anthrax. Raxibacumab has recently been approved for treatment of inhalation anthrax in combination with antibiotics (Tsai and Morris 2015).

Clostridium Toxin neutralization is also an effective strategy for *Clostridium botulinum*. There are seven toxins elaborated by *C. botulinum* BoNT/A-G. Of these BoNT/A is the most devastating toxin causing paralysis. A mixture of three IgG monoclonal antibodies with humanized regions, XOMA 3AB, is currently under development against BoNT/A (Nayak et al. 2014) (NCT01357213). The antibody targets different regions of the BoNT/A toxin. *Clostridium difficile*, which causes pseudomembranous colitis or diarrhea associated with antibiotics,

exerts its effects by production of toxins A (TcdA) and B (TcdB), which mediate damage to epithelial cells (Voth and Ballard 2005). A combination of human monoclonal antibodies to TcdA (GS-CDA1) and TcdB (MDX-1388) is currently in development by Medarex (NCT00350298). Similarly, Merck Sharp & Dohme Corp. is also developing actoxumab-bezlotoxumab (a mixture of monoclonal antibodies to TcdA and TcdB which has completed phase III clinical trial (NCT01513239, Yang et al. 2015)).

Staphylococcus *Staphylococcus aureus* produces many surface-associated as well as secreted virulence factors and toxins (such as proteases, adhesins, superantigens, leukocidins, autolysins) that serve as good targets for antibody therapy (Sause et al. 2016). Altastaph is a polyclonal IgG preparation directed against capsular polysaccharides 5 and 8 which has completed phase II trial (NCT00063089). Another polyclonal antibody in development (from Bristol-Myers Squibb) is Veronate which is directed against fibrinogen-binding protein, clumping factor A (ClfA) (DeJonge et al. 2007), but it failed to show any significant protection in the phase III trial (NCT00113191). Similarly a monoclonal Ab, Aurexis/tefibazumab (also developed by Bristol-Myers Squibb) directed against *ClfA* and aimed to be used in combination with antibiotic therapy has also completed phase IIa dose-escalation study (NCT00198289). Pagibaximab (Biosynexus Inc.) is a monoclonal antibody directed against lipoteichoic acids of staphylococcal cell wall and is in development for sepsis in neonates (NCT00646399). Aurograb (NeuTec Pharma.) has completed phase III trial for use in combination with vancomycin for treatment of methicillin-resistant *S. aureus* infections. Sanofi is developing SAR279356 (NCT01389700) a monoclonal antibody against the surface polysaccharide poly-N-acetylated glucosamine (PNAG) (Kelly-Quintos et al. 2006).

Escherichia coli Chimeric monoclonal antibodies to Shiga toxins 1 and 2 (Stx 1 and 2) are in development by Thallion Pharmaceuticals (NCT01252199) against bacteria that produce Shiga toxin such as "Shiga toxin-producing *E. coli* (STEC)." This organism can cause hemolytic uremic syndrome and acute renal failure.

Pseudomonas *Pseudomonas aeruginosa* is one of the most recalcitrant nosocomial pathogens. *Pseudomonas* pathogenesis relies on production of cytotoxins, surface adhesion molecules, and biofilms. Panobacumab (also known as KBPA-101 and Aerumab11) is a pentameric *IgM* monoclonal antibody targeting the O11 lipopolysaccharide of *P. aeruginosa* which is being developed by Kenta Biotech Ltd. and has completed phase IIa safety and pharmacokinetics trial (NCT00851435, Lazar et al. 2009; Que et al. 2014). *Pseudomonas* infections are particularly problematic in cystic fibrosis patients. KB001-A is a humanized monoclonal antibody targeting the pcrV structural component of the *Pseudomonas* type III secretion system, essential for translocation of *Pseudomonas* cytotoxins, and is being developed by KaloBios Pharmaceuticals (NCT01695343) for treatment of cystic fibrosis patients. MedImmune is developing MEDI3902 which is a bispecific antibody against *P. aeruginosa*. The antibody simultaneously targets the PcrV protein as well as Psl (a capsular lipopolysaccharide) and has shown promise for use independently as

well as an adjunct to existing chemotherapeutic regimens (DiGiandomenico et al. 2014). It has recently completed a phase I clinical trial (NCT02255760).

2.7 Advantages and Disadvantages of Antibody Therapy

Antibody therapeutics offer several advantages over antibiotic therapy. First and foremost, antibodies have a good safety profile (especially monoclonals) and hence are better tolerated. Owing to their specificity, they suffer less from off-target and nonspecific effects. Also due to their specificity, antibodies do not have adverse effects on normal microflora of the body. However, the extreme specificity of antibodies makes them very narrow-spectrum therapeutics which in turn makes them less attractive for pharmaceutical companies. Nevertheless, antibodies have multifunctional capabilities. They don't just exert their effects by directly blocking epitopes but also enhance immune function by mediating opsonization, agglutination, complement activation, and engagement of toxic cells by antibody-dependent cellular cytotoxicity (ADCC). Thus, they not only block pathogen attack but also aid in pathogen clearance from tissue spaces and bloodstream. When used in combination with antibiotics, they can work in conjunction with curtail pathogens and in some instances have even been shown to work synergistically to enhance the efficiency of antibiotics to which resistance has developed (DiGiandomenico et al. 2014). Another important advantage of antibodies is that they can be used not only therapeutically but also prophylactically.

Antibody therapies do suffer from several challenges as well. Besides being extremely narrow in their spectrum, they also pose the risk of being rendered ineffective if epitopes that they are directed against undergo a change. Bacterial and viral pathogens are known to show antigenic variation which can make antibodies ineffective. However in such a case, the paratope on the antibody may be modified to allow targeting of the new epitope. Further, since these antibodies are proteins, there is a chance that the immune system could mount a response against the antibody itself. Such anti-idiotypic antibodies can neutralize the therapeutic molecule, and they have been documented in treatment with therapeutic antibodies at least in case of rheumatoid arthritis (Isaacs et al. 1992). Additionally, certain types of antigens are only elaborated by select strains of the pathogen making such antigens less attractive for clinical development. Knowledge of the antigenic epitope is also crucial for developing an antibody therapeutic.

3 Development of Drug Resistance Through Inter- and Intracellular Communication

Cell-to-cell communication is the key to cellular adaptation and survival. Different types of cells have developed ways to communicate with each other. Initially, the intercellular communication was believed to be maintained by soluble paracrine and endocrine factors and by physical cell-to-cell contacts. The first of such contacts discovered were cell-cell synapses, gap junctions, plasmodesmata, and cellular projections (Abounit and Zurzolo 2012). In eukaryotes, one of these ways is through formation of nanotubular networks that provide valuable communicative means. Tunneling nanotubes, the long extensions of membrane between two cells placed distantly, and their networks were first discovered in plant and animal cells (Tarakanov and Goncharova 2009). In 2011, Dubey et al. showed how bacteria communicate with each other by nanotubes (Dubey and Ben-Yehuda 2011). Interestingly, bacteria can form nanotubes not only between same species but between different species as well (Dubey and Ben-Yehuda 2011; Pande et al. 2015). These nanotubes have been shown to be involved in cargo transport and antibiotic resistance (Dubey and Ben-Yehuda 2011; Pande et al. 2015). It is not clear if these bacterial nanotubes are just a communication mechanism or a survival strategy to counteract drug pressure.

Further, the molecular mechanisms of nanotube formation are still unknown. The study by Pande et al. has shown that bacteria form nanotubes during stress conditions such as nutrient starvation (Pande et al. 2015). These bacterial nanotubes reflect complex social behavior as bacteria from different species can form nanotubes to communicate (Dubey and Ben-Yehuda 2011; Pande et al. 2015). Hence, such nanotube formation may be a mechanism to exchange nutrients and other metabolites in bacterial communities such as biofilms. Biofilm-forming bacteria are known to survive drug pressure, and it is possible that in community some bacteria transfer metabolites to protect others from drug-induced pressure. The fitness advantage can answer how some bacteria survive drug pressure and acquire antibiotic resistance by staying metabolically quiescent for very long time. Further, mechanism of this vectorial transport also needs to be elaborated. One of the ways to maintain directionality is quorum sensing, the other could be electrical coupling. Also, concentration-dependent gradient between the two bacteria may help in determining the direction of flow.

Structurally, bacterial nanotubes resemble vectorial structure. Temperature may affect membrane motility, permeability, and membrane potential that in turn may affect nanotube composition, structure, and number. Another mode of intracellular communication is synapse, a link between nerve cells that are separated by a small gap across which neurotransmitters can be diffused through to generate an impulse. Synapse has also been proposed in plants and between immune and target cells, long after the corresponding notion of neuronal synapse in animals. Tunneling nanotubes have been shown to regulate neuronal synapses. These F-actin-based structures are involved in organelle transfer and electrical coupling between cells

(Wang and Gerdes 2015). These nanotubes may help drug-sensitive cancer cell to connect with drug-resistant mutant cells and survive drug pressure (Wang and Gerdes 2015). Although tunneling nanotubes are absent in bacteria, similar structural forms are involved in antibiotic resistance indicating divergent evolution.

Nanotubes have been shown to transport proteins, plasmids, and other macromolecules between the cells. This mechanism may confer high levels of resistance markers being transferred between the cells. Consequently, nanotubular structures will be an important target for designing new drugs to overcome development of drug resistance. These drugs can be given in addition to other drugs that kill the target cells. Thus, combination therapies can be developed targeting bacterial physiology as well as nanotube formation. These therapies would be sensitive as well as specific for a given disease, considering the fact that nanotubular structures are specific for a given cell type.

4 Exosome-Mediated Drug Resistance

4.1 Extracellular Vesicles as Carriers of Drug-Resistant Traits Across Pathologies

Drugs impart an evolutionary selection pressure on the target cells. The phenomenon of transmission of drug resistance across population involves multiple mechanisms and helps in selecting for resistant cells from heterogeneous population. One recently discovered mechanism that has garnered much attention is the transfer of these traits by means of secreted vesicles, also termed as extracellular vesicles (EVs) (Yanez-Mo et al. 2015). These vesicles are further subclassified depending upon their size and density as either large oncosomes derived from cancerous cells (ranging in size from 1 μm to 10 μm), microvesicles (100 nm to 1 μm), and exosomes (30–100 nm) (Minciacchi et al. 2015). While large oncosomes and microvesicles are the recent focus of attention, exosomes have long been studied, and sufficient evidence exist that implicate their role in pathologies (Robbins and Morelli 2014; Braun and Moeller 2015; Campos et al. 2015; Coleman and Hill 2015; Fujita et al. 2015; Mahmoudi et al. 2015; Schorey et al. 2015).

4.2 Vesicle-Mediated Drug Resistance in Noncommunicable Disorders

A major impediment in managing the epidemic of noncommunicable disorders (NCDs) is the emergence of resistance to the existing line of drugs. Drug resistance is a phenomenon that comes across disease boundaries including emergence of steroid-resistant asthma (Luhadia 2014), insulin resistance leading to non-insulin-

dependent diabetes mellitus, obesity, hypertension, dyslipidemia, atherosclerosis (DeFronzo 1997), and most importantly chemoresistance in cancers (Singh and Settleman 2010). While the direct role for exosomes has not been established for other conditions, their role in cancer drug resistance has been documented in several studies. Acquired and de novo resistance to existing therapies including chemotherapy, radiation therapy, and other targeted therapies has become a huge concern in the treatment of cancer (Meads et al. 2009), and exosomes have been implicated in several of these mechanisms (Azmi et al. 2013).

Radiation therapy employs high-energy waves, such as X-rays, electron beams, gamma rays, or protons for eliminating cancer cells, and theoretically this presents an attractive therapeutic choice; however, few cancerous cells were found to survive these high-energy radiations, the detailed cause for which is still being investigated. It has emerged recently that one of the mechanisms by which cancerous cells counters radiations is by secreting "survivin" which is a member of the inhibitor of apoptosis protein (IAP) family in exosomes (Khan et al. 2009). This creates a microenvironment that promotes further metastasis and drug resistance. Cancer cells have been shown to use exosomal pathway to physically efflux drugs, cisplatin, and doxorubicin that are one of the most widely used drugs (Shedden et al. 2003; Safaei et al. 2005). Similarly, in a breast cancer cell line that expresses Her2, the resistance to trastuzumab (monoclonal antibody-targeting Her2 receptor) arises due to exosomes that overexpress and secrete Her2. These exosomes are released by cancer-associated fibroblasts that increase cancer stem cells and induce several anti-apoptotic pathways (Ciravolo et al. 2012). Exosomes transferred from stroma to breast cancer cells lead to resistance against chemotherapy and radiation through antiviral and NOTCH3 pathways (Boelens et al. 2014). Tumor microenvironment has classically been associated with chronic hypoxia that can affect the DNA damage repair pathways and thereby induce DNA replication errors. This leads to genetic instability contributing to radiation resistance (Bristow and Hill 2008). Horizontal transfer of miRNAs and phosphorylated glycoproteins by drug-resistant breast cancer cell lines has also been suggested to be a novel mechanism of transmission of chemoresistance (Chen et al. 2014). Substantiating this principle, it was found that exosomes from docetaxel (DOC /exo)-resistant MCF-7 breast cancer cells can confer drug resistance in drug-sensitive variant MCF-7 cell line (MCF-7/S).

Exosomes also play a role in thwarting body's own defense mechanisms against cancerous cells as was demonstrated in several studies. It was reported that cancerous cells avoid complement-mediated lysis by exosomal secretion of protein mortalin (Pilzer and Fishelson 2005; Pilzer et al. 2005). Another study reported that TNF-α, which is secreted in association with exosomes, prevents cell death induced by activation of cytotoxic T cells (Zhang et al. 2006). Separately, lymphoma exosomes were found to protect target cells from attack of antibodies by releasing CD20 (Aung et al. 2011).

Exosomes carry several proteins that are unique to cancer cells. While some promote drug resistance, others have been used for cancer detection as biomarkers; for instance, exosome-associated glypican-1 (GPC-1) has been shown to sensitively

differentiate between healthy individuals and patients with pancreatic cancer (Melo et al. 2015). Apart from this exosome, encapsulated DNA has also been used for cancer detection as well as to determine mutational status of parental tumor cells (Skog et al. 2008; Guescini et al. 2010; Thakur et al. 2014). Thus, exosomes play multifaceted roles in several noncommunicable disorders.

4.3 Vesicle-Mediated Drug Resistance in Pathogenic Disorders

Most human pathogens are bacterial species that lack typical machinery required for exosome production; however, they are known to produce outer membrane vesicles (OMVs) that bud from their plasma membranes and are known to carry proteins, phospholipids, lipopolysaccharides, and nucleic acids. OMVs of bacteria play a variety of roles on extracellular activities within intra- and interspecies microbes. Experimental data suggests that these vesicles often act as bacterial virulence factor and play significant role in pathogenesis. They've also been implicated in transmission of drug resistance by bacterial species such as *S. aureus* and *P. aeruginosa* to entire polymicrobial community by transfer of vesicle enclosed β-lactamase enzyme that allows recipient gram-positive and gram-negative bacteria to survive in the presence of antibiotic penicillin (Ciofu et al. 2000; Lee et al. 2013).

Transfer of plasmid containing genes for antibiotic resistance has been a classic route by which drug resistance can rapidly spread across population. Several of the gram-negative, but not gram-positive, eubacteria-derived vesicles were shown to carry nuclease-protected linear or supercoiled DNAs (Dorward and Garon 1990). In a laboratory demonstration of similar phenomenon in malaria parasite *Plasmodium falciparum*, Neta Regev-Rudzki et al. demonstrated that strains that were separately transfected with plasmids conferring resistance to WR99210 or blasticidin S upon co-culture allowed parasite to grow in the presence of both the drugs, while culturing them separately didn't allowed growth of parasite (Regev-Rudzki et al. 2013).

Though much is known about the role of extracellular vesicles from bacteria and fungi in pathogenesis, the details of the role played by them in drug resistance is still an active area of research. Future research in extracellular vesicles is expected to shed new lights on the mechanisms of emergence and transmission of drug resistance across various physiological and pathological processes.

5 Structuromics

Transcription is one of the most important and basic steps for a cellular system. During transcription, RNA is formed using DNA as a template along with RNA polymerase and different kinds of modulator proteins. After transcription, primary transcript forms and additional modifications make mature RNAs, which are subsequently used for their respective functions. Mature RNA molecules retain some conserved sequences responsible for the formation of signature secondary structures for the interaction with different set of proteins. These secondary structures remain the same at a given time in the cell. But, these structures may change when RNA interacts with different set of proteins to maintain the physiology of the cell. Level of a given protein varies with time and requirement. It is intriguing to understand how the protein concentration varies for a given protein with time, keeping the sequence and hence the signature secondary structures the same. It suggests that RNA sequence or secondary structure is being modulated in response to specific signal. Thus, variations in different protein concentrations keep the cells alive and healthy in temporal environment (biotic and abiotic stress).

A highly informative concept came as "structuromics" by Howard Y. Chang's group, which gave the comprehensive analysis of in vivo RNA secondary structure. The approach which they named "in vivo click selective 2′-hydroxyl acylation and profiling experiment (icSHAPE)" utilizes biochemical modifications of unpaired RNA bases and determines the structural dynamics of whole-cell RNA modules (Spitale et al. 2015). Understanding of this machinery will provide information such as protein-binding sites within specific secondary structures.

5.1 Role of Structuromics in Deciphering the Drug Resistance Mechanism

Exploring the structure of RNAs and analyzing their interaction with protein molecules in vivo will shed light on intracellular regulatory networks. These mechanisms resemble hidden weapons of bacteria and they are activated by the cell when needed. If we know the way and time of their activation, we can target those modules by making them nonfunctional. For example, RNAIII of *S. aureus*, which is 514 nucleotides long RNA molecule, regulates many genes encoding exportins and cell wall-associated proteins (Waters and Storz 2009). RNAIII interacts with different sets of proteins with its 5′ and 3′ domains and regulate their expression. It also controls expression of many virulence factors; these factors keep them viable and give strength to fight against host (Morfeldt et al. 1995; Boisset et al. 2007; Novick and Geisinger 2008; Chevalier et al. 2010; Liu et al. 2011). Targeting the modules formed by RNAIII under specific conditions will help in reducing the virulence of *S. aureus*. Though most of these structures arise during infection of the host and, hence, are difficult to target technically. In such cases,

RNA structuromics might play a significant role, giving the vision of RNAIII complexes.

5.2 Role of Structuromics in Drug Designing

Till now, siRNA-mediated drugs have been used for some of the diseases like age-related macular degeneration (AMD), pachyonychia congenita (PC, a rare form of hereditary keratoderma that can affect the skin, mouth, hair, and eyes), and coronary artery disease and are also being used in HIV (Rossi 2006; Kleinman et al. 2008; Smith et al. 2008). Deciphering the structure of RNA-protein complexes (for some specific cases) either by structuromics or by cryo-electron microscopy will explore the mechanism of action of specific targets of siRNA molecules. This may play a crucial role in understanding the possible mechanisms of cells becoming resistant to an siRNA-based drug molecule. Subsequently, with the available information, chemical modifications within an siRNA molecule might provide additional information in its target inhibition. For example, addition of a drug (e.g., cross-linker which will inhibit the dissociation of protein and RNA molecule at its specific time of action) with siRNA will improve its stability as well as its action. Thus, RNA structuromics is a revolutionary aspect of RNA-based drug discovery.

6 Posttranslationally Modified Protein Networks as Drug Targets

In cellular information transfer, the most important transducers are proteins that define vital nature of the cell. For long, the central dogma of molecular information was thought to be DNA to RNA to protein. However, the translated proteins are often only the preprocessed forms which need further modifications to make them the elegant biological devices. The most common post-transnational modifications are phosphorylation, acetylation, glycosylation, and ubiquitinylation. Interestingly, these conserved modifications modify the protein activity, localization, and stability in both prokaryotes and eukaryotes. Therefore, these modifications and the enzymes responsible for carrying out these modifications are considered one of the most promising drug targets against many diseases such as cancer, malaria, and tuberculosis, among others. Specific posttranslationally modified (PTM) events are crucial in clinical manifestations and successful infections in most infectious disease. Therefore, targeting these modifications can be more effective than aiming a single drug target.

In *M. tuberculosis*, there are 11 Ser/Thr protein kinases, 2 Tyr phosphatases, and 1 Ser/Thr phosphatase. The genome also encodes at least one protein lysine acetyl

transferase and one deacetylase (Nambi et al. 2010, 2013; Singhal et al. 2015). These enzymes together modify at least 400 important proteins reversibly to help the bacteria handle the stress and adapt by physiological changes (Prisic et al. 2010; Singhal et al. 2015). Another related pathogen *M. ulcerans* that causes Buruli ulcer has a kinase present in pathogenicity-related plasmid pMUM001 (Arora et al. 2014). This kinase phosphorylates some other structural proteins, also coded by virulence-associated plasmid, and helps the pathogen in successful infection (Arora et al. 2014). Interestingly, while proteins such as PrkC or PknB are conserved in most gram positive and actinomycetes, there are some other kinases that are exclusive and possess dual specificity (Arora et al. 2012, 2013). Two such kinases present in *B. anthracis*: one is the first DYRK-like kinase of prokaryotes PrkD and another is Ser-/Thr-/Tyr-specific enzyme PrkG (Arora et al. 2012). *B. anthracis* has evolved for its pathogenic lifestyle needs, lost tyrosine kinases, and expressed dual specificity protein kinases that are important for growth and possibly pathogenicity of the organism (Arora et al. 2012). Interestingly, all Ser/Thr protein kinases, dual specificity protein kinases, Tyr kinases, and acetyltransferases are shown to modify and regulate key physiological pathway proteins such as glycolysis, protein translation, and one-carbon metabolism (Arora et al. 2010, 2012, 2013; Singhal et al. 2013, 2015; Maji et al. 2015; Pereira et al. 2015; Prisic et al. 2015; Sajid et al. 2015). Several of these modifications regulated the key metabolic events in overlapping manner. The network of such signaling events forms nodes that fine-tune cellular response and help in adaptation and thriving under different stress conditions. Therefore, identification and targeting such nodes will be key to novel drug discovery.

7 Conclusions

The human genome has 46 chromosomes and around 20,000–25,000 protein-coding genes. Interestingly, the total number of human diseases known is estimated to be 10,000–30,000. In the years before the discovery of antibiotics, the general perception was humankind will never be able to successfully combat the pathogens such as tuberculosis, malaria, and cholera. In the next hundred years, we have achieved an unparallel understanding of life at the molecular level. Medical science has provided limited but effective solutions to combat major diseases. However, drug-resistant disease forms have constantly challenged our success. The emergence of drug-resistant forms has resulted in prolonged illness and increased rate of death even for common infections. According to WHO estimate, people infected with drug-resistant form of MRSA are 64 % more likely to die than compared to nonresistant forms. To end our misery from palpable drug-resistant diseases, in the future we will have to adapt cooperative drug discovery strategies and benefit from emerging themes such as immunotherapy, pharmacogenomics, and structuromics to annihilate these enemies of mankind. It is a necessity to use new and powerful beacons that target different disease forms. To achieve this, discovery of effective

therapeutic agents that can provide protection against both acute and chronic forms along with better disease management is urgently required. With the experience of combating these diseases in last century, we must not rely on conserved drug target strategies and may have to develop multiple subordinate strategies each potent enough to strike pestilence effectively.

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