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A Review of In Silico and In Vitro Approaches in the Fight Against Carbapenem-Resistant Enterobacterales

Muhammad Absar[1] | Abdul Rahman Zaidah[1] | Amer Mahmood[2] | Sajjad Ahmad[3] | Hasan Ejaz[4] | Naveed Ahmed[1,5] | Nik Haszroel Hysham Nik Hashim[1,6] | Chan Yean Yean[1,6]

[1]Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia | [2]Department of Anatomy, Stem Cell Unit, King Saud University, Riyadh, Saudi Arabia | [3]Department of Health and Biological Sciences, Abasyn University, Peshawar, Pakistan | [4]Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Jouf University, Sakaka, Saudi Arabia | [5]Department of Assistance Medical Sciences, Applied College, University of Tabuk, Tabuk, Saudi Arabia | [6]Hospital Pakar Universiti Sains Malaysia, Kelantan, Malaysia

Correspondence: Nik Haszroel Hysham Nik Hashim (haszroel@usm.my) | Chan Yean Yean (ychan@usm.my)

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ABSTRACT

Objectives: The rise in carbapenem-resistant Enterobacterales (CRE) has reinforced the global quest for developing effective therapeutics. Traditional drug discovery approaches have been inadequate in overcoming this challenge due to their resource and time constraints.

Methods: English literature was searched by structured queries related to our review between January 1, 2020, and December 31, 2024.

Results: The key resistance mechanisms in CRE, such as enzymatic hydrolysis, decreased permeability, and efflux pump overexpression, have been examined in this review. Computational technologies have become pivotal in discovering novel antimicrobial agents with improved accuracy and efficiency. Besides this, the review highlights the advances in structure- and ligand-based drug discovery approaches for identifying potential drugs against CRE. Recent studies demonstrating the use of such in silico techniques to develop targeted drugs against CRE have also been explored. Moreover, this review also underscores the significance of integrating both in silico and in vitro techniques to counter resistance in Enterobacterales, supported by the latest studies. However, these promising computational technologies have a few major drawbacks, such as a lack of standardized parameterization, potentially false positives, and the complexity of effective clinical translations. The drug regulatory barriers also restrict the progress of new antimicrobials for market approval.

Conclusion: The use of computational technologies for antimicrobial inhibitor discovery is gaining popularity, and it can be expedited by refining computational techniques and integrating them with reliable in vitro validation. The use of innovative hybrid in silico and in vitro technologies is the need of the hour to tackle CRE and mitigate the global threat of antimicrobial resistance.

1 | Introduction

The order Enterobacterales contains the family Enterobacteriaceae, and with over 250 species is among the most taxonomically diverse groups of bacterial families. Most common bacterial pathogens in the family Enterobacteriaceae include *Escherichia coli*, *Shigella* spp., *Salmonella* spp., *Enterobacter* spp., and *Klebsiella pneumoniae* [1, 2]. These pathogens are key causative agents of severe healthcare-associated infections (HAIs) having very limited treatment options. The antibiotic abuse has led to the rise of resistance in the Enterobacterales [3]. Notably, the global spread of

Summary

- Explores advances in computational tools for antimicrobial drug discovery.
- Discusses the resistance mechanisms in carbapenem-resistant Enterobacterales.
- Integrates in silico and in vitro approaches for antimicrobial validation.
- Highlights limitations and prospects in combating antimicrobial resistance.
- Emphasizes artificial intelligence (AI) and machine learning (ML) advances for drug design and resistance prediction.

carbapenem-resistant Enterobacterales (CRE) has become a formidable public threat [4, 5]. The nosocomial infections such as urinary tract infections, septicemia, and pneumonia caused by CRE often lead to inpatient hospitalization and higher mortalities [6]. In light of this emerging threat, the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) have recognized CRE as among their top priorities for research and development of new antimicrobial therapeutics [7, 8].

Nonetheless, developing novel therapeutic agents against CRE is considerably arduous. These notorious pathogens have rapidly evolved various sophisticated resistance mechanisms that render the carbapenems (the most powerful class of β-lactam antibiotics) utterly ineffective against them [5]. Briefly, these carbapenem-neutralizing adaptations include enzymatic degradation, exclusion from entering the bacterial cell, alteration of the binding sites, omission or mutation of the porin proteins, and overstimulation of the efflux pump (EP), as well as modifications of the penicillin-binding proteins (PBPs) [9]. Adding to the complexity, these resistance traits are often on mobile parts of the bacterial genetic material, such as plasmids, that facilitate swift spread across the bacterial population, thus exacerbating the challenge of resistance [10]. The conventional approaches of drug discovery are laborious, costly, and often poorly address the rapid evolution of antimicrobial resistance (AMR) [11]. The failure to develop effective treatments for CRE prompts crucial questions regarding the type of drug discovery and development strategies required to restore healthcare systems.

Several in silico technologies, such as molecular modeling and machine learning, can expedite and facilitate the discovery of antimicrobials against CRE [12, 13]. The optimization of the probable drug candidates can also be performed through these techniques [14]. Once screened, the drug candidates can be subjected to various in vitro/in vivo tests to validate their cytotoxicity and efficacy [15, 16]. This review emphasizes the significance of computational technologies in developing as well as discovering antimicrobials against CRE. Furthermore, various latest research works that successfully implied combined in silico and in vitro techniques against CRE are also highlighted in this review. This study offers new opportunities for novel and innovative antimicrobial drug discovery using computational techniques, hence promoting strategies that can effectively address the global challenges of CRE as well as other pathogens.

1.1 | Literature Review

The academic databases that were utilized for obtaining data for this study included Web of Science, EMBASE, Scopus, ScienceDirect, Google Scholar, PubMed, and Cochrane Library. Structured queries were created for retrieving studies regarding carbapenem resistance mechanisms such as "carbapenem," "carbapenem resistance," "carbapenem resistance in Enterobacterales," "carbapenemases." Furthermore, the search terms used for curating data related to the application of in silico and in vitro technologies included but not limited to "computer aided drug discovery," "combined in-silico and in-vitro analysis," "antimicrobials for Enterobacterales," "drug discovery against CRE." The selected articles were published between January 1, 2020, and December 31, 2024. Articles published in languages other than English were strictly not entertained.

2 | Mechanisms of Carbapenem Resistance

Carbapenems are a clinically significant class of antibiotics, which have a broad spectrum of activity against several gram-positive and gram-negative pathogens including *Pseudomonas aeruginosa*, *Streptococci*, *Staphylococcus aureus*, *Acinetobacter* spp., and *Enterobacteriaceae* [17–19]. Carbapenems are somewhat like other β-lactam antibiotics apart from containing a 5-carbon ring fused with their β-lactam ring [20]. This peculiar structural dissimilarity makes carbapenems resistant to hydrolysis by common β-lactamases or even extended spectrum β-lactamases (ESBLs) [19]. Their mode of action involves inducing bacterial cell death by interacting with their PBPs and inhibiting cell wall synthesis [21, 22]. Imipenem, meropenem, ertapenem, and doripenem are four approved common clinical drugs. Carbapenems were previously favored as the last line of defense against infections which could not be treated by other antibiotics. However, the efficacy of carbapenems is now facing critical decline due to the emergence of resistance in Enterobacterales [23]. The carbapenem resistance is driven by the multifaceted mechanisms represented in Figure 1.

2.1 | Enzymatic Hydrolysis of Drugs

Enzymatic breakdown of antibiotics has been the most common resistance mechanism in bacterial pathogens. Originally, only hydrolyzing penicillin (by penicillinases), these enzymes have now significantly evolved over time. The carbapenem resistance in Enterobacterales directly arises from the production of carbapenemases [24–26]. In most members of the CRE, the resistance is solely conferred by these enzymes without requiring any other strategies. Carbapenemases are a subclass of β-lactamases having versatile hydrolytic potential [26, 27]. The genes encoding carbapenemases such as bla_{KPC}, bla_{NDM}, and bla_{VIM} are found on both bacterial plasmids and chromosomes

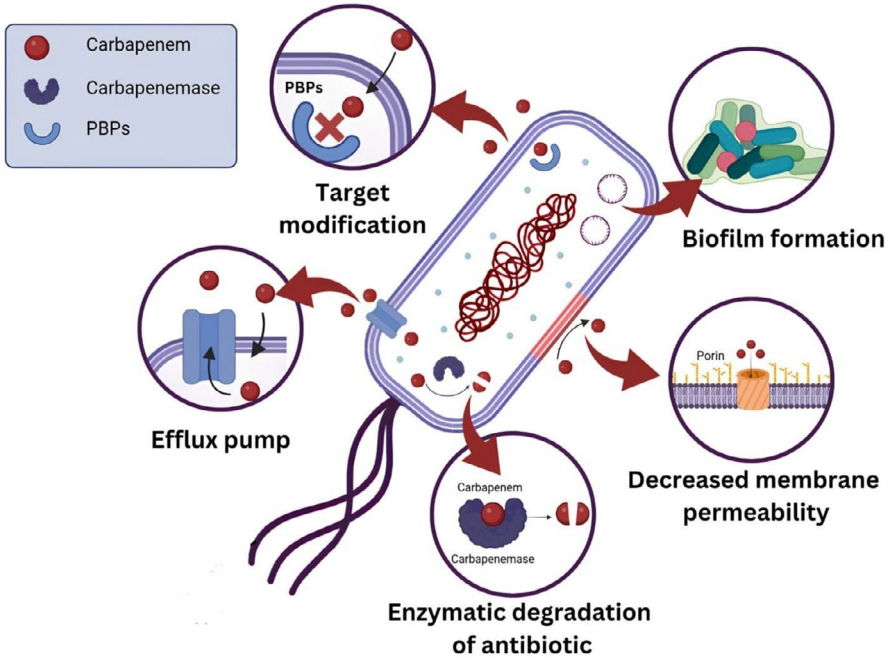

FIGURE 1 | Mechanisms of carbapenem resistance in Enterobacterales. PBPs, penicillin-binding proteins.

and can be disseminated to other pathogens by various mechanisms of horizontal gene transfer [25]. This plasmid-mediated spread of carbapenemases contributes to increasing resistance not only against carbapenems and cephalosporins but also against aminoglycosides and colistin [28].

According to Ambler's classification, all the members of class A, B, and D β-lactamases can hydrolyze carbapenems; thus, they are considered carbapenemases [5]. Based on active site fold, carbapenemases are broadly categorized into two groups. The first group comprises the class A penicillinases and class D oxacillinases, which contain serine in their catalytic sites and are often inhibited by clavulanic acid [29]. On the other hand, zinc metal is present at the active sites of the metallo-β-lactamases, which can be inhibited by EDTA-like chelating agents [29].

Popular members of class A carbapenemases are Guiana extended-spectrum β-lactamase (GES), *Klebsiella pneumoniae* carbapenemase (KPC), *Serratia marcescens* enzyme (SME) and imipenem hydrolyzing β-lactamase/non-metallo enzyme carbapenemase (IMI/NMC) [29, 30]. The OXA (oxacillinase) represents the class D carbapenemases, with OXA-48 being predominantly involved in carbapenem resistance in CRE [29–31]. Several widely distributed metallo-β-lactamases have been implicated in carbapenem resistance. Among them are New Delhi metallo-β-lactamases (NDM), imipenemases (IMP), and Verona integron-encoded metallo-β-lactamases (VIM) are commonly associated with serious clinical implications [26, 28, 30]. Other infrequent but noteworthy members include German imipenemases (GIM), Seoul imipenemases (SIM), Australian imipenemases (AIM), and L1 carbapenemases [9]. Table 1 summarizes information about the structure and activity profiles of the five most frequent and thoroughly investigated carbapenemases of CRE. Ikenoue et al. [32] observed that the effectiveness of the carbapenems can be reclaimed by synthesizing other drugs that can inhibit these enzymes.

2.2 | Active Expulsion of Drug

In Enterobacterales, another mechanism of carbapenem resistance is the overexpression of efflux pumps responsible for the expulsion of such drugs from bacterial cells [27]. For example, the carbapenem expulsion is performed by the RND (Resistance-Nodulation-Division) efflux family, commonly known as the AcrAB-TolC efflux system, in *E. coli* and *Salmonella enterica* [33, 34]. In this system: AcrA is an anchoring protein that embeds the pump in the periplasm; AcrB is the central component, localized in the inner membrane; and TolC is an outer membrane protein that forms the channel [34, 35]. When antibiotics are complexed to AcrB, the structure is changed to force them through TolC, facilitated by AcrA, thus ejecting the drugs out of the cell [36]. The rise of multidrug-resistant (MDR) pathogens is primarily caused by the presence of RND-like efflux pumps [33, 36, 37]. Thus, inhibiting the efflux pumps can lead to increased sensitivity of the CRE strains and better treatment strategies.

2.3 | Porin Channels Modifications

Alterations in the porins channels also significantly reduce the influx and permeability of carbapenems into the bacterial cells [27, 38]. These small, water-soluble antibiotics generally enter the bacterial cell through the porin channels. However, this antibiotic influx is greatly influenced by the size, structure, and quality of these outer membrane porins (OMPs) in the gram-negative pathogens [37].

Zhou et al. [39] emphasized the serious impact of certain OMPs of the CRE in declining the effectiveness of the treatment strategies. OmpA, OmpC, OmpF, OmpW, and OmpX-like porins are important for membrane stability and nutrient transport; moreover, these are also implicated in the development of antibiotic resistance [38]. Table 2 shows the properties of some OMPs essential for exacerbating resistance in gram-negative pathogens.

TABLE 1 | The five clinically relevant carbapenemases.

Enzymes	Origin	Clinical significance	Gene location	Notable variants	Key features	Mode of transmission	Available treatment strategies
IMP	Japan	Common in hospital-acquired infections; can also increase resistance to other antibiotics	Plasmid/ Chromosome	IMP-1, 4, 6	Broad hydrolytic spectrum; resistant to most β-lactams; frequent in Enterobacterales and *Pseudomonas aeruginosa*; frequently associated with other resistance genes	Acquired in hospital facilities	Limited; polymyxins or tigecycline; poor outcomes
KPC	North Caroline, USA	High mortality and morbidity rates; treatment failure in cases of severe infections	Plasmid	KPC-2, 3, 4	Most popular carbapenemase; Susceptible to β-lactamase inhibitors; often identified in *K. pneumoniae* and other Enterobacterales	Mostly hospital-acquired; may be transmitted by direct contact.	Combination therapies using β-lactamase inhibitors (Ceftazidime/ Avibactam)
NDM	India, South Asia	Spreading rapidly within the healthcare facilities; a critical public health threat	Plasmid/ Chromosome	NDM-1, 5, 7	Resistant to carbapenems; reported in *E. coli* and *K. pneumoniae*	Transmittable as nosocomial infection, particularly in the third-world countries.	Restricted; may require colistin or tigecycline, but often difficult to treat
OXA	Turkey	Increases the incidence of nosocomial infections; impacts the efficacy of antibiotics; also associated with other resistance genes	Plasmid/ Chromosome	OXA-23, 48, 51	Resistant to penicillin and carbapenems; weak activity against cephalosporins; widespread in Middle East and North Africa	Linked with healthcare associated infections; highly transmissible mediated by plasmid	Limited treatment options; combination therapies often prove more effective
VIM	Italy	Associated with severe infections in immunocompromised patients	Plasmid	VIM-1, 2, 4	Often co-transferrable with other resistance genes; widespread in *Pseudomonas aeruginosa* and selected Enterobacterales; integron-associated	Hospital and other healthcare facilities acquired; rapid plasmid mediated spread to other pathogens	Like other carbapenemases; combination therapies are often required

TABLE 2 | Major OMPs and their role in conferring antibiotic resistance.

Key OMPs	Organisms	Regulation factors	Resistance mechanisms	Impact on antibiotic permeability	References
OmpF, OmpC, LamB and Pho	*E. coli*	Sigma factors such as RseA and LPS factors such as GmhB	Deregulation of DegP protease via RseA mutation or GmhB deletion causes OMP degradation	Upregulation of the CTX-M β-lactamase enzymes, increase resistance to carbapenem	[3]
OmpK35 (OmpF analog), OmpK36 (OmpC analog)	*K. pneumoniae*	Insertion or deletion in OmpK35/36 genes	Insertion sequences are IS903, ISEc68 and IS1 as well as Gly115-Asp116 insertion impacts OmpK36	Reduces carbapenem permeability by 75%	[40, 41]
Omp35, Omp36	*Enterobacter aerogenes*	Two sRNAs: MicC and MicF	Overproduction of MicC and MicF decreases OMPs production; point mutations impair protein function	Increases carbapenem resistance; minor effect of sRNAs on MIC	[42]

2.4 | Alterations in Drug Target Sites

PBPs play a critical role in synthesizing the peptidoglycans of the bacterial cell walls [43]. Carbapenems form acylated complexes by interacting with these PBPs, thus inhibiting cell wall biosynthesis. Carbapenem resistance can also occur if the genetic structure of the target proteins is mutated [44]. These genetic variations can change the conformation or availability of the PBPs, hinder the carbapenem's ability to reach its site of action, or inhibit its activation and thus decrease the drug efficacy [27, 42, 44]. Genetic modifications can also lead to structural changes in the PBPs, synthesis of new PBPs as well as reduced binding affinity to the carbapenem [43].

Le Terrier et al. [45] observed that the decrease in carbapenem susceptibility in *E. coli* was associated with mutations in the PBP2-encoding *mrdA* gene. Moreover, when this mutation coexists with changes in the PBP3-encoding *ftsI* gene, the carbapenem resistance of *E. coli* is substantially increased [46]. Strikingly, PBPs modifications often require additional factors such as decreased permeability or overstimulation of efflux pumps to actively induce carbapenem resistance in CRE. Jiang et al. [47] reported that targeting PBPs could effectively reduce carbapenem resistance (particularly induced by the ESBL/PBP pathway) in *K. pneumoniae*.

2.5 | Biofilm Barriers

Biofilms are complex clusters made by bacteria as they adhere to a self-produced polymeric matrix [48, 49]. These entities protect bacteria against various antimicrobial agents and unfavorable environmental conditions. The penetration of carbapenem is drastically reduced in the presence of bacterial biofilms [11]. Sauer et al. [50] defines biofilms as structures with distinct phases essential for bacterial communication, gene exchange, and defense. The LPS (lipopolysaccharides), flagella as well as type I and III fimbriae are characteristic components of a bacterial biofilm [7, 51]. Modifying these components helps protect bacteria when exposed to carbapenems; therefore, facilitating antibiotic resistance.

The biofilm production in *K. pneumoniae* is regulated by capsular polysaccharides, which aid in intracellular signaling, and type III fimbriae, which facilitate cell-to-cell adherence [52, 53]. When exposed to meropenem, *K. pneumoniae* reduces the production of pili and flagellar proteins as its key resistance mechanism [51]. Al-Bayati et al. [54] credited the biofilm-associated genes' upregulation concomitant with IMP and NDM carbapenemases' production in greatly enhancing the antibiotic resistance and virulence of *E. coli* and *K. pneumoniae*. Clinical isolates of carbapenem-resistant Enterobacterales were observed to have a strong propensity to form biofilms, which was directly associated with NDM-1 production [55].

3 | Computational Strategies for Antimicrobial Discovery

Although advances in combinatorial chemistry produce surplus compound libraries, only a small fraction of them contribute to

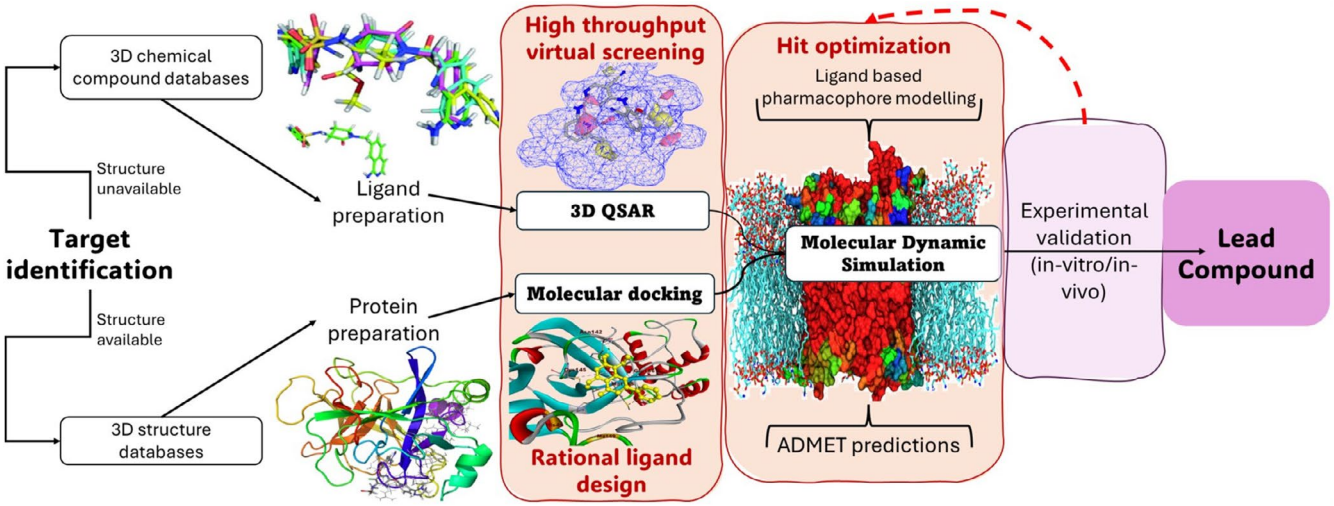

FIGURE 2 | Workflow of ligand and structure-based approaches for drug discovery. ADMET, absorption, distribution, metabolism, excretion, and toxicity; QSAR, quantitative structure–activity relationship.

the drug market. The intensive time and financial demands of conventional validation approaches are primarily responsible for this gap, creating a great bottleneck in the antimicrobial discovery pipeline. Computer-aided drug design (CADD) approaches are shifting the paradigm of antimicrobial discovery by rapidly screening the potential lead compound. Its main uses include but are not limited to identifying "hits" or "leads" for a particular target from a small molecule library, evaluating the selectivity of compounds towards the targets, improving leads to increase their affinity to the target, their ADMET (absorption, distribution, metabolism, excretion and toxicity) profiles, and finally, designing new chemotypes for library synthesis and biological testing [12, 56]. This section describes the computational techniques commonly used for discovering drugs against CRE. Figure 2 summarizes the basic workflow of CADD.

3.1 | Ligand-Based Drug Discovery

Ligand-based drug discovery (LBDD) is a powerful approach in drug discovery, particularly useful when the 3D structure of the biological target is unknown [57, 58]. Its operating principle is that the molecules with structural similarities tend to display related bioactivities [59]. Thus, rather than focusing on the target itself, LBDD focuses on the ligands or known "actives" to identify compounds with comparable structures or physicochemical properties [56]. In this way, the geometric or chemical features of a compound essential to interact with the target can be identified.

LBDD measures the similarities between molecules using Tanimoto coefficient-like metrics [57]. These metrics simply compare the structural fragments of known active molecules with those of the virtual libraries. LBDD techniques, such as Quantitative Structure-Activity Relationship (QSAR) and pharmacophore modeling, often categorize descriptors into three classes: 1D for basic molecular features such as weight, atom numbers, or types; 2D for structural topology or molecular connectivity; and 3D for spatial arrangement and molecular conformations [57, 60].

3.1.1 | Pharmacophore Modeling

Pharmacophore modeling identifies the set of structural features of a compound required for its optimal interaction with the target [59]. The pharmacophore model depicts the spatial arrangement of these key features such as hydrogen bond donors and acceptors, and ionizable regions, which are essential for producing a biological effect after binding to the target [58, 61]. As the exact atomic details are ignored, only the essential chemical features are identified. This facilitates recognizing similarities across structurally diverse compounds sharing a common biological target [62]. Thus, a pharmacophore model serves as a reference for screening new drugs, with the potential to interact with a specific target, from vast compound libraries [60, 63].

Pharmacophore models can be developed using either ligand-based or structure-based approaches. The ligand-based approach is employed when the 3D structure of the target protein is inadequate or unavailable, but information regarding active ligands exists [62]. So, the chemical properties of these known ligands are used to identify structural motifs relevant to target binding [64]. Contrarily, in the structure-based approach, the features of the binding site are directly mapped from the available structure of the target protein, which is used for searching compounds with complementary structural features [61]. A pharmacophore model can be generated using the steps depicted in Figure 3 [12, 63].

Pharmacophore modeling plays a significant role in the fight against CRE, aiding the identification of structurally diverse compounds with similar antimicrobial activities. Behera et al. [65] developed ligand-based pharmacophore models using Phase (Schrödinger) to screen *E. coli*-AcrB efflux pump inhibitors. Their training set consisted of 32 FDA-approved efflux pump inhibitors and constructed 10 pharmacophore models. Their virtual screening based on the finally developed pharmacophore model showed that argatrobam can actively inhibit the AcrB efflux protein of *E. coli*. Almuhayawi et al. [66] generated pharmacophore models based on three cytoplasmic proteins to target *E. xiangfangensis*. They utilized this model to screen

a large library of phytochemicals retrieved using online databases. Their study concluded that only 5 out of 2500 screened phytocompounds could effectively bind to the target proteins and therefore, can be used to inhibit *E. xiangfangensis*.

3.1.2 | Quantitative Structure–Activity Relationship

QSAR is a highly valuable in silico drug discovery approach that predicts the biological activities of chemical compounds by developing models based on their structural features [67, 68]. QSAR quantitatively describes how molecular descriptors of certain compounds relate to their bioactivities. These models facilitate selective filtering and optimizing of potential drugs for improved activities in terms of affinity, selectivity, and reduced side effects [69]. QSAR identifies structural modifications of a molecule that can enhance its biological activity. Table 3 summarizes the properties of QSAR models.

QSAR models utilize both statistical and multivariate techniques, such as logistic regression and pattern recognition methods, to analyze the structural features such as electronic, spatial, and hydrophobic properties and relate these to the pharmacokinetic, toxicity, and bioactivities of the compound [70, 71]. QSAR

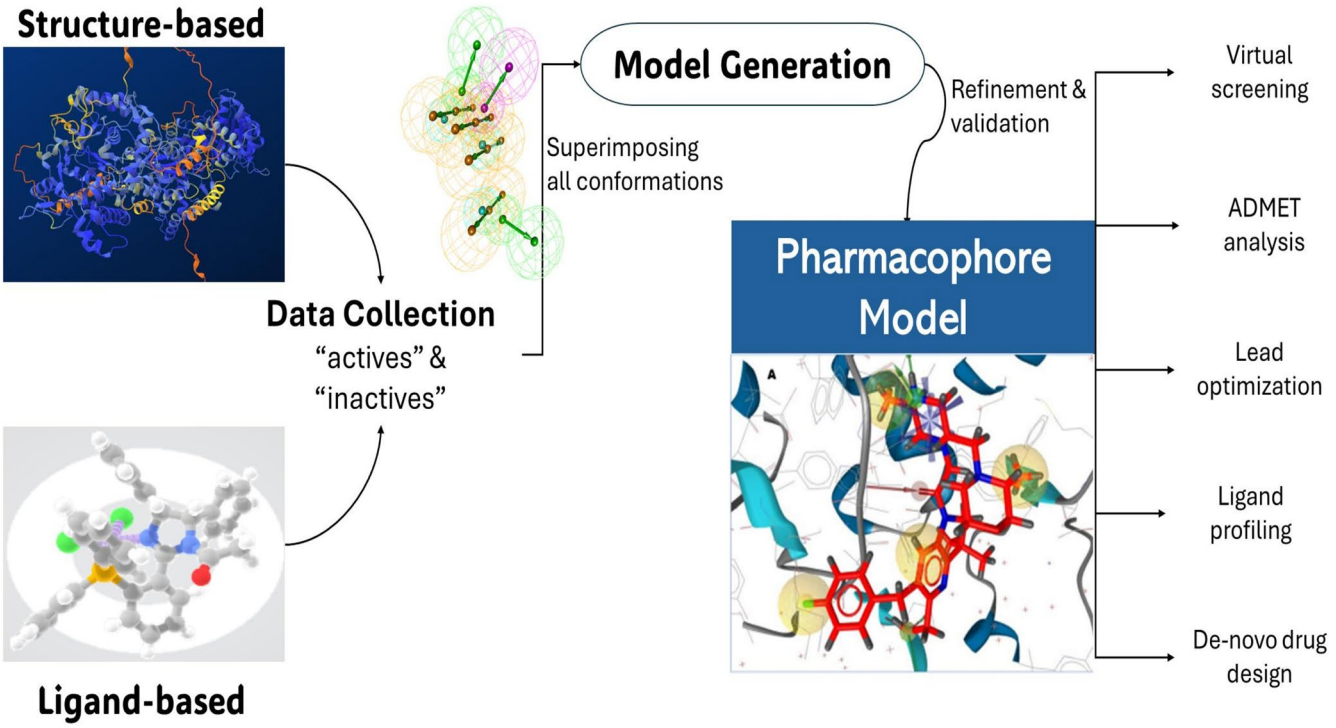

FIGURE 3 | Steps involved in pharmacophore modeling. ADMET, absorption, distribution, metabolism, excretion, and toxicity.

TABLE 3 | Classification of QSAR models.

Classes	Descriptors	Significance	Limitations
1D QSAR	Correlating activity with global molecular or physiochemical properties	_	Degeneracy issues
2D QSAR	Correlating activity with geometry, structural patterns, and topology of molecule	Gives information about possible conformations	No representation of 3D structures of stereochemistry
3D QSAR	Correlating activity with non-covalent interaction fields surrounding the molecules and its structural patterns	Most sensitive to structural variations	Structural alignment
4D QSAR	Reflects ensemble of ligand configurations or a set of conformers in 3D-QSAR	Adequately describe the chemical structure	—
5D QSAR	Explicitly represents different induced-fit models in 4-D QSAR models	Less biased than 4D-QSAR models	—
6D QSAR	Incorporates different solvation functions in 5-D QSAR models	Assists in analyzing various solvation models	—

models are particularly useful in the process of virtual screening to rapidly evaluate vast compound libraries, expediting the drug discovery [67]. These models also help in estimating the ADMET (absorption, distribution, metabolism, excretion, and toxicity) profiles of compounds and excluding those with undesirable pharmacokinetics early in the process [69, 72]. Software such as PharmQSAR, ADMEWORKS Model Builder, DeepAuto QSAR, ezqsar, QSAR-Co, Spartan, MOE, and GUSAR are commonly employed to construct QSAR models, generally following this scheme, Figure 4 [70, 73].

QSAR modeling has become quite popular in the discovery of antimicrobial agents to inhibit CRE. Since NDM-1 neutralizing drugs are quite few currently, this study therefore employed QSAR models to screen promising inhibitors [74]. Yu et al. [74] collected IC_{50} values of over 600 NDM-1 inhibitors from the ChEMBL database and analyzed 12 molecular fingerprint sets using a machine learning approach. The QSAR model revealed that aromatic, carbonyl, and aliphatic regions can potentiate the inhibition of NDM-1. Such studies provide key information regarding lead optimization for developing NDM-1 inhibitors, advancing the combat against CRE.

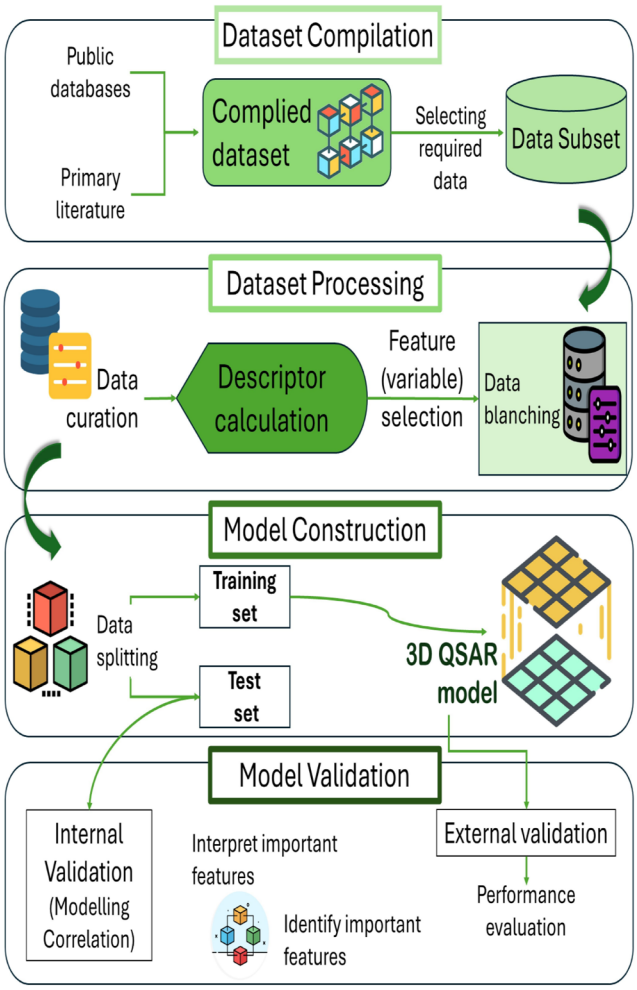

FIGURE 4 | Quantitative structure–activity relationship (QSAR) models.

3.2 | Structure-Based Drug Discovery

Structure or receptor-based drug discovery (SBDD) uses the 3-dimensional structures of target molecules—typically proteins—to predict and score the potential drugs (ligands) [75, 76]. SBDD determines the binding affinities and stability of the complexes by simulating the ligands' orientations in the active site of the target protein. One of the widely used SBDD techniques is molecular docking, which can practically measure and predict the binding modes, prioritizing the compounds with the highest affinities [60].

SBDD advances antimicrobial discovery by leveraging the well-defined 3D structural properties of target molecules for designing and optimizing the ligands for desired bioactivities [75]. Briefly, this approach is concerned with locating the protein binding sites, preparing the ligand compound libraries, performing the molecular dynamics simulations, and ultimately estimating the binding energies [76]. SBDD also provides insight into understanding the resistance mechanisms at the molecular level, promoting the development of targeted antimicrobial drugs [77].

3.2.1 | Molecular Docking

Molecular docking can predict the most favorable binding mode and affinity between a ligand (small chemical compounds) and a target receptor (proteins) [78]. Docking or search algorithms stimulate how a ligand would fit in the binding site of the target protein and identify optimal binding orientations. Then the scoring function ranks these orientations as binding affinities in terms of their hydrophobicity and structural complementation [79]. Molecular docking requires high-resolution 3D structures of the target proteins, which can be obtained from experimental techniques (NMR or X-ray crystallography) or homology-based computational techniques [80]. With the help of AI-powered tools like AlphaFold, the 3D structures of proteins, previously difficult to characterize, can be predicted with high accuracy [75].

Consequently, molecular docking has become indispensable in antimicrobial drug discovery for rapidly screening large compound libraries and identifying potential inhibitors against bacterial proteins, which can be experimentally validated [81]. The detailed molecular interactions between ligands and their protein targets can also be modeled through docking algorithms. AutoDock vina, Discovery studio, FlexX, GOLD, LigandFit, UCSF DOCK, Glide, rDOCK, and FRED are commonly used software and algorithms for generating and scoring multiple orientations of protein-ligand complexes [78, 82]. The steps of the general docking scheme are presented in Figure 5 [82–84]:

Wu et al. [85] identified a new variant of OXA carbapenemase in a clinically isolated strain of *E. coli*. They predicted the structure of this OXA-1041 by homology modeling using ClustalW based on the already known structures of other closely related OXA enzymes (OXA-780, 427, 1037). Molecular docking was then performed to analyze the binding interactions of several known carbapenemase inhibitors and OXA-1041. Tran et al. [86] also

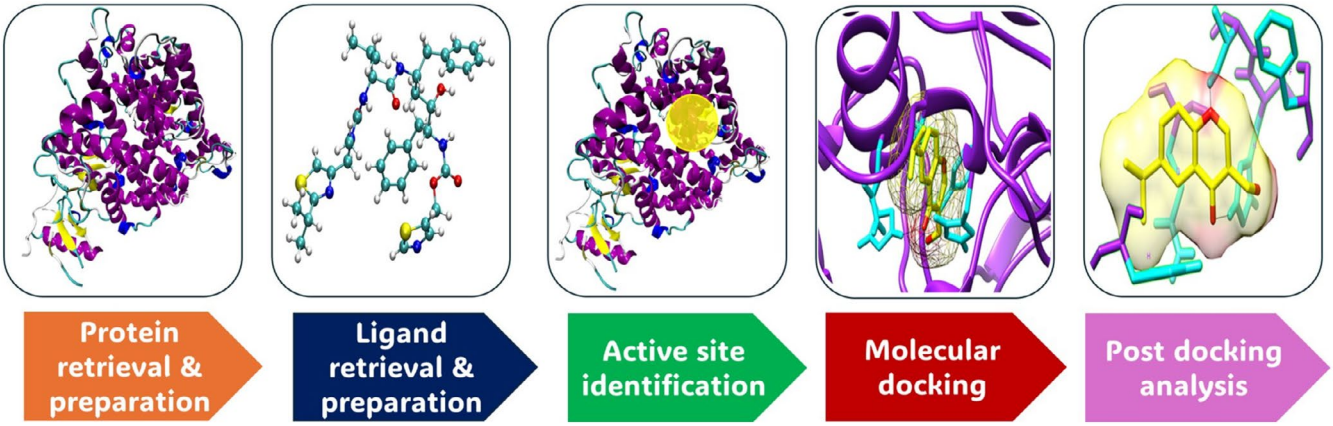

FIGURE 5 | Process of molecular docking.

performed a similar study in which they evaluated the effects of one-point mutations in NDM-1 on its carbapenem hydrolysis abilities. For this purpose, they selected seven mutants of NDM-1 that increased the binding affinities with inhibitors by 50% and subjected these complexes to further simulation. Tran et al. [86] concluded that amino acid substitutions at the 122 and 124 positions of wild-type NDM-1 significantly influence its binding with antibiotics.

3.2.2 | Molecular Dynamics Simulation

Molecular dynamics simulations (MDSs) are now widely applied in antimicrobial drug discovery as they provide detailed insights into the atomic changes within the molecular systems occurring due to physical interactions or docking [80]. MDS captures the gradual changes in the structures of interacting molecules, which are difficult to obtain when conducting experiments only [87]. It has become an effective approach for probing complex issues related to protein misfolding or aggregation and uncovering new therapeutics for various disorders [16]. MDS improves the SBDD process by considering protein flexibility, representing its original biological conformation, which is an inherent limitation of experimentally determined static protein models [88]. It enables the development of multiple conformations of protein structure and identifies more accurate druggable sites for ensemble docking.

MDS process begins with the system preparation, where both ligand and protein are optimized by energy minimization and a dual-stage equilibration that ensures complex stability [88, 89]. Then, simulations are performed to monitor the atomic interactions over time, mapping the binding interactions, flexibility, and stability of the protein-ligand complex [79]. MD programs such as AMBER, DESMOND, GROMACS, NAMD, and CHARMM utilize force fields to accurately represent the molecular interactions and atomic forces, producing detailed MDS trajectories [57, 80]. The simulation trajectories are further analyzed based on specific parameters, summarized in Table 4. Despite its efficiency, MDS requires robust computational resources and is often limited by the accuracy of force fields and simulation periods, which may not properly record long-term dynamics [87].

Major facilitator superfamily (MFS) is a class of efflux pumps that can actively expel antibiotics from bacterial cells, significantly contributing to resistance in CRE. MDS revealed the conformational transitions of MFS proteins, identifying an intermediate state between its inward and outward conformations [90]. Li et al. [90] reported that in *E. coli*, this inward transition is initiated by protonation on the periplasmic side, which enhances the hydrophobic interactions and structural changes. On the other hand, the reverse transition is maintained by hydrophobic membrane interactions. Concurrently, the changes in cytoplasmic bridges restrict substrate entry. These findings develop insights regarding efflux pump dynamics in CRE and contribute to the screening of inhibitors to overcome resistance.

3.3 | ADMET Profiling

ADMET analysis determines the safety and efficacy of potential therapeutic candidates [91]. With billions invested annually by pharmaceuticals for new drug development, about half of these compounds fail due to their poor pharmacokinetic properties or undesirable toxicity [80, 92]. Although several predictive models are available that can determine the toxicity of the drug candidates (Figure 6), their use raises serious ethical concerns. In contrast, computational tools overcome these challenges and facilitate early-stage ADMET profiling with higher efficiency, lower cost and time, and limited reliance on animal models [93].

The potential drug candidates must follow some of the predefined rules for drug-likeness. One significant rule is the Lipinski Rule of Five (RO5) which focuses on molecular properties such as molecular weight (< 500 Da), partition coefficient (≤ 5), polar surface area (< 140), hydrogen bond donors (≤ 5) and acceptors (≤ 10) [94]. Additional rules include the Ghose filter, which determines molecular weight, lipophilicity, and molecular refractivity, and the Veber rule, which considers molecular flexibility and polar surface area [95]. Despite being valuable, all such rules are not absolute, and drug discovery approaches often integrate them with computational tools for better predictions [91].

Online tools such as admetSAR, SwissADME, ADMETlab, and DrugMint have expedited drug discovery by predicting the

TABLE 4 | Post-MDS analysis of ligand–protein complexes.

Parameter	Full form	Purpose	Interpretation	Significance
RMSF	Root mean square fluctuation	Analyzes flexibility or fluctuations of individual amino acid residues	High RMSF indicates more flexible and unstable regions; Low RMSF indicates stability	Provides information about individual residue contribution to docking as well as highlight flexible or rigid protein regions
RMSD	Root mean square deviation	Analyzes overall structural stability of the docked complexes	High RMSD indicates significant deviation and unstability; Low RMSD indicates stable interactions	Measures how much protein structure deviates from its native conformation over time
SASA	Solvent accessible surface area	Measures the surface area of the target protein accessible to interact with ligand & solvent	Higher SASA values represent large surface areas available for interaction; Lower SASA represents compact structure	Determines stability, folding and potential binding patterns of proteins based on solvent accessibility
Rg	Radius of gyration	Quantifies the rigidity of protein structure	Higher Rg shows protein aggregation; Lower Rg shows compact or rigid structure	Monitors the structural rigidity, folding stability and conformational changes upon ligand binding
Number of hydrogen bonds	—	The number of hydrogen bonds formed as well as their stability	Stable bonds indicate strong interactions; Fluctuations indicate dynamic interactions	Corresponds to stability and strength of protein–ligand as well as within protein interactions

permeability and toxicity of potential therapeutic drugs [16, 80]. Moreover, some of the AI-powered platforms, such as LiveDesign by Schrödinger, have revolutionized the drug optimization process [96]. The advances in structural modeling of proteins with critical roles in drug metabolism have enhanced the accuracy of these ADMET predictions, reflecting realistic clinical outcomes [93]. The integration of multi-omics techniques in precision medicine has also improved ADMET predictions [95]. Moreover, by incorporating proteomics, genomics, and metabolomics, the inter-individual variability in drug response can be considered, facilitating more personalized therapies [76].

3.4 | Successful Applications of In Silico Approaches

Computational tools have been indispensable in the search for antimicrobials targeting CRE, as shown in Table 5. In this section, we have explored some of the latest studies employing the use of computational techniques to overcome the carbapenem resistance mechanisms of Enterobacterales.

The study by Elbaramawi et al. [104] is particularly interesting in this aspect. In this study, Elbaramawi et al. [104] screened and developed novel inhibitors against Methionine tRNA synthetase (MetRS) of *P. mirabilis*. MetRS has a crucial role in the biosynthesis of several metabolic proteins of *P. mirabilis*. As the 3D structure of the target protein was unavailable, Elbaramawi et al. [104] used MOE software to construct its homology model. Then, structure-based pharmacophore models were developed to screen potential inhibitors against *P. mirabilis*. The inhibition activities of screened compounds were evaluated by molecular docking and simulation analyses. This study concluded that Val235, Lys334, Asp52, and Glu27 of *P. mirabilis* MetRS are key amino acids that should be targeted to develop its novel inhibitors.

Oyedara et al. [105] conducted systematic in silico analysis to determine the inhibitory activities of several phytocompounds from selected Mexican medicinal plants against *Salmonella* AcrB efflux pump protein. For this purpose, they performed molecular docking of 71 phytochemicals and the target protein using Open Babel and AutoDock vina. The top-scoring phytochemicals were then screened for their ADMET properties, and only those having desirable drug-like properties were chosen for further testing. The selected protein-ligand complexes were subjected to 50 ns simulation using GROMACS software. Oyedara et al. [105] reported naringenin, licarin A, and methoxypsoralin as potential inhibitors of the efflux pump of *S. enterica*, as these compounds actively bound the distal deep pockets of the AcrB protein.

Computational technologies are also valuable in identifying novel target sites of CRE. In this essence, Jamil et al. [103] emphasized targeting proteins of *K. pneumoniae* essential for host interactions. Initial screening of over 400 bacterial proteins identified 16 candidates based on their non-homology and pathogenicity. 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase (DapD), which is a key protein of the lysine metabolic pathway of *K. pneumoniae*, was selected as the target molecule. Subsequently, docking-based virtual screening of

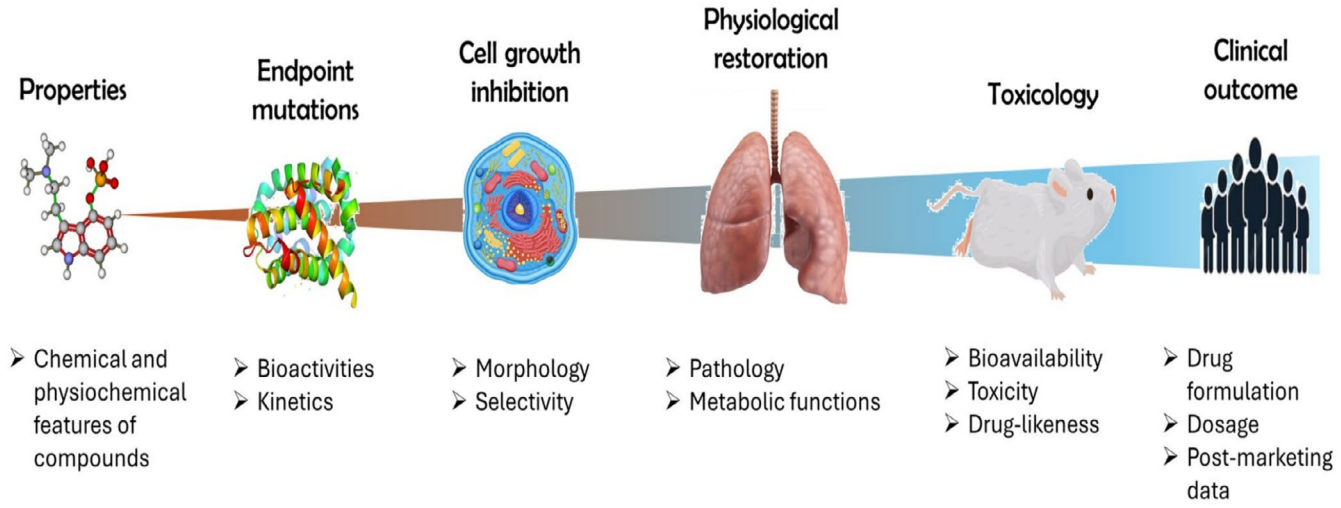

FIGURE 6 | Predictive models used to analyze various properties of drug candidates.

9000 FDA-approved compounds was performed using AutoDock vina. Thus, Jamil et al. [103] identified 15 promising candidates to inhibit this novel target protein of *K. pneumoniae*, which could be further experimentally validated.

Similarly, Ahmed et al. [106] identified inhibitors against a novel target protein of *S. typhi*. They generated protein-ligand interaction fingerprint (PLIF)-based pharmacophore models to screen natural compound inhibitors against *S. typhi*. LpxC, a metallo amidase responsible for the synthesis of Lipid A (bacterial endotoxin) was selected as the target protein. The toxicity and ADME properties of the top-scoring compounds were also analyzed after docking. MD simulation of 100 ns was performed using YASARA software, identifying three inhibitors of the *Salmonella* LpxC enzyme.

4 | Combined In Vitro and In Silico Validation of Antimicrobials

The in silico and in vitro combination therapy has become the new reference for drug development in the face of increasing antibiotic resistance. This iterative approach integrating the speed and accuracy of computational techniques with the precise in vitro/in vivo validation provides a reliable and effective drug discovery process against CRE [14, 107]. The analysis of molecular factors influencing the effectiveness of a chemical compound derives the discovery of novel and more potent analogs [12, 108]. Such analogs can then be tested by rigorous experiments for their antimicrobial efficacy specifically targeting the resistance mechanisms. Various latest research works involving such combined approaches are briefly summarized in Table 6.

In a comprehensive study by Abuelizz et al. [109], three Benzoquinazoline derivatives were identified as potent inhibitors of carbapenem-resistant *K. pneumoniae*. In their study, Abuelizz et al. [109] determined the antibacterial activity of the benzo-[g]-quinazolines through in vitro XTT reduction assay, and the results showed that the compounds inhibited the growth of *K. pneumoniae* 60% more effectively than the selected antibiotics. The docking results further confirmed the effective binding of the designed compounds with the OXA-48 β-lactamase of *K. pneumoniae*. The stability of the docked complexes was evaluated by MD simulation of 10 ns. Thus, both in silico and in vitro analyses of the benzoquinazoline derivatives highlighted their antimicrobial activities not only against *K. pneumoniae* but also against methicillin-resistant *Staphylococcus aureus* and fluconazole-resistant *Candida albicans*. Moreover, these compounds exhibited very low cytotoxic activity against normal human lung fibroblast cells (WI-38 cell line).

In another compelling study by Abdel-Halim et al. [111], the inhibitory activities of coumarin targeting *K. pneumoniae* were analyzed using various in silico and in vitro techniques. In this study, the presence of certain carbapenemase-encoding genes (bla_{OXA}, bla_{NDM}, bla_{VIM}) was detected in 6 different clinical isolates of *K. pneumoniae* through PCR. These carbapenemases were then selected as target enzymes docked against coumarin. The broth microdilution assay showed that the growth of *K. pneumoniae* is inhibited in the presence of coumarin. Strikingly, the checkerboard assay exhibited the synergistic activities of coumarin with meropenem against the selected pathogen. Such studies highlight the discovery of coumarin and other such compounds that can not only actively inhibit CRE but can also revive the efficacy of carbapenems against them.

Medicinal plants produce several secondary metabolites that exhibit antimicrobial, anticancer, and antioxidant activities. Thus, in a study by Mehta et al. [114], the potential of various medicinal plants, namely Centella asiatica, Ocimum sanctum, Momordica charantia, *Zingiber officinale*, and Ziziphus mauritiana, to inhibit the AcrAB-TolC efflux pump of *S. typhimurium* was evaluated. For this purpose, the phytocompounds of the selected medicinal plants and RamR (key protein of EP) were subjected to molecular docking employing AutoDock vina. The in silico analysis screened the presence of certain compounds such as Lariciresinol exhibiting high binding affinities for the target proteins. The in vitro microdilution analysis also confirmed the antimicrobial potential of selected plants. For analyzing their biofilm inhibition potential, the EtBr cartwheel assay was also performed. The integrated in silico and in vitro study concluded that *Z. officinale* had comparatively the highest antibacterial

TABLE 5 | Computational discovery of inhibitors against CRE.

CRE					
Organisms	**Target**	**Ligand**	**Method**	**Software/tools used**	**References**
K. pneumoniae	Porin protein	Zinc oxide nanoparticles	Docking, QSAR, MDS	Schrödinger Glide, HyperChem Professional, Schrödinger Desmond	[97]
Enterobacterales	Class A carbapenemases	Relebactam, enmetazobactam, QPX7728	Homology modeling, Docking, MDS	SWISS-MODEL, AutoDock vina, AMBER	[98]
S. flexneri	Dihyroorotase	Natural compounds	Homology modeling, Docking, MDS, ADMET	I-TASSER, MOE, Schrödinger Desmond, pkCSM, PreADMET	[99]
S. marcescens	FabI	Bergamot essential oil	Homology modeling, Docking, MDS, ADMET	SWISS-MODEL, XP docking, Schrödinger Desmond, SwissADME, pkCSM	[100]
E. coli, *K. pneumoniae*	SHV-1, NDM-1, OXA-48, KPC-2	Oleanolic acid, protocatechuic acid, tannin	Docking, MDS, ADMET	AutoDock vina, WebGro, PROTOX-II, admetSAR	[101]
K. pneumoniae	KPC-2, OmpK37	Thiadiazol derivative	DFT, Docking	Gaussian 16 package, BIOVIA Discovery Studio	[102]
K. pneumoniae	DapD	FDA approved inhibitors	Docking	AutoDock vina	[103]

TABLE 6 | Integrated experimental and computational studies discovering antimicrobials against CRE.

CRE		In-silico analysis			In vitro/in vivo analysis				
Organism	**Source**	**Drug**	**Target**	**Methods (tools)**	**Antimicrobial assays**	**MIC/MIC$_{50}$ (μg/mL)**	**Cytotoxicity assays**	**Results/ IC$_{50}$**	**References**
K. pneumoniae	ATCC BAA-2342	Benzoquinazoline	OXA-48	Docking (MOE-Dock), Dynamic simulation (NAMD)	XTT reduction assay	3.9/0.67	Viability assay (WI-38 cell lines)	Weak <400	[109]
Enterobacter cloacae	CCBH10892	Thiosemicarbazone	NDM-1	Docking (GOLD), Dynamic simulation (GROMACS)	Microdilution assay	31.25 μM	—	—	[110]
K. pneumoniae	Clinical isolates	Coumarin	NDM-1, VIM-2, OXA-48	Docking (MOE-Dock)	Microdilution assay, carbapenemase inhibition assay	1000, $>50\%$ enzyme inhibition	—	—	[111]
E. coli, *K. pneumoniae*	Plasmid constructed *E. coli* BL21 (DE3), ATCC BAA-2146	Emerione A, aspoquinolone E	NDM-1	Virtual screening, Docking (SYBYL)	Microdilution assay, Microscale thermophoresis assay	32–64, $>60\%$ enzyme dissociation	Hemolysis assay	$\leq 3\%$ blood lysis	[112]
S. flexneri	MTCC-1457	Rutin	Sep A, iNOS	Docking (FRED), Dynamic simulation (AMBER), Network pharmacology (Cytospace)	Disc diffusion assay, Microdilution assay	24 ± 0.25 mm, ≥ 0.25 mg/mL	—	—	[113]
S. typhimurium	Plasmid constructed	Lariciresinol	RamR, RamA	Docking (AutoDock vina)	Microdilution assay, EtBr efflux inhibition assay	64 ± 0.02, Low fluorescence post-treatment	—	—	[114]

(Continues)

TABLE 6 | (Continued)

CRE		In-silico analysis			In vitro/in vivo analysis				
Organism	**Source**	**Drug**	**Target**	**Methods (tools)**	**Antimicrobial assays**	**MIC/MIC$_{50}$ (μg/mL)**	**Cytotoxicity assays**	**Results/ IC$_{50}$**	**References**
E. coli, *K. pneumoniae*, *S. typhimurium*	—	Cefuroxime derivatives	PBPs	Ligand designing (ChemDraw), Docking (AutoDock vina)	Microdilution assay	12.5	Brine shrimp lethality bioassay	Minor lethality (LC$_{50}$: <900 μg/mL)	[115]
K. pneumoniae, *S. flexneri*	MTCC	Nisin	NDM-1, KPC-2	Ligand designing (QSAR chemosophia) Docking	Agar well diffusion assay, Microdilution assay, Anti-biofilm assay	>11 mm, 25–50, >90% inhibition	MTT assay (NHDF cells)	<100 μL	[116]
S. marcescens	MTCC97	Coumarin	AHL synthase	Docking (Schrödinger Desmond) Dynamic simulation (Schrödinger Maestro)	Antibiofilm assay	>50% inhibition	—	—	[117]
E. coli, *S. flexneri*	—	Campanulin, Epifriedelanol	PBPs	Docking (AutoDock vina)	Agar well diffusion assay	6–18 mm	—	—	[118]

and anti-biofilm activities against carbapenem-resistant *S. typhimurium* [114].

5 | Future Perspectives

In silico technologies have transformed the conventional drug discovery process by being rapid and cost-effective. Techniques such as QSAR, molecular dynamics, pharmacophore modeling, and docking have been indispensable, particularly in the development of antimicrobials targeting CRE. These tools have facilitated not only the identification of "hit" or lead compounds but also the optimization of drug candidates, substantially reducing the time for experimental stages. Nevertheless, these advanced technologies have their own drawbacks. In silico models often face parameterization challenges, can produce false positive or negative results, and lack the subtility to accurately predict ligand-protein interactions.

Recent advances in artificial intelligence (AI) and machine learning (ML) have the potential to revolutionize the antibacterial drug discovery process by making quicker, more accurate predictions based on massive datasets of chemical substances [119, 120]. These models can precisely and efficiently predict receptor-ligand docking interactions [121]. Moreover, the high-throughput screening and optimization of pharmacokinetic properties of chemical compounds are also facilitated by these models [122]. Python-based AI models such as DeepChem (https://github.com/deepchem/deepchem) and DeepTox (http://www.bioinf.jku.at/research/DeepTox) can screen vast chemical compound libraries and predict their toxicity [121]. ORGANIC (https://github.com/aspuru-guzik-group/ORGANIC) and other molecular generation tools aid in designing chemical compounds having desired drug-like properties [123] DeltaVina (https://github.com/chengwang88/deltavina) and PotentialNet (https://pubs.acs.org/doi/full/10.1021/acscentsci.8b00507) use different scoring functions to predict the binding affinity of ligands and receptors [124]. Moreover, AlphaFold (https://deepmind.com/blog/alphafold), RoseTTAFold (https://github.com/RosettaCommons/RoseTTAFold) and trRosetta (https://yanglab.nankai.edu.cn/trRosetta/download/) use neural networks to predict the protein structures based on residue-to-residue distance and contact [125]. To enable individualized treatment methods, future research should concentrate on improving AI systems to anticipate resistance patterns with greater precision [126]. Integrating AI and ML techniques with in vitro and in vivo experiments can expedite drug validation and enhance clinical outcomes [127]. Additionally, these techniques offer real-time solutions for optimizing drugs by facilitating the development of adaptive learning models that evolve with emerging antibacterial resistance [128]. However, handling enormous data and ensuring ethical considerations will be crucial in utilizing the full potential of AI and ML-based approaches in antimicrobial drug discovery.

The future of in silico drug discovery lies in bridging the gap between computational predictions and their clinical applications. For this purpose, the predictive capabilities of these computational technologies must be integrated with reliable experimental validation.

Author Contributions

Muhammad Absar: conceptualization, writing – review and editing, writing – original draft, investigation, formal data analysis. **Abdul Rahman Zaidah:** data curation, writing – review and editing. **Amer Mahmood:** data curation, writing – review and editing. **Sajjad Ahmad:** writing – review and editing, data curation, formal analysis. **Hasan Ejaz:** writing – review and editing, data curation, formal analysis. **Naveed Ahmed:** writing – review and editing, data curation, formal analysis. **Nik Haszroel Hysham Nik Hashim:** writing – review and editing, data curation, formal analysis. **Chan Yean Yean:** conceptualization, writing – review and editing, supervision.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors have nothing to report.

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