Novel Beta-Lactam Antibiotics Derivatives: Their New Applications as Gene Reporters, Antitumor Prodrugs and Enzyme Inhibitors

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Abstract: Since the antibiotic properties of penicillin were first noticed in the beginning of last century, beta-lactam based antibiotics have been well developed as miracle drugs for the therapy of bacterial infectious diseases in clinics. Recently, these "old" antibiotics and their relevant derivatives have also found new applications as gene reporters, anti-cancer prodrugs and enzyme inhibitors. In this review, we will introduce the latest developments in the study of these new applications based on literatures reported over the last decade. The first section covers the recent developments of β-lactam antibiotics as drugs against bacteria, the second section briefly discusses the occurrence of bacterial resistance and mechanistic studies of β-lactam resistance in bacteria, the third section presents the current development of fluorogenic cephalosporin based β-lactam probes for real-time imaging of gene expression, and the fourth section describes relevant studies on β-lactam based substrates as anti-tumor prodrugs. Beta-lactam substrates as protease inhibitors will be also described in the fifth section. The final section summarizes future perspectives for β-lactam antibiotic derivatives as scaffolds in the fields of molecular imaging, drug delivery and enzymatic assays.

Key Words: Beta-lactam antibiotics, antibiotics resistance, β-lactamase, fluorescence imaging, gene expression, antibody-directed enzyme prodrug therapy, protease inhibitor.

BETA-LACTAM BASED PENICILLIN AND CEPHALOSPORIN ANTIBIOTICS AGAINST BACTERIA

The discovery and development of beta-lactam antibiotics are among the most powerful and successful achievements of modern medicinal science and technology. The first documented β-lactam antimicrobial agent, penicillin, was discovered accidentally in fungus *penicillium notatum* by Alexander Fleming in 1929. Since then, β-lactams rapidly became the "wonder drug" against the growth of infectious bacteria and contributed to the reduction of pain and suffering of people throughout the world. There are a large number of different β-lactams that have been developed, which are structurally related to a common core structure, the β-lactam ring (Fig. (**1**)). All the β-lactam antibiotics have structural similarities with the binding sites of bacterial substrates which can inhibit the transpeptidases involved in bacterial cell wall formation [1]. Penicillin, as the first discovered β-lactam antibiotic, was introduced into medical practice in the 1940s for the treatment of bacterial infections caused by susceptible, usually gram-positive organisms. Cephalosporins, another group of β-lactam antibiotics, are a class of broad-spectrum antibiotics derived from the fungus *cephalosporium acremonium*. Cephalosporins are widely and successfully used in medicine and have the same mode of action as penicillins. This group of antimicrobial agents, however, has different bi-cyclic β-lactam structures and tends to be more.

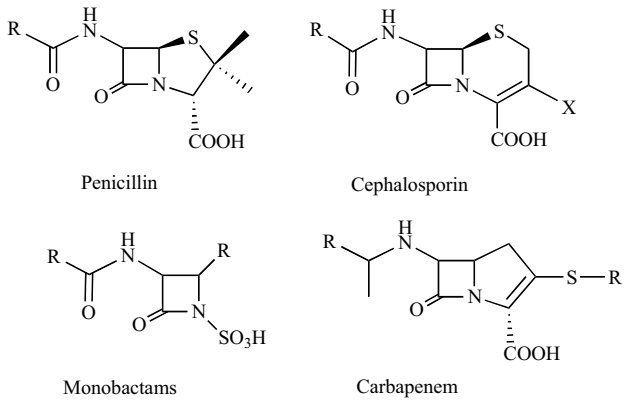

Fig. (1). Structure of the β-lactam antibiotics.

stable than penicillin Cephalosporins are often grouped into "generations" based on their antimicrobial properties. Normally, each newer generation has significantly improved gram-negative antimicrobial properties than the preceding generations [2, 3] (Fig. (**2**)).

Following on the heels of the development of penicillins and cephalosporins, extensive efforts have also been made to improve the existing β-lactam antibiotics with respect to potency, breadth of spectrum, activity against resistant pathogens, stability and pharmacokinetic properties. All the advances provide more antibiotics available to medicine. Today, the β-lactam antibiotics family is comprised of not only penicillin and cephalosporin, but also some natural and synthetic carbapenems, oxapenams, carbacephems, oxacephems and monocyclic β-lactams as well [4, 5] (Fig. (**2**)).

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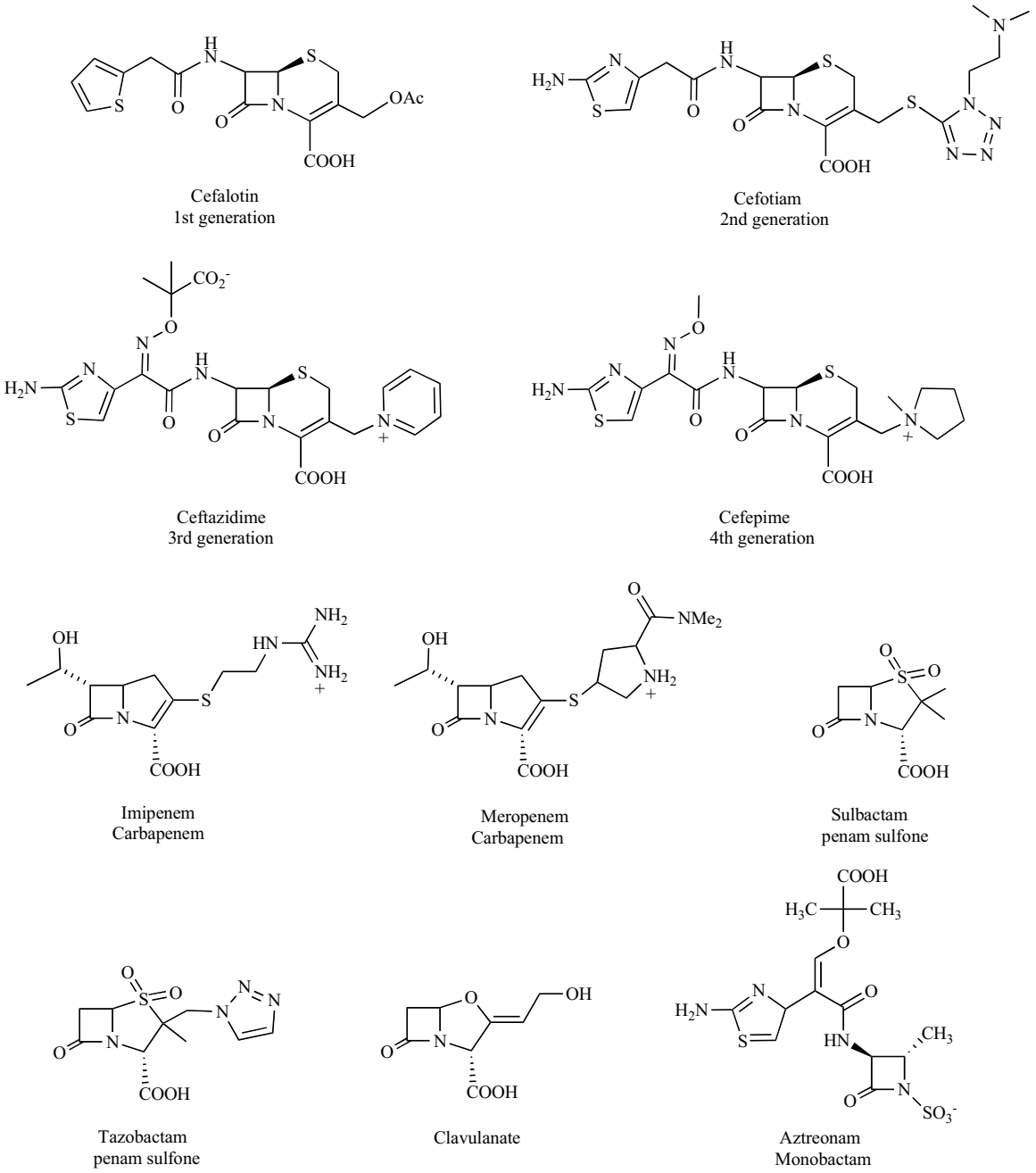


Fig. (2). Carbapenems, oxapenams, carbacephems and oxacephems, monocyclic β-lactams [3-5].

All these β-lactam antibiotics exhibit varying antibacterial activities and have a wide range of clinical applications [6, 7]. For example, natural product carbapenems, in which sulfur is replaced by a carbon atom (with a double bond present), are able to treat a wide range of infections with gram-positive and negative bacteria [8, 9]. Meropenam, another non-penicillin β-lactam antimicrobial agent has an improved safety profile compared with imipenam [8]. Then there are other antibiotics, which, when used together with penicillins, increase their antibiotic effectiveness. Clavulanic acid would be one such example. When combined with penicillin, it becomes a potent drug able to overcome certain types of bacteria's resistance to antibiotics [10]. When used in association with penicillins, sulbactam and tazobactam are able to suppress β-lactamase, an enzyme produced by bacteria that destroys antibiotics [11]. But bi-cyclic β-lactam structure antibiotics are not the only types of effective antibiotics available. Some monocyclic β-lactam structure antibiotics are also potent bacterial inhibitors. Aztreonam is a synthetic monocyclic β-lactam antibiotic originally isolated from *chromobacterium violaceum* and is used in clinics against gram-negative bacteria. Other new semisynthetic monocyclic β-lactams especially those designed to inhibit bacterial infections and combat resistance development, are

being studied and examined in clinical trials [12, 13]. All these lactam antibiotics have proven themselves to be invaluable in the treatment of bacterial infections caused by several clinically relevant pathogens. Furthermore, the combination of these β-lactam based antibiotics with bacteria resistant inhibitors has been proven as a promising therapy alternative, as the similar structure of these inhibitors with the lactams help to restore drug activity and minimize bacterial resistance. Therefore, despite being more than 70 years old, β-lactams continue to remain efficient in combating bacterial infections.

OCCURRENCE OF BETA-LACTAM ANTIBIOTIC RESISTANCE AND RELATED MECHANISMS

The introduction of penicillin into the healthcare system in the early 1940s gave humans the upper hand in the fight against bacterial infections. Today, β-lactams remain the most widely used antibiotics due to their high effectiveness, low cost, ease of delivery and limited side effects. However, increased bacterial resistance to antibiotic treatment has been extensively documented and has now become a generally recognized problem for clinicians worldwide, in both hospital and community settings [14].

The mechanisms of antimicrobial drug resistance, particularly the resistance of β-lactam antibiotics have been discussed in several authoritative reviews [13-16]. In general, bacteria resist β-lactam antibiotics *via* one or more of the following three mechanisms. One common mechanism is the production of enzymes that hydrolyze or modify the antibiotics before they can reach the appropriate target site. This is the process seen in a wide variety of clinically important bacteria [15-18]. The second mechanism is the modification of penicillin-binding proteins, the target site for the antibiotic binding. This modification is usually performed by β-lactam resistant cell wall transpeptidases [19]. This allows several pathogens, including the problematic gram-positive *Staphylococcal* and *Streptococcal* species [20-22], to resist the antibiotics. The third mechanism is the up-regulating pumps in bacteria that expel antibiotics from the affected cells, decreasing the antibiotics' access to bacterial targets [23-26]. In the following section, we will limit our discussion to the molecular structural basis for β-lactam resistance.

In general, bacteria can become resistant to β-lactam antibiotics by obtaining a resistant gene from other adjacent or even unrelated genera of organisms. Some bacteria can also develop resistance *via* mutation and natural selection. In such cases, bacterial strains carrying resistance-conferring mutations are selected for survival. While this movement is at the expense of other susceptible strains, it allows the resistant-conferring strains to develop antibiotic immunity.

The most common and efficient mechanism of bacterial resistance to β-lactam antibiotics is the acquisition of plasmid-encoded β-lactamases such as TEM-1 or TEM-2 β-lactamase [27-29]. Recently, the evolution of a new generation of β-lactamase, which bears an extended substrate spectrum, have been found following the widespread application of some β-lactams [30]. All these β-lactamases can destroy penicillin, cephalosporin and other β-lactam based antibiotics before they reach the appropriate target site. Normally, the acquisition of resistant genes is a result of microbial populations sharing genetic materials among themselves. This process has now been speeded up with the β-lactamase genes' integration with genetic elements such as plasmids or transposons, which facilitates the transfer and incorporation of acquired resistance genes to adjacent bacteria. Some bacteria may develop acquired-resistant nucleic acid segments (containing entire genes) from other bacteria that have released their DNA complement into the environment [31].

The *blaZ* gene encoded β-lactamase expression is often induced by β-lactams *via* a novel regulation system that consists of the receptor BlaR and the repressor BlaI (Fig. (**3**)). BlaR is a transmembrane protein with an extracellular sensor

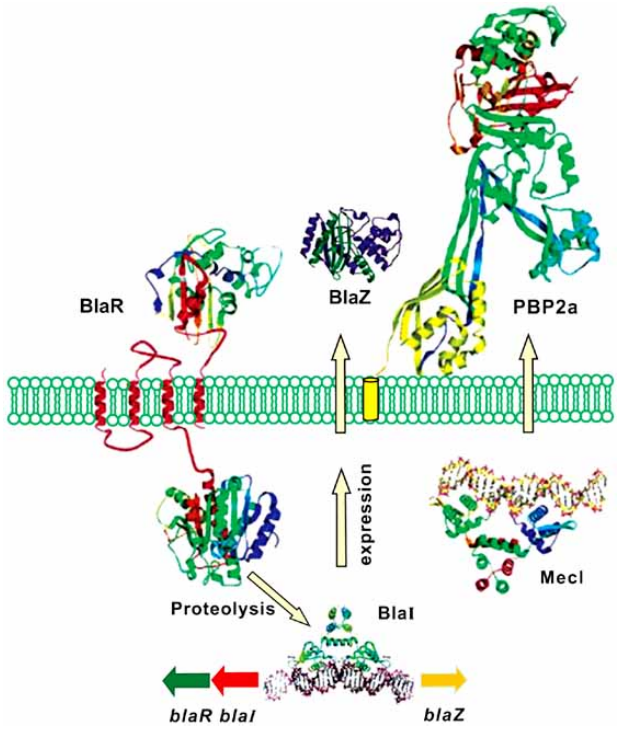


Fig. (3). Scheme of beta-lactam induced expression of β-lactamase and penicillin-binding protein 2a (PBP2a) in antibiotics resistant bacteria [15] (Copyright ACS).

domain on the surface of its plasma membrane that can bind with β-lactam antibiotics through the acylation of the lactam rings. The transmembrane domain contains four membrane-spanning helices and transduces the β-lactam-binding signal across the membrane. There is another domain in BlaR, a zinc metalloprotease domain located in the cytoplasm that is activated by autoproteolysis upon acylation of the sensor domain. The cytosolic repressor BlaI forms a dimer and binds an operator in the *bla* divergon that encodes BlaI, BlaR and the β-lactamase structural gene. Once activated, the BlaR zinc metalloprotease domain will hydrolyze the repressor BlaI protein and then induce the cleavage of BlaI in its dimerization domain, which precipitates transcription of the gene and prevents operator binding, allowing β-lactamase

expression [34-36]. Based on homology and biochemical characteristics such as molecular weight or substrate specificity of the amino acid sequence, the expressed beta-lactamases can be divided into four classes: three serine-dependent enzymes classes (A, C and D) and one metal-dependent class (class B) [37]. Of these classes, class A, to which the plasmid-mediated TEM-1 enzyme belongs, is the most prevalent in clinical isolates. All of these different types of β-lactmamses can catalyze the hydrolysis of β-lactam antibiotics and generate bacterial resistance.

In addition to the production of β-lactamase that resists antibiotics, another beta-lactam-insensitive cell wall transpeptidase, penicillin-binding protein 2a (PBP2a), can be expressed by a similar process [38-39]. The repressor BlaI dimer will not only bind with the *bla* operator but also bind the operator sequence for the *mec* divergon as well, which will allow BlaR and BlaI to mediate the expression of PBP2a. In processes involving encoded enzyme expression, bacteria can also become resistant to antimicrobial agents through new mutations [40]. Spontaneous mutations can cause resistance by modifying or eliminating the active binding sites of the target protein to which the beta-lactams bind, which results in their inability to inhibit the growth of the bacteria. Recent development of antibiotics and other means of bacterial infection control have demonstrated that gram-negative bacteria are more resistant to a large variety of antimicrobial agents than gram-positive strains. It is recognized that the main contribution to this resistance is the presence of efflux pumps that expel the drugs from gram-negative cells [25-28].

NOVEL FLUOREGENIC BETA-LACTAM SUBSTRATES FOR REAL-TIME FLUORESCENCE IMAGING OF GENE EXPRESSION

As β-lactamases (Bla) can efficiently hydrolyze penicillin and cephalosporin-based antibiotics and make bacteria resistant to these antibiotics, considerable effort has been expended in clinical studies to develop new antibacterial agents with different modes of action which can evade the bacterial resistance caused by β-lactamase expression. There have also been efforts to combine the existing β-lactam antibiotics with β-lactamase inhibitors to retain antibiotic effectiveness. However, putting aside the looming threat of dangerous antibiotic-resistant "superbugs", we might be able to consider some other aspects of β-lactamase that will make it a nearly ideal tool for certain biological applications. Upon enzymatic cleavage of a cephalosporin derivative, the relevant products will undergo a facile rearrangement which can result in the fragmentation of the molecule. This feature of enzyme-catalyzed fragmentation makes the enzyme an ideal reporter for optical measurement of gene expression in single living cells [41].

Cells respond to the intrinsic development programs and extrinsic stimuli through the precise and selective regulation of gene expression. Understanding the regulation process, will greatly benefit from an assay with high sensitivity and fidelity at the single cell level. Live-cell fluorescence based assays are among the most powerful tools for *in vitro* and *in vivo* detection of the proteins or the enzymes. Current assays based on the reporter genes such as secreted alkaline phosphatase [42], β-galactosidases [43] and firefly luciferase [44] typically require cell permeabilization or lack single cell resolution. Although the green fluorescent proteins (GFP) are nearly ideal labels for fluorescent tagging of recombinant proteins in living cells, as reporters of gene expression they are not perfect due to their lack of enzymatic amplification [45-46].

TEM-1 β-lactamase (Bla), one of the monomeric isomer products of the ampicillin resistance gene ('*amp'*), has proven to be an attractive biosensor for detecting biological processes and protein-protein interactions *in vitro* and *in vivo* because of its desirable properties, such as small enough (29kD) to be easily expressed in eukaryotic cells without noticeable toxicity and with no interference from mammalian enzymes [47-48]. The technological breakthrough that first enabled the application of Bla as a catalytic reporter was the chemical synthesis of the sensitive and membrane-permeable fluorogenic cephalosporin-type substrate, CCF2/AM (Fig. (**4**)) [41]. The basic design of the substrate relies on the intramolecular Fluorescent Resonance Energy Transfer (FRET). The coumarin donor is connected in the 7'-position of cephalosporin in CCF2/AM. Fluorescein, as the acceptor, is attached at the 3'-position through a stable thioether linkage. After diffusion of nonpolar CCF2/AM through the plasma membrane, four labile esters are hydrolyzed by non-specific intracellular esterases to generate CCF2. If the cell is not expressing the TEM-1 Bla reporter enzyme, the intact molecule of CCF2 will emit green fluorescence at 520nm when excitated at 409 nm, owing to FRET between the coumarin donor and the fluorescein acceptor. However, if Bla is present in the cell, CCF2 will be readily hydrolyzed, resulting in the breaking of the FRET and an increase in the blue coumarin fluorescence (447nm). CCF2 exhibits reasonable catalytic properties and can detect 5fM of lactamase readily *in vitro* (Table **1**). Noninvasive imaging of blue and green fluorescence generates a ratiometric signal that is minimally influenced by diversities in cell size and substrate uptake. As the first developed fluorogenic probe for Bla, CCF2/AM was able to function as a biosensor to examine the promoter/ regulator activities in living mammalian tissue culture cells [49] and to monitor constitute and inducible proteins interactions [50-52].

Recently, Rao reported that CCF2/AM could image tetrahymena ribozyme splicing activity in live cells. This reporter system allowed high throughput screening with flow cytometry of single living mammalian cells for a direct and facile selection of desired ribozyme variants *in vivo* [53-54]. Lippard also demonstrated the high throughput screening over 3600 reaction products of platinum based antitumor drugs for their ability to inhibit transcription of Bla in the BlaM Hela cell line by monitoring the cleavage of CCF2/ AM. Four species were identified among the reaction products from this screening. Three of them have been previously determined to be active cisplatin analogues and another one represents a new kind of antitumor drug candidate similar to ZD0473, a recently reported antitumor analogue [55]. Similarly, Zuck [56] presented a cell-based reporter gene assay for the identification of inhibitors of hepatitis C virus replication by using CCF2/ AM. Leppla and Bugge [57] developed

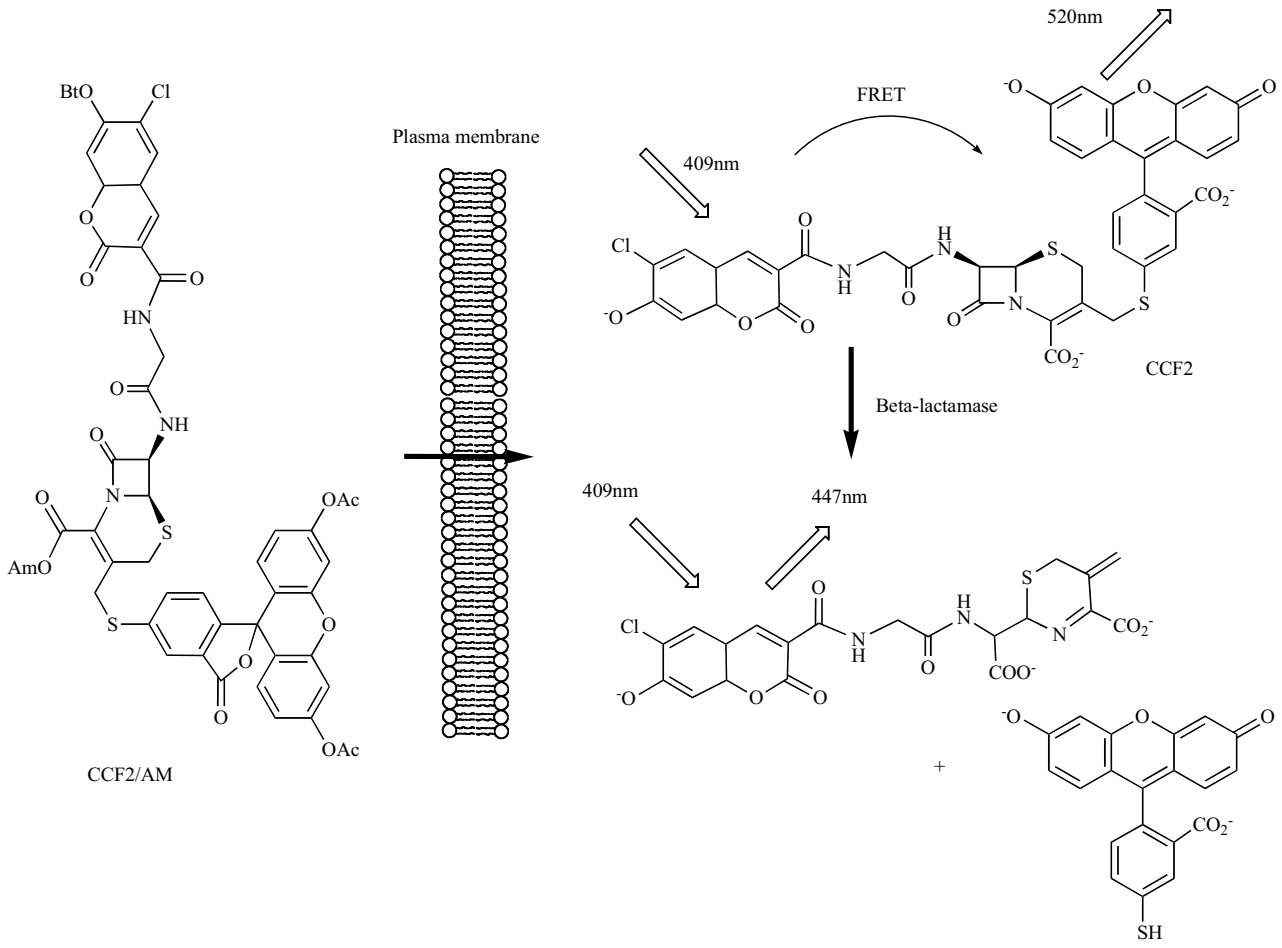


Fig. (4). Mechanism of action in the FRET in CCF2/AM [41].

a simple, sensitive and noninvasive assay that used reengineered anthrax toxin-β-lactamase fusion proteins with altered protease cleavage specificity to visualize specific cell-surface proteolytic activities in single living cells. The assay could be used to specifically image endogenous cell-surface furin, urokinase plasminogen activator metalloprotease activity. Cunnningham and his coworkers explained that polystyrene beads could be successfully used for establishing fluorescence-activated cell sorting to sort cells with the Bla reporter gene by using the substrate CCF2 [58]. This is a very useful whole-cell assay for the high throughput screening of drugs.

Based on the recent trends in the reporter technology, lactamase will likely continue to grow in popularity as it is adapted for applications in a great deal of systems. Rao [59] suggested a new class of small fluorogenic substrates that

Table 1. Kinetics Constants for β-Lactamase Catalytic Hydrolysis of Different Cephalosporin Fluorogenic or Bioluminogenic Substrates [41, 58, 59, 61, 62]

Compound	K_m(μM)	k_{cat} (s^-)	k_{cat}/K_m ($s^- M^-$)
CCF2 [41]	23 ± 1	29 ± 1	1.26×10^6
CC1 [59]	70 ± 7	52 ± 1	7.4×10^5
CC2 [59]	350	10	2.86×10^4
CR2 [59]	17 ± 3	114 ± 12	6.70×10^5
CNIR1 [61]	6.7 ± 1.5	0.8 ± 0.1	1.19×10^5
Bluco [62]	90	1.0	1.11×10^4

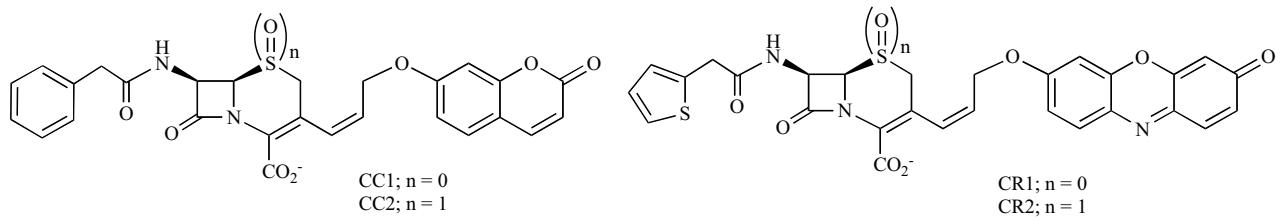


Fig. (5). Structures of CC1, CC2, CR1 and CR2 [59].

would expand the application of Bla as a biosensor. As shown in Fig. (**5**), these small fluorogenic substrates can work by releasing a phenolic fluorescent dye from a vinylogous cephalosporin. In their design, 7'-hydroxy of umbelliferone or resorufin were connected to the 3'-position of cephalosporin. Intact parent substrates exhibited no fluorescence due to alkylation of the phenolic group of the fluorescent dyes. Treatment by β-lactamase leads to spontaneous release of fluorescent molecules and the production of fluorescent signals. These types of substrates exhibit fast kinetics toward Bla and their stability can be further improved by oxidation of sulfide to the sulfoxide in the six-membered ring of the cephalosporin nucleus (Table **1**). Moreover, a membrane-permeable acetoxymethyl ester of CR2 (CR2/ AM) was able to image the Bla activity in Bla-stably transfected C6 Glioma cancer cells as shown in Fig. (**6**).

All of these small molecular weight β-lactam fluorogenic substrates are ideal for imaging β-lactamase *in vitro* and in cell cultures. However, none of them works in intact tissues or whole organisms because of the poor tissue penetration and light scattering of the short excitation and emission wavelength. Over the past decade, infrared and/or near-infrared spectroscopy has attracted a great deal of academic attention in molecular imaging studies for living subjects because its long wavelength leads to less photo damage to cells, produce less autofluorescent background and provide better sensitivity and tissue penetration [60]. Rao and his coworkers [61] reported a comprehensive study on the design and preparation of a new class of small cell-permeable near-infrared (NIR) fluorogenic β-lactam substrates for β-lactamase detection and their application in imaging gene expression in living mammalian cells.

As shown in Fig. (**7**), one carbocyanine dye Cy5 with maximum emission at 670nm and another quenching group Qsy21 with a peak absorbance at 660nm was chosen as a FRET pair. Cy5 was connected to the 7'-amino of the cephalosporin through a glycyl linkage, Qsy21 was attached to the 3'-position *via* a linker of amino thiophenol and cysteine residue. Introduction of one fully acetylated D-glucosamine in the cephalosporin nucleus (CNIR4) was found to improve the staining of the Bla stably transfected C6 glioma cells presumably with involvement of endocytosis in the uptake (Fig. (**8**)). In CNIR4, two sulfoxide functional groups were added into Qsy21 to enhance the aqueous solubility. All the NIR fluorogenic β-lactam substrates were stable in aqueous buffer and exhibited reasonable catalytic efficiency toward enzyme treatment. Through modification of the NIR lactam substrates' structure by the introduction of another longer wavelength NIR fluorescent dye Cy5.5, Rao *et al.* demonstrated the successful real-time imaging of Bla expression in a C6 glioma tumor in living mice (unpublished work). In addition to fluorogenic substrates, after coupling the D-Luciferin, one commonly used substrate for the bioluminescent enzyme firefly luciferase (fLuc), to the 3'-position of cephalosporin *via* ether bond, Rao and his team [62] recently reported the first bioluminogenic substrate (Bluco) for β-lactamase activity. The *in vivo* imaging results indicated that

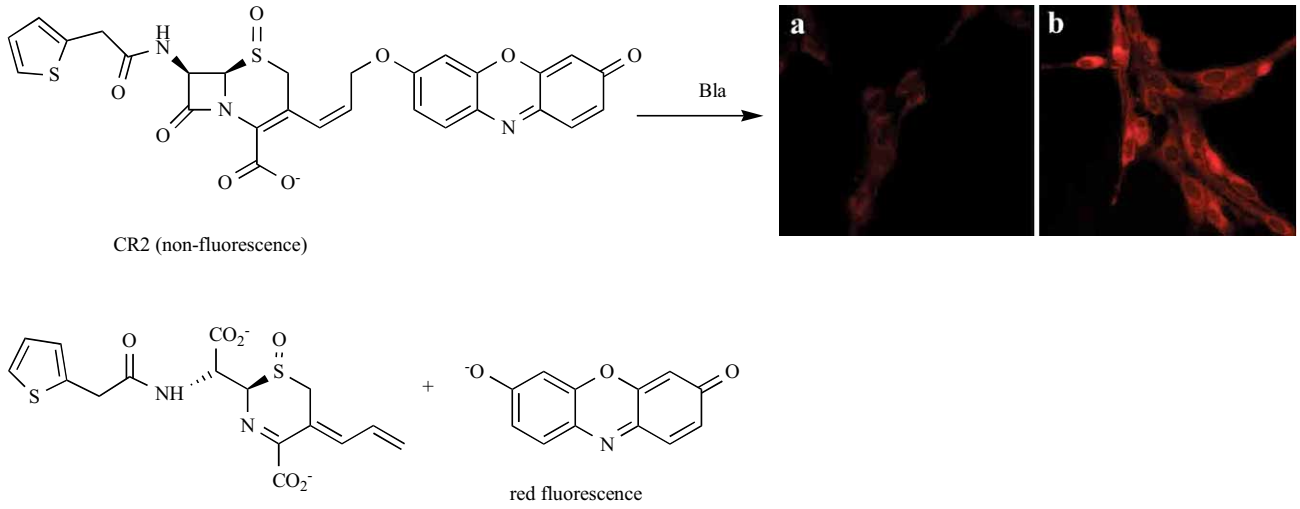


Fig. (6). Fluorescence images of wild-type (a) and Bla-stably transfected (b) C6 glioma cells incubated with CR2/AM [59].

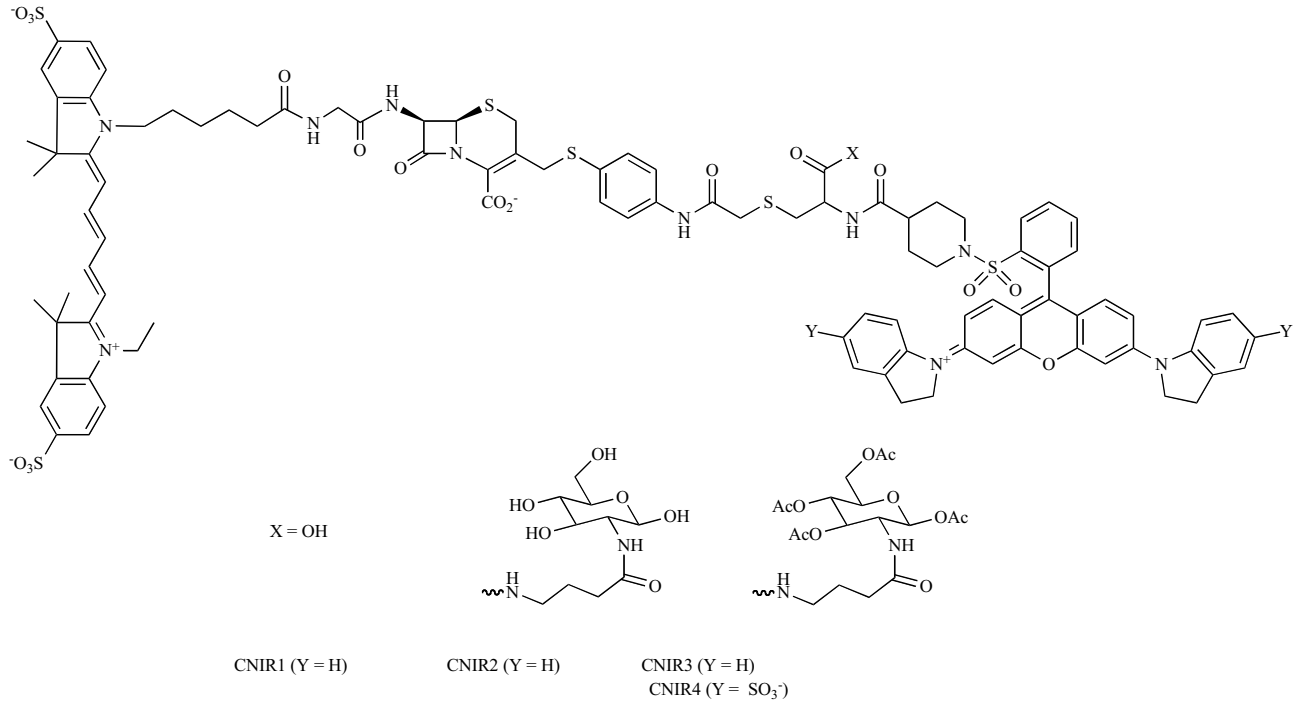


Fig. (7). Structures of CNIR1, CNIR2, CNIR3 and CNIR4 [61].

about 15-25 folds signal contrast could be identified after administration of Bluco *via* tail vein injection into mice which had been implanted tumors respectively on the left and right rear thigh with the Bla and fLuc cotransfected or fLuc transfected COS7 cell line (as shown in Fig (**9**)). The maximum emission could be detected at about 30 min post-injection, which could last for approximately 30 min. As the first developed bioluminogenic substrate, Bluco was proven to be useful in the practical application for *in vivo* imaging of the β-lactamase expression.

In comparison to traditional organic fluorophores, nanocrystal quantum dots (QDs) have extreme advantages in their high quantum yields and photostability, size tune-up fluores-

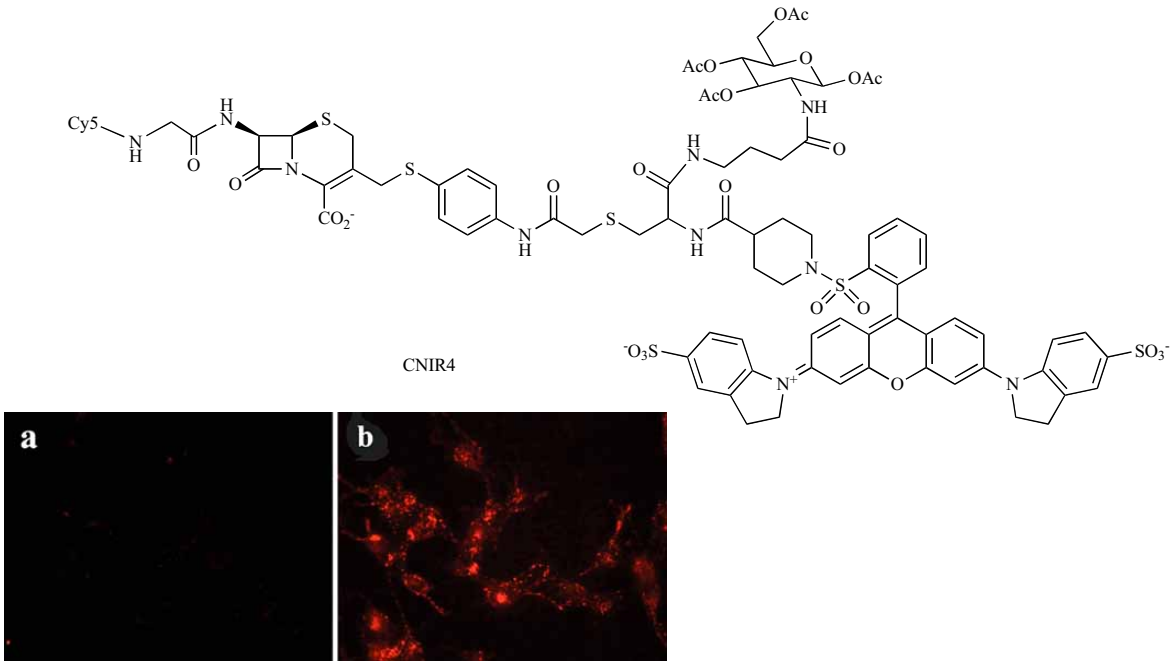


Fig. (8). Structure of CNIR4 and fluorescent images of wild-type (a) and Bla stably transfected (b) C6 Glioma cells staining with CNIR4 [61].

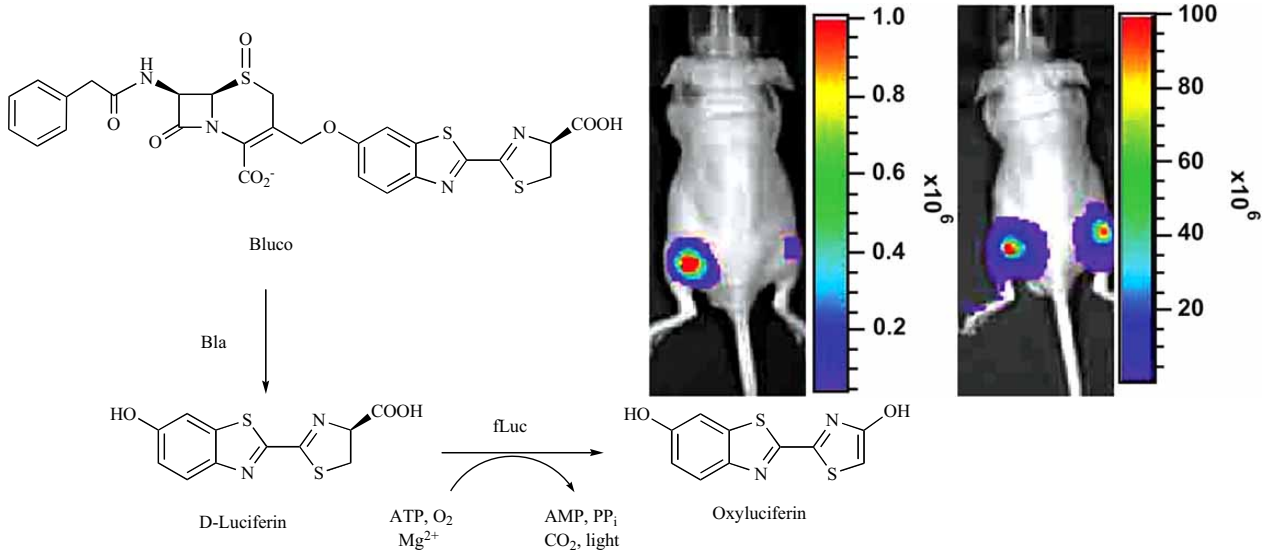


Fig. (9). Structure of Bluco and *in vivo* bioluminogenic imaging Bla activity in living mice. COS7 cells (1×10^6) were cotransfected with Bla and fLuc and injected into left rear thigh of a nude mouse, and the same number of cells transfected with fLuc only were injected at the right rear thigh [62].

cence, extensive applications in near infrared windows as well as their broad excitation features but narrow emission properties which are suitable for optical imaging and multiplexing [63]. Very recently, Xu [64] reported a biotinylated β-lactam substrate which was labelled with a NIR dye Cy5 and immobilized on the surface of quantum dots through binding pre-coated biotin to streptavidin (as shown in Fig (**10**)). Based on FRET, they found that the distance between substrates and the quantum dot surface, and the density of substrates were important for the enzyme function. The self-assembled quantum dot probe exhibited excellent properties for detecting β-lactamase activity.

All the recent developments of fluorogenic and bioluminogenic β-lactamases substrates indicate that the "old drug" β-lactam antibiotics can be used as novel biosensors for efficient reporting of the biological pathway *in vitro* and *in vivo*. In addition, it may have potential in cancer diagnosis, high throughput drug screening as well as imaging-guided surgery and therapy.

BETA-LACTAMASE DEPENDENT ANTITUMOR PRODRUGS

One of the most efficient approaches in clinical cancer therapy lies in tumor cell-selective cytotoxicity with minimum systemic drug exposure. It has been applied successfully in clinical studies to target all kinds of tumors and increase local drug concentration by using monoclonal antibodies (mAb) as drug conjugates. This strategy has proven to be useful but still has several shortcomings; for example, there are a limited number of drug molecules available to attach to mAb, there is unspecific release of the drugs before anchoring on the surface of the tumor, and mAb conjugates themselves have limited cell penetration properties. Recently, an alternative targeting approach using antibody-

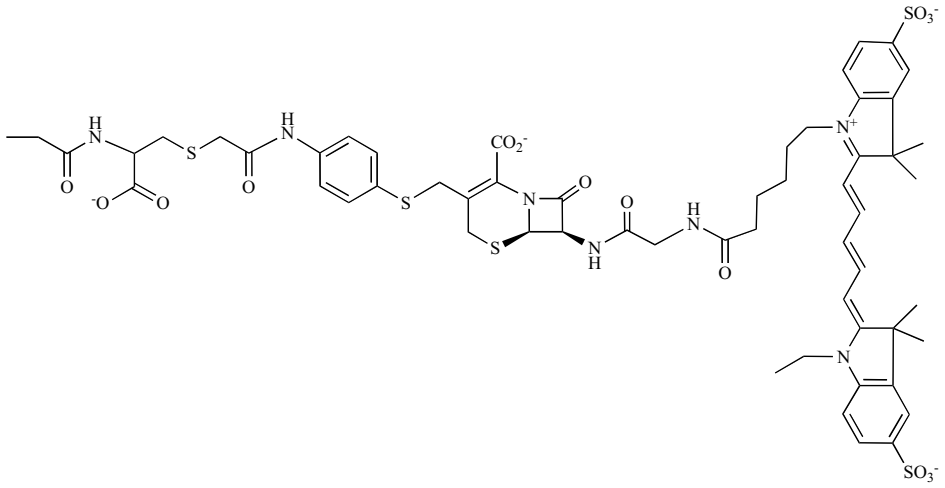


Fig. (10). Structure of QDot-based nanosensor for detecting Bla [64].

directed enzyme prodrug therapy (ADEPT) has received considerable attention [65-66]. ADEPT is a two-step approach to drug delivery in cancer therapy, where the enzyme is firstly identified and then engineered to the mAb which localizes on the targeted cancer cell surface. Subsequently, the tumor-selective localization or expression of the prodrug-activating enzyme can produce relatively high local concentrations of the active drug at the tumor site after administration of the prodrug. In this section, we will mainly focus on the recent development of β-lactamase/prodrug combinations as ADEPT in antitumor research.

Beta-lactamases are particularly useful for ADEPT systems due to their unique substrate specificity, through hydrolytic cleavage of the β-lactam ring in penicillins, cephalosporins and some closely related structures. This enables the activation of a variety of lactam-based prodrugs with high plasma stability, a relatively low risk of toxicity in patients, and most importantly, minimal interference from mammalian enzymes. Thus far, utilization of β-lactamase substrate moieties allows for the covalent attachment of a wide variety of potent antitumor agents. These highly desirable properties and the extensive knowledge surrounding cephalosporin and penicillin chemistry offer the possibility of exploiting the β-lactam based prodrugs for potential applications in ADEPT [67-76]. Fig. (**11**) lists the structures of some prodrugs for β-lactamase. All these cephalosporin-

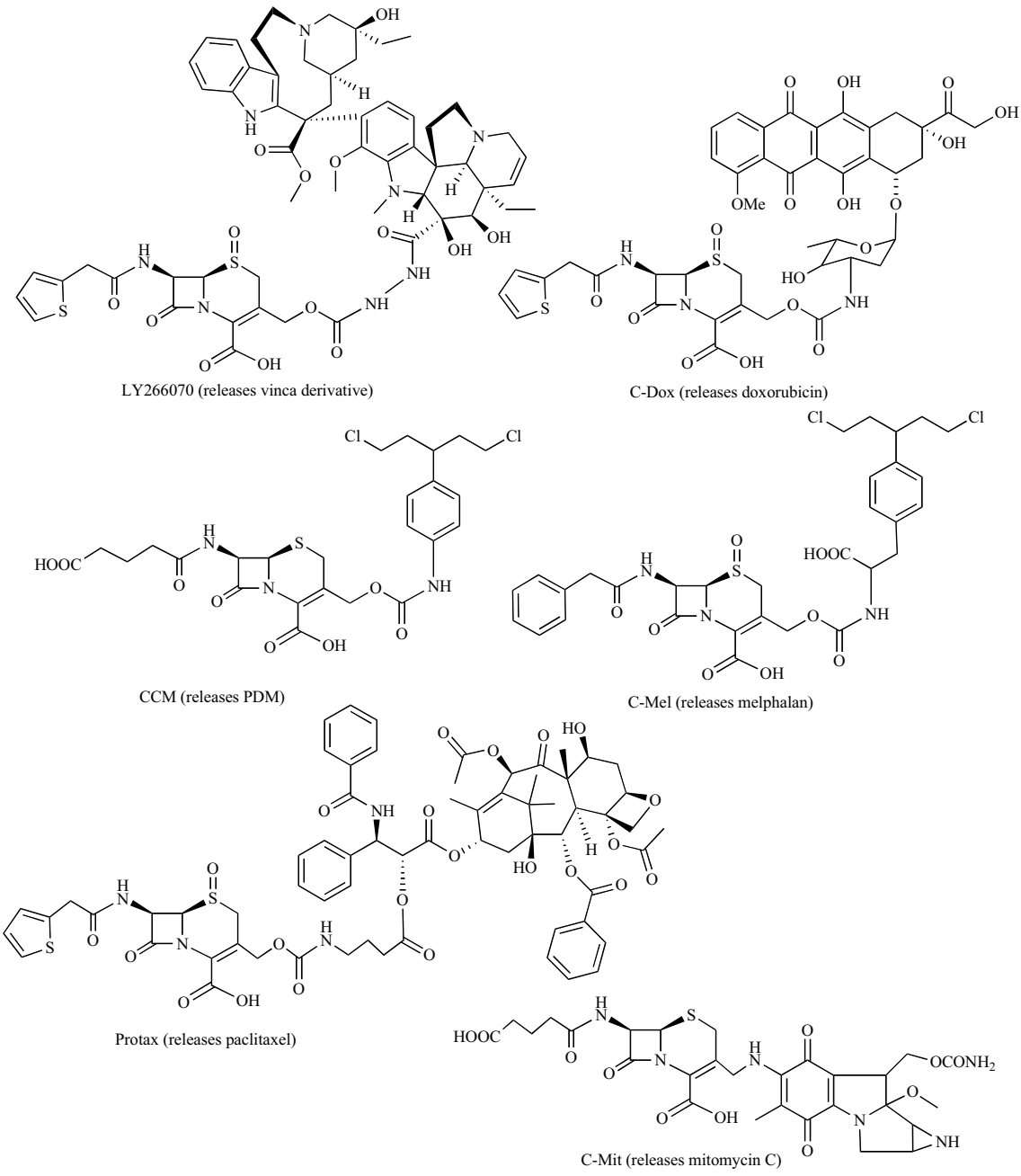


Fig. (11). Structures of some β-lactamase targeted prodrugs.

Table 2. Kinetics Constants and IC_{50} Values for β-Lactamase Hydrolysis of Different Cephalosporin Based Prodrugs [71-74, 76]

Compounds	K_m(μM)	k_{cat} (s^-)	Mean IC_{50} (μM)	
			Bla (-)	Bla(+)
LY266070 [71]	160	1700	10^{-2}	2×10^{-3}
C-Mit [72]	119 ± 20.6	248 ± 15.1	30.1 ± 12.7	3.0 ± 1.2
C-Dox [72]	38 ± 7.8	30 ± 3.0	7.4 ± 1.2	0.75 ± 0.17
CCM [73]	31.1	116	25 ~ 50	1.5
Protax [74]	$k_{cat}/K_m = (1.4 \pm 0.1)\times10^5$ (s M)$^{-1}$		10 fold less toxic	
C-Mel [76]	218	980	53	1.3

prodrugs displayed favourable properties and pronounced antitumor effects in *in vitro* and/or *in vivo* tests (Table **2**).

The first published example of a β-lactam based anticancer prodrug concerned cephalosporin methotrexate (MTX) derivatives (shown in Fig. (**12**)), which were identified by Jungheim and Shepherd in the early of 1990's [69]. They covalently attached the antitumor agent methotrexate (MTX) to the C-3' position of the cephalosporin nucleus through the esterification of the γ-carboxylic acid on the MTX. This antitumor prodrug was then treated in aqueous solution with β-lactamase and free MTX was released rapidly. However, the cytotoxicity of this prodrug could not be distinguished from that of free MTX due to the nonspecific cleavage of the acetate ester in the cell culture test. Similarly, Jungheim and Shepherd also developed another vinca alkaloid based antitumor prodrug as showed in Fig. (**11**) [71]. Vinblastine is a commonly applied anticancer agent used to treat certain kinds of cancers such as Hodgkin's lymphoma, non-small cell lung cancer, breast cancer and testicular cancer. One potent analogue of this agent, desacetylvinblastine hydrazide (DAVLBHYD) was attached to the cephem C-3' position through the *p*-nitrophenyl carbonate moiety in the intermediate. The carbonate group in the intermediate structure was then replaced readily by the potent antitumor drug. In order to improve the kinetic stability and aqueous solubility of the prodrug, the sulfur atom on the C-1' of the cephalosporin nucleus was oxidized into sulfoxide. Hydrolysis of this prodrug by β-lactamase could release the anticancer agent rapidly. The results of tumor cell tests in LS174T human colorectal tumor cell lines indicated a reproducible 5–fold difference in cytotoxicity between the prodrug and free DAVLBHYD. In addition, the prodrug exhibited less toxicity within the short incubation period. The therapeutic effects of the enzyme–mAb conjugates in combination with the DAVLBHYD prodrug were studied in mouse xengraft and significant regression of established tumors has been observed. Doxorubicin is a widely used DNA-interacting antitumor agent in chemotherapy. Jungheim and Shepherd prepared a carboxamide type doxorubicin based anticancer prodrug [69-70, 72]. When used in combination with enzyme–mAb conjugates, the prodrug displayed a broad spectrum of antitumor activity. However, the cytotoxicity in tumor xenograft studies was found similar to that of the original drug, doxorubicin. Alexander described a cephalosporin nitrogen mustard antitumor prodrug based on an enzyme antibody catalysis system [67]. They prepared a series of cephem carbamates with the antitumor agent nitrogen mustard at the C-3' position. Their work was extended in several laboratories to other nitrogen mustard derivatives [68]. All these substrates were activated by the broad scale β-lactamase enzymes from *Enterbacter cloacae*. *In vivo* colorectal xenograft tumor

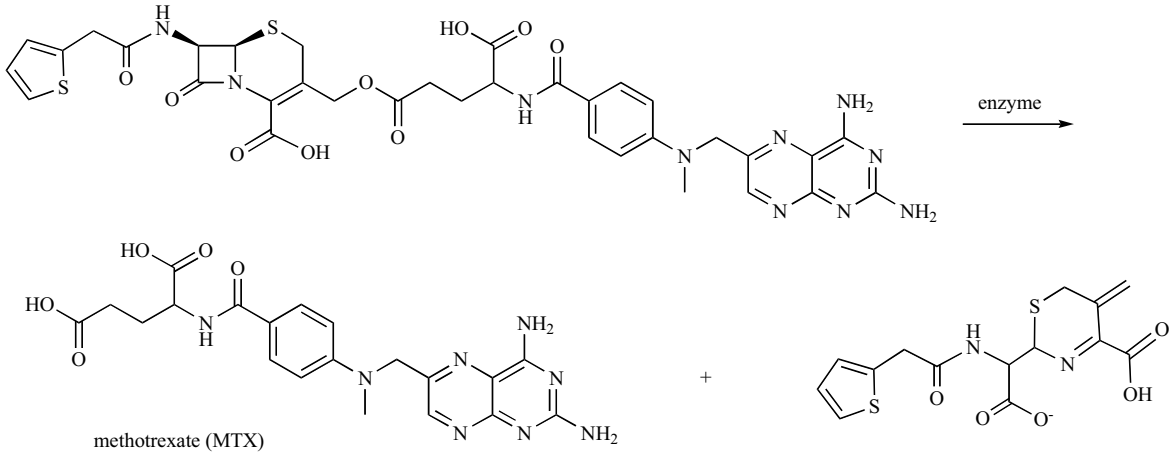


Fig. (12). Structure and reaction scheme of cephalosporin methotrexate prodrug [69].

model studies indicated that these nitrogen mustard prodrugs exhibited significant efficacy in tumor growth suppression. Paclitaxel and mitomycin are two other well known cytotoxic agents used to treat cancer diseases. Both of these two drugs can be used as antitumor β-lactamase prodrugs after conjugation of the free drugs at the C-3' of the cephalosporin nucleus [72-73]. In the cephalosporin-paclitaxel prodrug, protax, one γ-aminobutyric acid (GABA) was incorporated as a linker due to the bulky structure of the free drug molecule. When exposed to β-lactamase in phosphate buffered saline solution, paclitaxel is released slowly from the prodrug protax. *In vitro* SK-BR-3 cancer cell tests demonstrated that the prodrug exhibited ~10 times less toxicity than the free paclitaxel [74]. The Bristol-Myers group explored the possibility of delivering mitomycin through the antibody directed enzyme prodrug process. Cytotoxicity assays performed on H2987 lung adenocarcinoma and clone 62 melanoma cell lines indicated that the mitomycin prodrug was approximately 40- and 10- times less toxic than the free drug. It was also demonstrated that the less toxic prodrug was activated in an immunologically specific manner by L6-F(ab')-β-lactamase and 96.5-F(ab')-β-lactamase conjugates selective to H2987 and clone 62 cells respectively [75]. Hanessian [77] reported the synthesis of a platinum based antitumor beta-lactamase prodrug. He used C-3' hydroxyl cephem as the starting material and DACCP, a well known platinum antitumor agent, conjugated at the C-3' position through the corresponding diacid derivative as the linker to afford the desired prodrug. However, no *in vitro* and *in vivo* activities for this prodrug were documented yet.

Recently, Smyth and coworkers demonstrated [78] another standard cephalosporin structure which incorporated an S-aminosulfenimine side chain at the 7'-position, and behaved as a β-lactamase-dependent dual-release prodrug (Fig. (**13**)). They initially prepared the cephalosporin based dual–release prodrug by conjugating two enzyme inhibitors aminoglutethmide at the 3'-position and coumate *via* the S-aminosulfenimine at the 7'-position. Both inhibitors were used clinically to reduce the production of estrogen in hormone–dependent breast cancer. Addition of the enzyme led to the rapid hydrolysis of the intact cephalosporin structures and to the rapid release of both inhibitors at 3'-position and the S-amino moiety. These estrogen forming enzymes were present in significantly higher concentrations in the tumor tissues than in the area considered as normal, therefore, it might be appropriate for the ADEPT mode of targeted drug delivery.

Penicillin can also be used as a prodrug to induce the release of the cytotoxic agents upon reaction with the β-lactamase. Smyth [79] reported penicillin derivatives by conjugating S-aminosulenimine R(R')NSN= at the 6-amino position and established one reaction pattern not previously encountered in penicillin chemistry (Fig. (**14**)). This special and stable structure could lead to the cleavage of the β-lactam ring and the release of the sulfur-attached moiety as R(R')NH rapidly and quantitatively. Another reported β-lactamase-dependent 'prodrug' was reported by the same group. Attachment of 7-hydroxy-4-methylcoumarin was conducted as the releasable group by incorporation of a vinyl ester linkage at C-6' to afford a penicillin structure that could function as a fluorescence-based reporter substance/diagnostic for the presence of low levels of β-lactamase enzyme in solution [80].

Penicillins and cephalosporins are prototypic examples of novel classes of β-lactamase dependent prodrugs in which the enzyme catalyzes the cleavage of the lactam ring to induce the rapid release of free drugs at the 6-, 7-, or 3-position. Certain N-methylthio-substituted β-lactam antibiotics have also shown promise, having exhibited direct DNA-damaging and apoptosis-inducing activities in various tumor cells. For example, Kazi demonstrated that this lactam potently inhibited the colony formation of human prostate cancer cells and triggered programmed cell death in the hu-

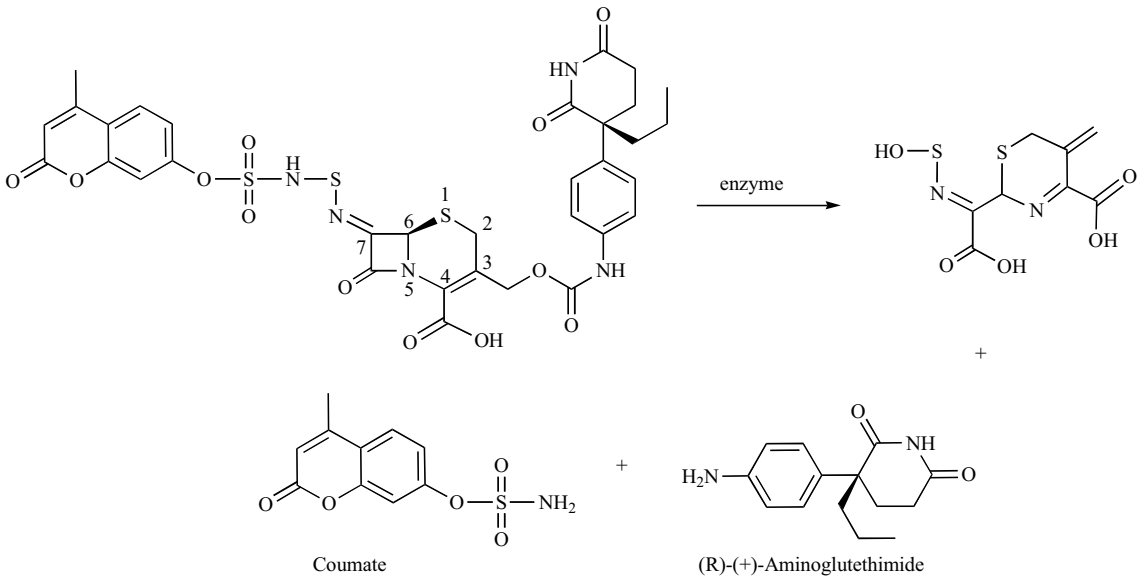


Fig. (13). Structure of dual release cephalosporin prodrug based on S-aminosulfenimine[78].

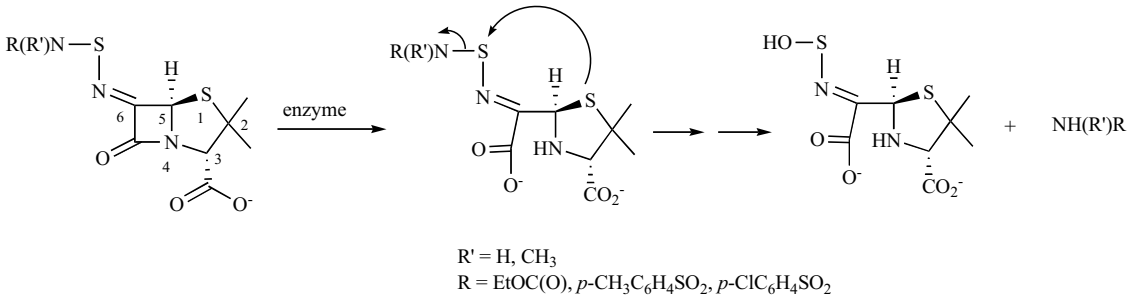


Fig. (14). Structure and reaction scheme of S-aminosulfenimine based penicillin prodrug.

man breast, prostate, head and neck cancers [81-82]. Banik [83] revealed that stereoselective synthesis of β-lactams by the Staudinger reaction would generate the potent activity against nine human cancer cell lines. Recently, Rothstein [84] documented a new property of the β-lactam antibiotics to activate the gene for a neurotransmitter transporter, which was the first evidence of stimulatory pharmaceutical modulation of glutamate transport. Although the mechanism of action was as yet unknown, this study provided a new pathway for manipulation of glutamate transmission in disease. All these results indicate that synthesis and evaluation of β-lactams are a promising area for further development in anticancer research.

BETA-LACTAM ANTIBIOTIC DERIVATIVES FOR PROTEASE INHIBITION

The β-lactamases catalyze the hydrolysis of the β-lactam amide bond of a variety of β-lactam antibiotics destroying their antibacterial activity by utilizing of a serine hydroxyl as a key nucleophile at the bacterial enzymes' active site. These β-lactamase inhibitions act in the same irreversible manner as the reactions that acylate serine hydroxyl in serine protease inhibition and cysteine thiol in the inhibition of cysteine protease. It is reasonable to hypothesize that the acyl enzyme formed by the nucleophilic attack on the β-lactam nuclei and the ring opening of the β-lactam nuclei may last long enough to function effectively as inhibitors to inhibit other serine and cysteine proteases [85-86].

The serendipitous discovery of benzyl clavulanic acid to inhibit protease was reported by Zimmerman in 1986 [87]. As the first β-lactam inhibitor of mammalian serine proteases, the benzyl ester was found to have good activity against human leukocyte elastase (HLE). Knight and his team [88] proposed one mechanism for inhibition of HLE based on the 7-methoxy-substituted cephalosporin derivatives. As shown in Fig. (**15**), in the first step, the enzyme active site Ser 195 attacked the β-lactam ring to form a tetrahedral intermediate. This intermediate collapsed and resulted in the opening of the lactam ring and elimination of the leaving group on C-3'

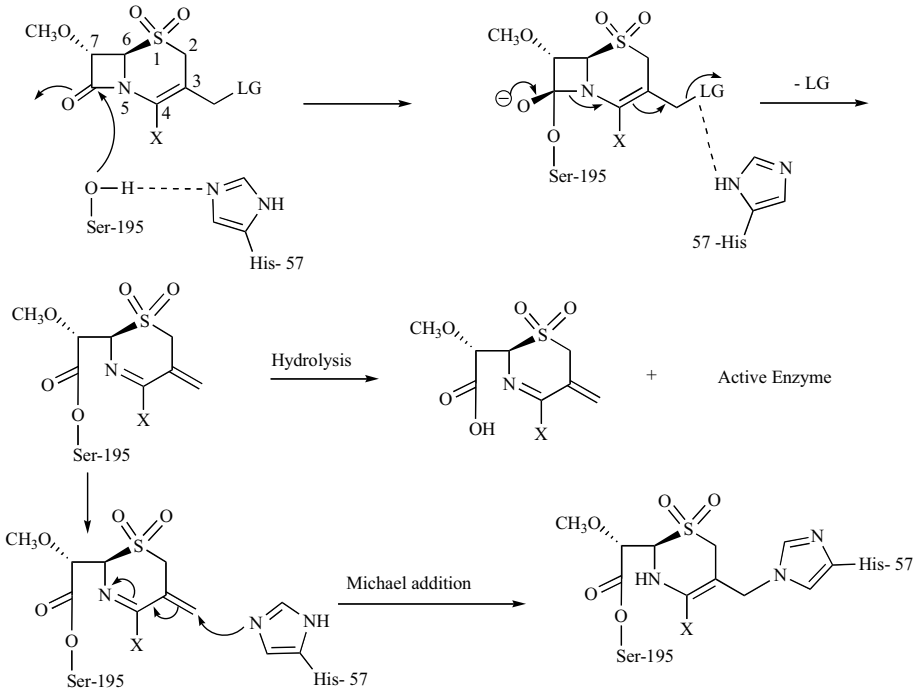
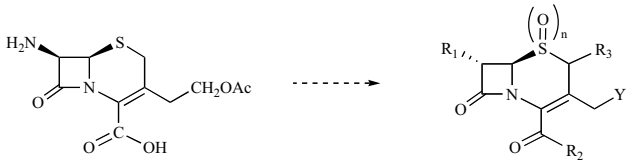
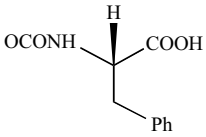
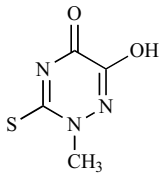
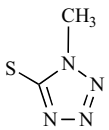
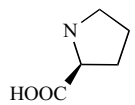


Fig. (15). Proposed chemical mechanism for the inhibition of HLE by the cephalosporin derivative [89].

position. Then the acyl enzyme intermediate underwent hydrolysis to produce the active enzyme, followed by Michael addition at active site His 57 in C-3' position to form a stable inhibitor-enzyme complex. Such mechanistic studies led to the discovery of cephem derivatives not only behaving as a specific inhibitor against HLE, but also exhibiting a similar effect *in vivo*, blocking the pathologic processes caused by the excess amounts of this enzyme. Table **3** summarizes some currently synthesized cephem derivatives as potent inhibitors for HLE [89]. Buynak [90] discovered the HLE inhibition of some cephalosporins derivatives with various modifications at C-7' position such as 7-alkylidenecephalosporin (shown in Fig. (**16**)). Modelling studies indicated that the S1 pocket of the enzyme could accommodate the alkylidene substituent readily, which could provide favourable binding energy for the unoxidized cephems and subsequent

Table 3. Inhibitory Properties of HLE by Some Cephalosporin Derivatives [89]



R_1	R_2	R_3	Y	n	$K_{obs}/[I]$ $M^{-1}s^{-1}$
H	OBu-*t*	H	OAc	0	ND
Cl	OBu-*t*	H	OAc	0	ND
Cl	OBu-*t*	H	OAc	2	161000(9500)
OCH_3	OBu-*t*	H	OAc	0	ND
OCH_3	OBu-*t*	H	OAc	2	19000 (1500)
OCH_3	OCH_2Ph	H	OAc	2	ND
OCH_3	HN-Bu-*t*	H	OAc	2	2200 (100)
OCH_3	$N(CH_3)CH_2Ph$	H	OAc	2	9600 (800)
OCH_3	OBu-*t*	H	SOPh	2	16800(1600)
OCH_3	*t*-Bu	H		2	63900 (4000)
OCH_3	OBu-*t*	H		2	8600(500)
OCH_3	OBu-*t*	H		2	1880(60)
OCH_3		H	OAc	2	18000 (100)
OCH_3	OCH_2Ph-p-CO_2tBu	α-CH_3	OAc	2	118000(3900)
OCH_3	OBu-*t*	α-CH_3	H	2	125200 (100)

ND = Not determined

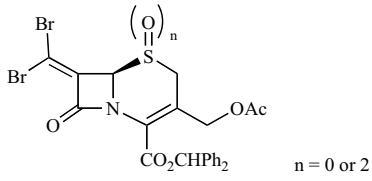


Fig. (16). Structure of 7-alkylidenecephalosporin derivatives [90].

participation of His-57 in the nucleophilic substitution of bromide.

All the cephem derivatives behaved not only as specific inhibitors for HLE, they also served as potent inhibitors to some extent toward other mammalian proteases such as porcine pancreatic elastase (PPE), α-chymotrypsin and human cytomegalovirus proteases [91-95]. With the crystallographic structure of tert-butyl 7α-chlorocephalosporanate sulfone complex with porcine pancreatic elastase (PPE), Navia [96] proposed another slightly different mechanism than in HLE, as shown in Fig. (**17**). After attacking the cephalosporin derivative by active Ser195, the lactam ring was opened and released the acetoxy group at the C-3' position. The acyl enzyme then underwent elimination of HCl to generate the unsaturated form of the enzyme. Finally, the acyl enzyme would either undergo hydrolysis to afford the active enzyme or react with another active site at His 57 to afford a second covalent bond between the enzyme and substrate.

As efficient enzyme inhibitors, the structural alterations for the cephalopsporin derivatives were prerequisite for their molecular recognition toward the active site of the target enzyme. Usually, the substituents at the C-7' α position must be small and short in order to fit into the shallow S1 binding pocket of enzymes. Oxidation of 1-S sulfides or sulfoxides into sulfones could improve inhibitory potency and enhance more hydrogen bonding opportunities. Substitution at the C-2' position would lead to both an increase in inhibitory property against target enzymes and the stability of the obtained cephem structure. The substituents at C-3' positions had a positive effect on the reactivity of β-lactam. Replacement of acetoxy at C-3' with spacious electron-withdrawing and leaving groups significantly enhanced the enzyme inhibitory properties. Modelling indicated that functional groups at the C-4' position of the β-lactam ring would not interact strongly with the enzyme, however, these functional groups could be released upon inhibition and had an important influence on the *in vivo* activity [92, 97- 98].

Like cephalosporin, penicillin can also serve as a template for the inhibition of serine proteases such as human leukocyte elastase or cysteine proteases such as calpains [89, 92]. Some typically modified penicillins proteases inhibitors are listed in Fig. (**18**). As inhibitors against serine proteases, the binding of penicillin to serine proteases was not so sensitive to the size of the side chain in the enzymes. Therefore, in contrast to the most effective cephalosporins inhibitors, penicillins derivatives exhibited less pronounced inhibitory properties and poorer hydrolytic stability.

Besides cephalosporin and penicillin bicyclic β-lactam structures, some monocyclic β-lactam [99-100] and other cyclic lactam systems (a few chemically related structures such as gamma- [91] or delta-lactams or azetidines [101, 102], pyrrolidine-t-lactams [103-107]) have also been proven to be very promising sources of inhibitors for enzymes including protozoan enzymes (e.g. cruzain, falcipain), plant enzymes (papain) and mammal enzymes such as human leucocyte (neutrophyle) elastase etc. All these derivatives likely have the same site and mechanism of action. The related structures are listed in Fig. (**19**).

CONCLUSION AND FUTURE PERSPECTIVE

Significant advances have been achieved in developing various β-lactam antibiotics derivatives as potent drugs to improve the activity against bacteria and to act as efficient inhibitors to overcome bacterial resistance. β-lactam antibiotics will continue to grow in popularity as important antimicrobial agents in the treatment of bacterial infections in hu-

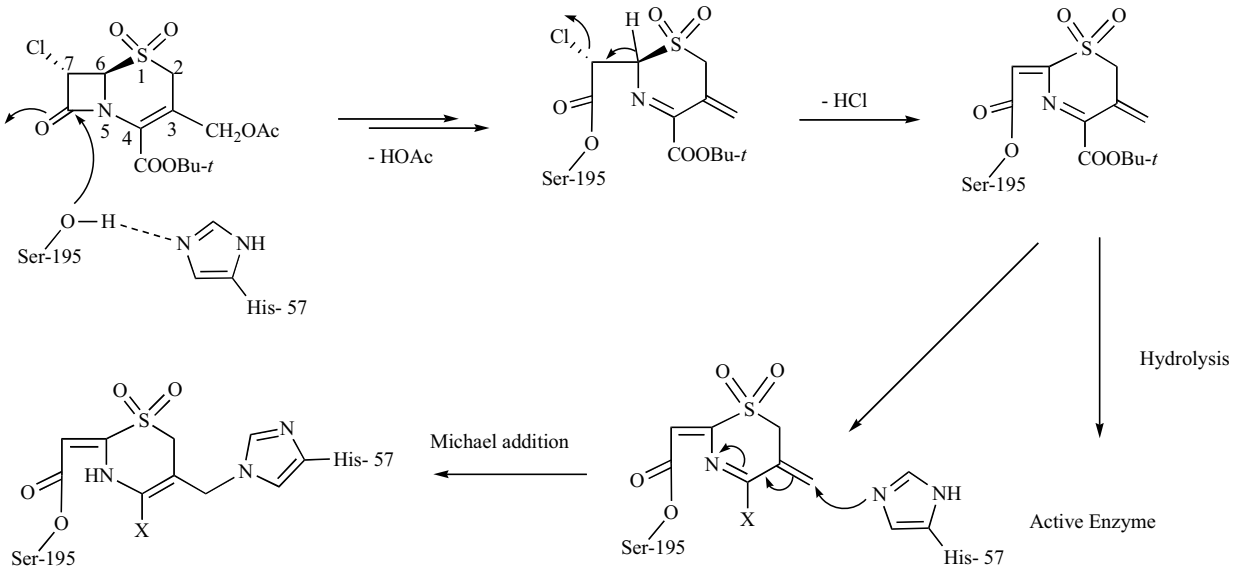
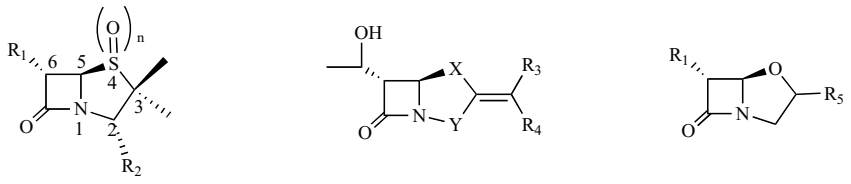
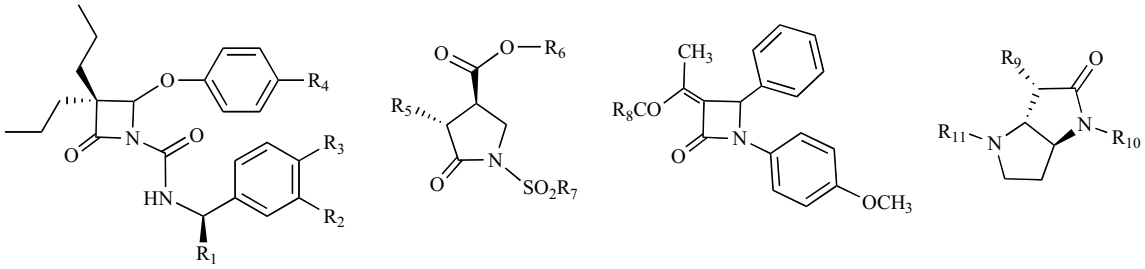


Fig. (17). The chemical mechanism for PPE inhibition [90, 96].



R_1 = MeO, AcO, Cl, CF_3CONH etc
R_2 = COOBn, COOBu-t, CH_2OCOCH_3 etc
R_3 = COOBu-t, H; R_4 = H, COOBu-t
R_5 = $CONH(CH_2)_{11}CH_3$; etc
n = 0, 2; X= C or S; Y = C or S

Fig. (18). Structures of penicillins derivatives [89, 92].



R_1 = Pr, H; R_2 = H; R_3 = H, CH_3; R_4 = COOH, CH_2COOH
R_5 = Me, Et; R_6 = H, Me, Et; R_7 = C_6H_4-CH_3-4, C_6H_4-CF_3-4 etc
R_8 = Ala-OCH_2Ph, Ile-OMe, Phe-OCH_2Ph etc
R_9 = H, Me, vinyl etc; R_{10} = COOMe, COMe, CONHMe etc
R_{11} = $PhOCH_2CO$, N-Me-2-PyrrolylCOCO

Fig. (19). Structures of monocyclic β-lactam and other cyclic lactam systems [100-107].

mans. Meanwhile, the development of more β-lactam antibiotics derivatives with respect to their nonantibacterial applications as novel reporters in gene expression, the specific antitumor prodrugs in ADEPT or the potent inhibitors of proteases will open new pharmacological perspectives for these "old " antimicrobial agents.

Based on recent trends in fluorescence technology, β-lactam derivatives have already been shown to be very promising fluorescent imaging probes for visualizing gene expression in an ever-greater variety of systems. However, the existed fluoresecent substrates for imaging β-lactamases have some shortcomings such as high molecular weight, difficult to prepare, poor cell permeability and limited imaging contrast etc. The small but powerful fluorescent probes will still be highly desirable. Furthermore, the conjugation of magnetic particles or radioisotopes with β-lactam derivatives may offer powerful tools for many other exciting applications such as magnetic resonance imaging (MRI) or nuclear medicine imaging techniques (e.g. positron emission tomography (PET) or SPECT etc). Therefore, with more new substrates available, β-lactamases will be able to serve as a "unified" reporter to noninvasively image the gene expression *in vitro*, in the cell culture as well as in the living subjects, which may be helpful in the detection of tumors under their early stages, high-throughput screening the antitumor drugs and monitoring the therapeutic intervention in real-time.

Considerable attention has been directed to apply β-lactam substrates as prodrugs in ADEPT systems for drug delivery. Upon enzyme treatment, antitumor agents are released in high concentrations to tumor tissue while minimizing systemic drug exposure, which exhibited the specific and potent cytotoxicity towards different tumors. However, many challenges have to be addressed before β-lactam based ADPET prodrugs become a useful tool for cancer therapy. For example, one main disadvantage of such ADEPT system is that it may elicit immune response in humans. Currently, β-lactam as prodrug in ADEPT in animals is just in the experimental stage of development and testing; there is still a long way before it can be applied to humans. However, as one potent approach for the efficient drug delivery in ADEPT, it will still be promising for evaluation of the cancer treatment status and screening the antitumor drugs in a high throughput fashion in living animals.

β-lactam can be developed as a potent and selective inhibitor for different proteases. The major advantage of lactam based protease inhibitors is based on their ability to form a stable covalent intermediate with nucleophiles in the active site of the enzymes. This reaction mechanism provides an alternative to noncovalent binding of traditional protease inhibitors. Development of more stable, cell-permeable lactam inhibitors will not only allow us to discover antiprotease drug candidates with reasonable efficacy and improved pharmacokinetics, but it will also pave the way towards new therapies with potent drug delivery options and less toxicity for the treatment of cancer, hepatitis and other relevant diseases.

ACKNOWLEDGEMENTS

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