Organic Chemistry In Biology And Drug Development
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Lecture - 51
Mechanistic Studies of Beta- Lactamase

Welcome back. In the last session we just started the topic that how the bacteria acquires resistance against the β-lactam antibiotics. And one of the major cause for that is that the bacteria produces enzymes which are classified as β-lactamases and these β-lactamases are so named because they hydrolyze the β-lactam ring. And if you can break the β-lactam ring, then all the β-lactam antibiotics are going to lose their activity.

So, now the question is what is the difference between transpeptidase and β-lactamase? That should be made very clear. Transpeptidase is a serine based enzyme, where the serine attacks the β-lactam ring, breaks the β-lactam ring, makes an acyl-enzyme complex and that stays there for a long time. On the other hand, there are different classes of β-lactamases; one of them utilizes a serine. So, if it utilizes a serine, again the mechanism is virtually same, the serine attacks the β-lactam ring, forms the acyl-enzyme complex.

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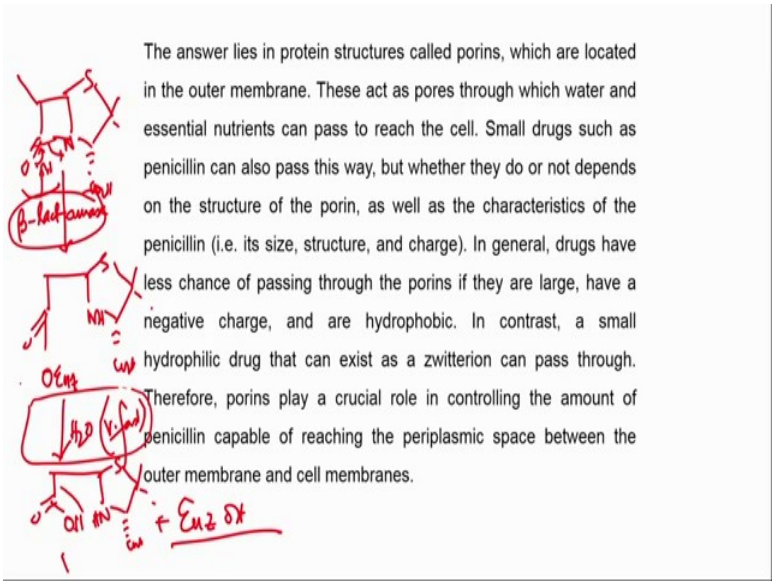

However now, the attack by water is very fast. So, this is the penicillin and β-lactamase hydrolyzes the β-lactam ring. So, the OH attacks the carbonyl, this breaks,

there is substituent here. So, O then the enzyme and then you have NH S and this. In case of β-lactamase, now water immediately comes, very fast and frees the β-lactamase enzyme and leave the hydrolyzed penicillin which is ineffective. For transpeptidase, this next reaction; that means, attack by water is very slow. So, this is the difference and this difference is causing havoc; that a β-lactamase enzyme has a very high turnover number. So, basically high turnover number means 1 molecule of β-lactamase can hydrolyze several molecules of penicillin. So, that makes it ineffective. And this is the major cause of concern in today's bacterial infection scenario.

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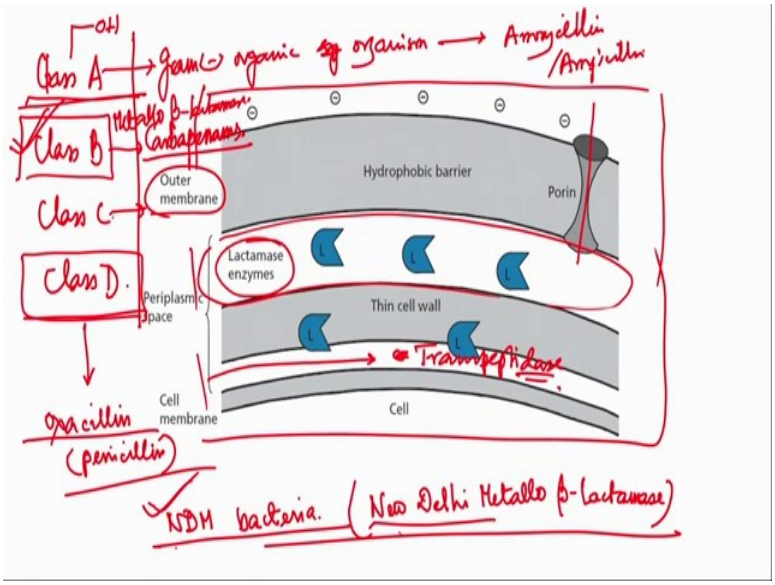

Because the β-lactamase enzymes are grouped into four classes; class A, class B, class C and class D. At least for today at this point of time, these are the four different β-lactamases. What is class A? Class A is basically the gram negative organisms, which were earlier treatable with ampicillin or amoxicillin type of drugs; that means, this class A β-lactamase hydrolyzes ampicillin or amoxicillin by a serine, the active site has a serine.

Class B hydrolyzes penicillins, it hydrolyzes cephalosporins; but the most important is that it hydrolyzes the carbapenems. We have not talked about carbapenems, I have showed you only the structure the skeleton; but carbapenems are powerful drugs which are in the market now. It is one of the last defenses that we have; that if the infection is very resistant, then people start giving the carbapenems. But there are now enzymes,

which are called class B enzymes, which utilizes a metal ion to hydrolyze the β-lactam ring.

So, it is not the serine OH, it is now a metallo β-lactamase. Then class C basically hydrolyzes the cephalosporin molecules and class D hydrolyzes the oxacillin; that is another type of penicillin which was in the market to have, which has got very good resistance earlier against this β-lactamases. But now there are enzymes which are called class D β-lactamases that hydrolyzes very good effective antibiotics like oxacillin. Which is a penicillin by the way, but it has got a different section.

So, these are the four classes; out of these, most difficult one is this class B, because it is a metallo β-lactamase. So, its mechanism is different from the other three and it extremely difficult to treat this type of infections which are caused by microorganisms producing class B. And, I think you should know this that one bacterial strain which produces the class B metallo β-lactamase enzyme is what is called the NDM bacteria, NDM harboring bacteria. What is NDM? New Delhi β-lactamase.

So, this strain was originated maybe 10 years back, New Delhi metallo β-lactamase. So, any NDM strain it will be *Klebsiella pneumoniae* is the one which is very dangerous; it causes pneumonia and they are now becoming very difficult to treat because of this NDM harboring bacteria. What is NDM, New Delhi metallo β-lactamase?

So, you see that gone are those era that when we are really safe, because we had many antibiotics in our hand; like erythromycin is one group of macrolide antibiotics, ciprofloxacin that is another group. I initially, I showed you one slide where the different targets have been shown; that some drugs target the nucleic acid, some target the transcription, some target the translation protein synthesis, some targets this synthesis of the cell wall.

So, for different targets, different antibiotics are there; ciprofloxacin, then you have the erythromycin that is macrolide antibiotics, you have cyclic peptide antibiotics, you have carbohydrates based antibiotics, amikacin; those type of molecules. Then you have this β-lactam class of antibiotics and you have vancomycin polymyxin. So, lot of antibiotics are there, but the problem is that many of these antibiotics have serious side effects; because they are targeting something which is also there in the host.

So, that is where the problem lies, except for penicillin and possibly vancomycin; because vancomycin also targets the cell wall biosynthesis. Now let us see the major problem of this β-lactamase producing microorganisms, they are mainly with a gram negative bacteria. Gram positive bacteria are easier to treat, because the cell wall is exposed to the outside world. But in case of the gram negative, first you have this outer membrane which is hydrophobic; then you have a space which is where these bacteria produces the beta-lactamase enzyme.

So, they are basically traversing here; they are looking for molecules which come here and then if it is penicillin, they are going to destroy it, then the cell wall which is much thinner and actually here is your cell wall; that means, your transpeptidase is here. So, the problem is, that if penicillin molecule enters it cannot go to the transpeptidase; because it has to cross the layer, a periplasmic space, where the β-lactamase is present.

So, it is a very difficult or challenging task to treat the gram negative bacteria. So, if somebody says why gram negative bacteria are difficult to treat; this is because first of all the molecule has to cross this hydrophobic barrier. And that is difficult, because you need carrier molecules to take it through the hydrophobic barrier. However, there are certain channels, because bacteria also need some molecules, some nutrients which has to be given from outside. So, that goes through pores which are called porins or these are the channels through which small molecules enter and go there.

So, if penicillin by chance enters here, now the β-lactamase is here; that is going to destroy the penicillin. So, that was the major problem till late 70s; then cephalosporins came into the market and lot of new cephalosporins were having resistance to this β-lactamase producing microorganism. So, cephalosporins became the savior, then first generation cephalosporin, second generation cephalosporin; but people were running out of options, because the bacteria is also changing its character.

Changing its characters, so new antibiotics were required, because it felt that yes it is time that we should discover new antibiotics, or at least we should discover molecules which takes care of this β-lactamase, ok.

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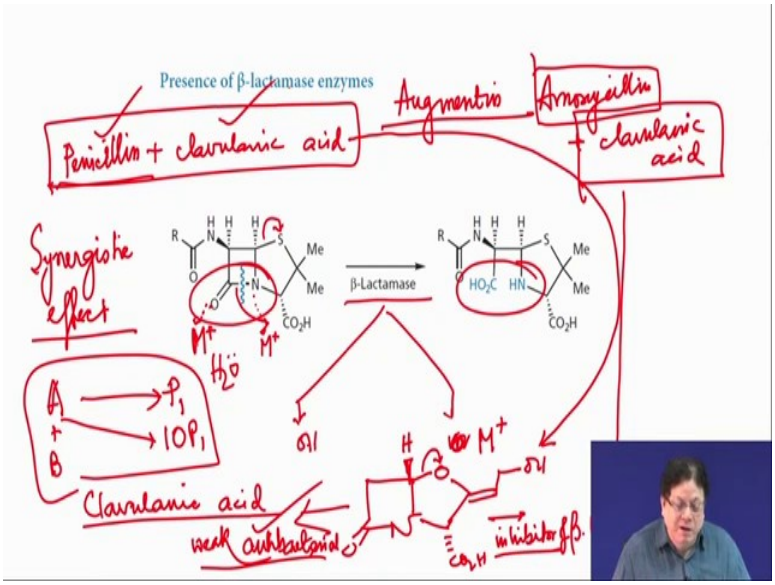

This is what the reaction is; β-lactamase hydrolyzes the penicillin; I already told you that there are various mechanisms it can do it by serine or it can do it by a metal ion. Like if it is M^+ so; obviously, the metal ion chelates here, like metallo-proteases you know. So, very similar mechanism, it activates the carbonyl and then this is broken very easily only by water.

So, the enzyme does not have to participate directly, and usually these metallo β-lactamase have two metal ions; one for the coordination to the carbonyl and another for this negative charge to be stabilized by the second metal ion. So, what I was telling that for the late 70s people, these various pharmaceutical companies frantically searched for molecules which have stability towards β-lactamase.

And what they found a molecule which is known as clavulanic acid. The structure is again a β-lactam compound with an oxygen and then an exocyclic double bond and this is what is clavulanic acid. So, what is the difference with penicillin? First of all it is an oxapenam and then it does not have the side chain here. And it has oxygen and this oxygen is very funny or very interesting, in the sense that there is a double bond here, so this has got a very good leaving group character; because it is with this O minus, then it can resonate with the double bond.

In case of penicillin, where you have sulfur, there is not much stability of the sulfur; although sulfur is quietly good leaving group, but it is not assisted by any double bond

like this. So, clavulanic acid is a molecule which was discovered again by Beecham pharmaceuticals and they found that this molecule is a very potent inhibitor of β-lactamase. But at that time, metallo β-lactamase was not there, protein inhibitor of β-lactamase means they are serine class of beta lactamase; and that was prevalent at that time.

However, this it has very weak antibacterial activity. So, it is an inhibitor of β-lactamase, but it is not a good antibiotic. So, if you take clavulanic acid, it will not treat you; because it will just inhibit the β-lactamase that is all. But the cell wall biosynthesis will take place because it is not a good antibiotic, if it is not a good antibiotic; that means, it targets the β-lactamase enzyme, but it does not target the transpeptidase.

So, quite interesting molecule, but then people are very clever at Beecham. So, what they thought that better take a penicillin which is hydrolyzed by β-lactamase and mix it with clavulanic acid. If you take this combination; then what will happen? Since clavulanic acid has got very high affinity for β-lactamase, so it is going to stop the β-lactamase from attacking the penicillin.

And, so the β-lactamase is busy with the clavulanic acid and by that way penicillin sneaks through and then attacks the transpeptidase and stops the transpeptidase enzyme. In fact, this combination is one of the lifesaving drugs today; it is called augmentin, which is a mixture of amoxicillin and clavulanic acid. So, it is a combination of drugs, and clavulanic acid targets β-lactamase, takes care of the β-lactamase; and amoxycillin now attacks the transpeptidase.

More interesting is that, in presence of clavulanic acid, amoxicillin becomes very powerful antibiotic; means earlier whatever the antibacterial activity amoxicillin had alone, when there was no β-lactamase, if you have to take 500 milligram to have the same effect now in presence of clavulanic acid, you need much less amount. That means, amoxicillin not only now targets the transpeptidase in presence of clavulanic acid, its power also is increased in presence of clavulanic acid this is called synergistic effect.

Synergistic effect is, that suppose you have a drug A and that has got a potency say P_1 and now you mix that with B; now the potency is say 10 P_1; that means, 10 times more potent in presence of B. How that does happen? Synergism can be very easily described by comparing with a real life scenario. This is not chemistry; but it is it can explain this

very well, the synergism. You know Lionel Messy, everybody knows him, he is a very good footballer, and he plays for Barcelona and scores many goals.

But when he plays for Argentina, he has not produced that form which he showed in Barcelona. Now instead of the molecule, you have the same man Messy, he plays, so well in Barcelona, but not that well for Argentina. So, there must be some synergistic effect going on when he plays for Barcelona and that is called synergistic effect; if you are a football fan you know that there is a there are players whose names are Iniesta and Xavi; they are the clavulanic acid of Barcelona.

So, they supply balls which Messy can convert into goals. So, that is this synergistic effect, that in presence of good footballers the other player shows much better efficiency than when those players are not there. So, clavulanic acid and penicillin combination is basically a miracle.

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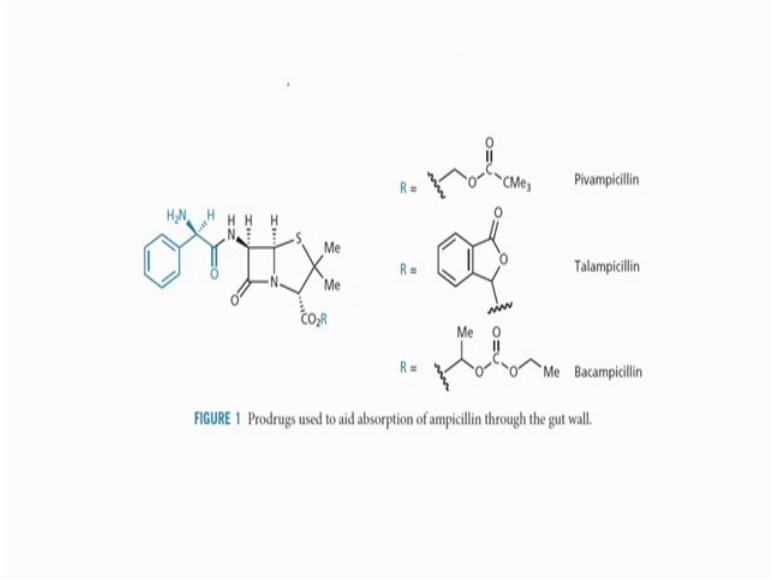

FIGURE 1 Prodrugs used to aid absorption of ampicillin through the gut wall.

But always remember, bacteria is also very clever; clavulanic acid was there. So, everybody thought that now the answer is there, but actually this battle between bacteria and human being will continue forever.

Because bacteria wants to survive, we also want to have a healthy life. So, bacteria wants to mutate, have different strategy and then like this advent of the metallo β-lactamase where clavulanic acid does not work. So, for treating the infection by metallo beta-

lactamase, carbapenem was introduced. I think, I have the structure of carbapenem possibly.

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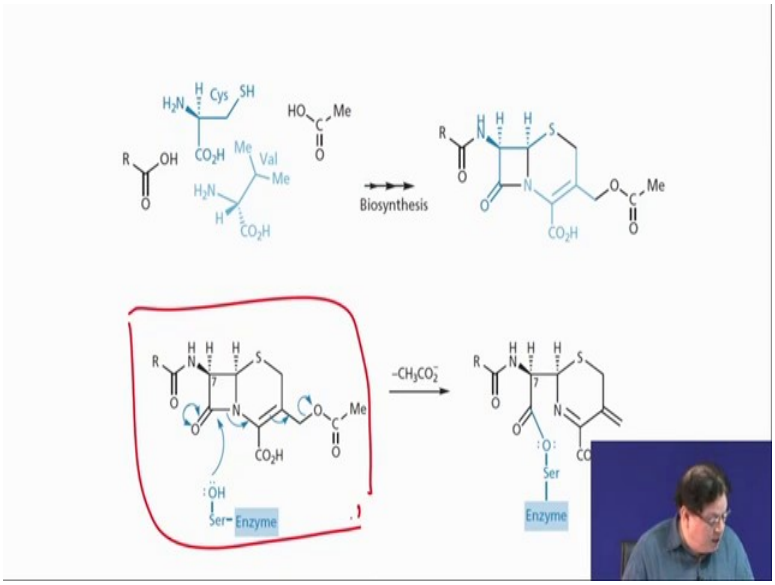

Let us see, this I told you about the about cephalosporin how it works.

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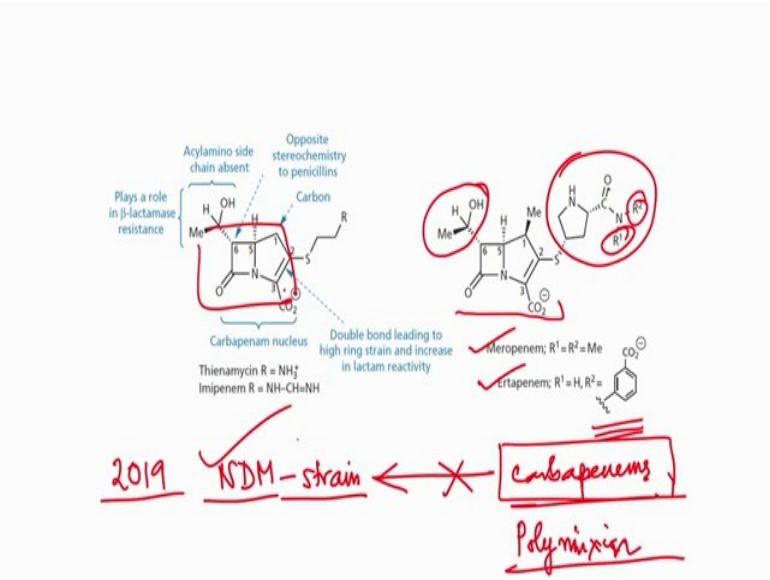

This is the Carbapenem that is in the market today. So, carbapenem antibiotics have different heterocyclic ring here and in the side chain it is always hydroxymethyl group. So, there are drugs which are called meropenem, ertapenem. Variations depending on

this R^2 R^1. So, lot of research is being spent on developing new types of carbapenem, but these are the ones which are in the market today.

Remember this alone can withstand the β-lactamase. This alone can target the transpeptidase, the same molecule takes care of, the β-lactamase, does not get hydrolyzed and then by the same molecule, the transpeptidase is inhibited. But today at 2019, the scenario is that, because of this advent of NDM strains, carbapenems are also not effective against this. Carbapenems also failed, it does not work. So, then what is the answer? This situation is very bleak; if people have this NDM infection then unless we get new antibiotics, I think the last answer is polymyxin.

But polymyxin is very toxic also. So, the patient may not survive, that is the problem; but polymyxin can kill the NDM strain, but the problem is that because of the toxic side effect, the patient may not survive. So, things are very grim, unless we have some mechanism to take care of these NDM problems. So, that is carbapenem again.

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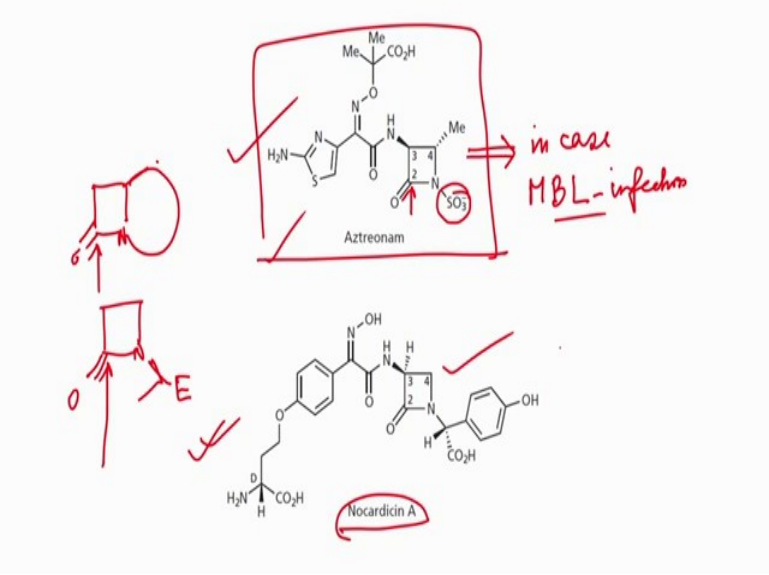

Now, the I told you that there are some drugs like aztreonam; this is also the one which is prescribed in case of NDM this metallo β-lactamase, MBL-infections; aztreonam is one. Now, you can actually ask that why, because earlier you have been told that it has to be a bicyclic network, to increase the electrophilicity of this carbonyl carbon.

But if you do not have bicyclic framework, how come that this is also opening very rapidly? You see the group here, you can bypass the bicyclic framework by putting an electron withdrawing group here; if you put an electron withdrawing group, then also, that is going to activate the opening of the β-lactam ring and exactly nature has done that by putting the sulphonic acid group here.

So, that is an electron withdrawing group and that helps the carbonyl to be attacked by the enzyme, that serine in the transpeptidase. There is another group of antibiotics which are called nocardicins. This is also a mono-cyclic compound and they are also having antibacterial activity so; that means, the myth that it has to be bicyclic is not true. They are called mono bactams because they have only the β-lactam group.

So, the mono-bactams are there which can show activity, but in order to do that, it is better that they have an electron pull from the nitrogen.

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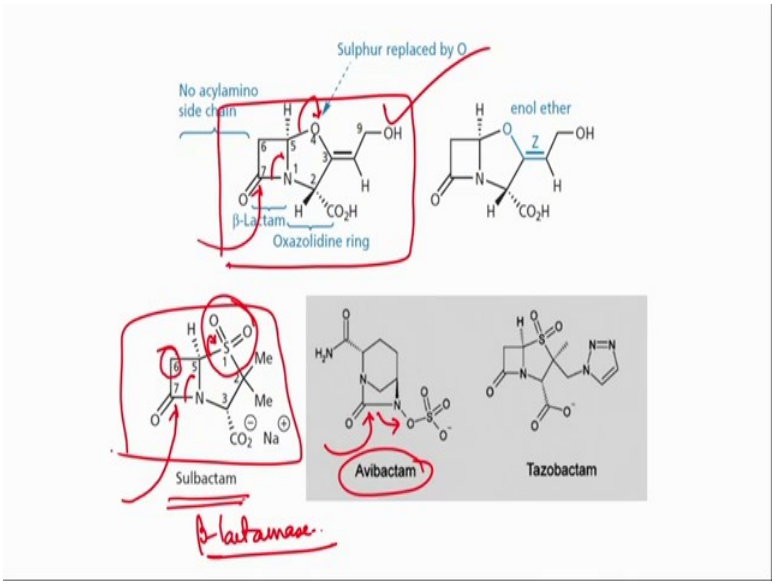

Every time you cannot use clavulanic acid, there you have to always have different varieties of inhibitors of β-lactamase. Clavulanic acid is very good; that is true; again it is a microorganism made compound.

Now there are synthetic compounds, sulbactam; what they did? It is basically a penicillin without any side chain, I think the numbering goes like this, but we will have

to check that whether different text follows different numbering. But whatever you follow, this becomes 6 in penicillin and this becomes 7 in cephalosporin.

Now, this is oxidized to a sulfone, sulbactam is a penicillin sulfone. So, what happens if you make a sulfone; that means you are making this a good leaving group, much better than your only sulphur? Because that is similar to a withdrawing group, it is now pushing, so if the nucleophile attacks here, that goes there, that opens up. Now question is after opening up what happens, I will go to tell you next. In clavulanic acid, the same thing happens; this is a very good leaving group, so that leaves; but what happens after that, why the enzyme is inhibited; that is the million dollar question.

You have another compound avibactam, which is entirely different; but again it is a bicyclic compound and again the nitrogen is attached to a sulphone. So, this is opened up by the nucleophilic serine –OH in transpeptidase or in β-lactamase. So, they are actually good β-lactamase inhibitor; do not get confused. Some molecules which are β-lactamase inhibitors they are very weak antibiotics like clavulanic acid, like sulbactam, like avibactam, like tazobactam; these are only beta lactamase inhibitors except the carbapenems.

Carbapenems are the ones which are not attacked by β-lactamase; or even if they are attacked by β-lactamase, the β-lactam enzyme is inhibited; at the same time they are very good antibiotics. So, that has to be very clear. So, now, the question is what is the mechanism of this inhibition of β-lactamase by clavulanic acid?

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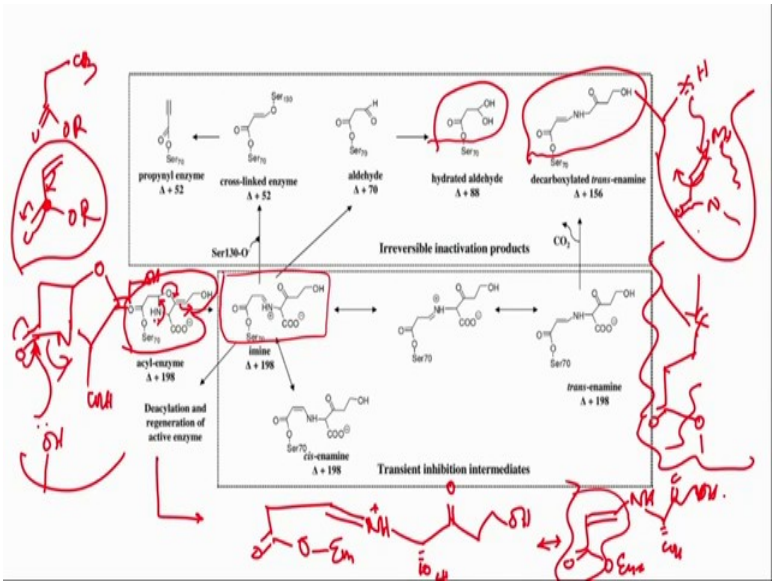

We take the clavulanic acid as the example. So, in clavulanic acid molecule, I right here N O, forget about the stereochemistry for the time being; double bond CH_2OH, this is clavulanic acid. So, the first β-lactamase attacks the carbonyl, this goes here comes back and that opens up.

So, from here we can start. So, the serine has attacked and opened the clavulanic acid β-lactam ring; then what happens, then as I told you that this is a very good leaving group that oxygen, so the lone pair goes here that comes here and this takes up a proton.

So; that means, you get this iminium ion. Now there are several mechanisms, because it is all done by mass spec and some IR spectroscopy, several mechanisms were investigated. So, what they did? They added that β-lactamase and tried to identify different metabolites which are obtained from clavulanic acid by mass spec; and one of the mechanisms says that you get once this happens. So, you get O enzyme; and then you have this iminium ion NH plus then you have CO_2H here you have a carbonyl here and you have this OH; that is the same compound which is written here. Now there are many things that can happen, this is a β-keto acid.

So, decarboxylation can take place and if you look at this slide, decarboxylation has taken place here, so it can decarboxylate. So, what they got? They got this type of species, which means, it is just only three carbon; but without going into such details what is generally believed is that this iminium ion now tautomerize into the

enamine. You know the enamine- imine tautomerism; that means, double bond is here now and this becomes NH and CO_2H and then the rest carbonyl and this OH.

Now, this has become a good Michael acceptor,. So, what will happen? The enzyme which is acyl enzyme, there are many things that can happen. First of all, if I ask you which ester is hydrolyzed faster? A propionic ester or an acrylic ester? The answer is that the acrylic ester will be slower because of this conjugation.

So, that reduces the electrophilicity of this carbon. So, now, when the double bond is here, the enzyme cannot free it because, hence the hydrolysis will be slow. Further, the enzyme is actually a very big molecule; see what happens here, here it is only written as *E* and *Z*; but actually the enzyme is like this, whatever is happening is inside the cavity of the enzyme. So, then the double bond, then you have this NH and then other part of the clavulanic acid. So, what can happen, this is a very good Michael acceptor.

So, if there is a nucleophilic amino acid in the enzyme that can come and attack this carbonyl and that undergoes a 1,4-addition. And finally, what you get is a covalent attachment with the enzyme. So, even if the enzyme, now frees here, water comes and hydrolyzes this; this is already attached, the other portion of the enzyme is attached to the hydrolyzed clavulanic acid. So, that means, it is covalently linked to the that modified clavulanic acid, so; that means, it is irreversibly inhibited.

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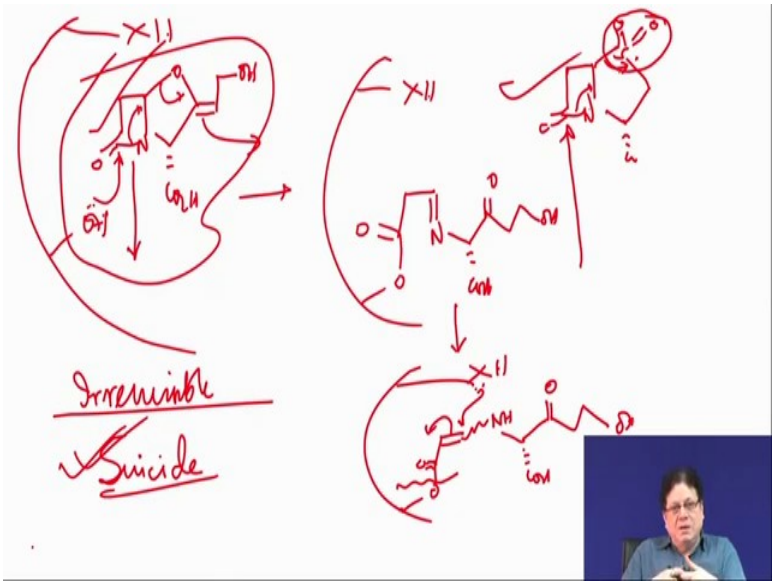

I can again write it in a better way, let me see whether there is any blank, yes. So, again I write this is your clavulanic acid, by the way this is one of the mechanism because the mass spec has given many peaks. Of course, that does not tell you, because in mass spec, there are may be fragmentation also that is not the sacrosanct that that has to happen. So, now, you have a β-lactamase like this; I write the big enzyme now and an XH here.

So, this is going to hydrolyze, that goes here, in one step you can show it and that comes here. So, if that be the case; that means, now XH and you have O, then the CO, then you have double bond N; what you have is a double bond here and then a carbonyl here CO_2H here and then this CH_2CH_2OH. Now this tautomerize into O CO double bond here, then actually you should not write any stereochemistry here; it could be cis or trans NH, and then you have this CO_2H CO and CH_2CH_2OH.

So, what I am saying now XH is present. So, X now attacks here and forms the covalent connection. So, even if it is hydrolyzed, the enzyme stays with this hydrolyzed clavulanic acid.

So, this is an irreversible inhibition, because covalent bonding is formed. Now the question is whether it a suicide inhibitor or is it an active site directed irreversible inhibition? This is a suicide inhibition, because the molecule has been accepted first as the normal substrate what the β-lactamase does; then it is transformed into a reactive species and that reacts with the active site, active site amino acid residues.

So, that is the best example of a suicide inhibition. So, this is the mechanism and all penicillin that sulfone whatever I said, tazobactam or your sulbactam they all work on this principle; that the β- lactamase comes attacks this, this opens up, and that goes out. As soon as this forms imine, that tautomerize to the enamine and then the enzyme active site adds on to the double bond. So, the trick to develop a new β-lactamase inhibitor, is to have a good leaving group at this site.

However molecule has to be accepted by the enzyme. Clavulanic is such a molecule, penicillin derivative is such a molecule. So, we have now done the penicillin chemistry in quite detail that how penicillin works and what are the different modifications, what are the different natural products which contain β-lactam, where you can use them, then what is the genesis of resistance in against the β-lactam antibiotics, and then how to

overcome them, and how to utilize β-lactamase inhibitor. So, next we will go to other topics in the next session let. So, this session ends here, ok.

Thank you.