EFFECT OF COMPLEX FORMATION BETWEEN Zn^{2+} ION AND THE AUREOLIC ACID GROUP OF ANTICANCER ANTIBIOTICS UPON THE STRUCTURE AND FUNCTION OF Zn(II)-CONTAINING ENZYMES

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ABSTRACT: Mithramycin (MTR) and Chromomycin A_3 (CHR) are two naturally occurring antibiotics belonging to the aureolic acid group of anticancer antibiotics that are isolated from *Streptomyces plicatus* and *Streptomyces griseus*, respectively. The biological action of the aureolic acid anticancer antibiotics, CHR and MTR is mainly attributed to their ability to bind bivalent cations and form high affinity $(D)_2$: metal ion complexes ('*D*' represents the antibiotic). Studies from our laboratory have shown the formation of two types of complexes with Mg^{2+} ion: complex I (represented as $[(D)Mg^{2+}]$) and complex II (represented as $[(D)_2Mg^{2+}]$). The transcription inhibitory activity of the antibiotics via reversible association of their metal complexes with nucleic acids is well documented. On the other hand, there is a lacuna of knowledge regarding the effect of their metal binding ability upon the structure and function of metalloenzymes. Such knowledge might lead to an extension of its pharmaceutical use as drugs particularly in case of diseases originating from metal dyshomeostasis. The abundance of reports on the therapeutic usage of metal chelators for the treatment of a number of different diseases and the mounting evidence of Zn(II)-metalloproteins as therapeutic targets further emphasizes the necessity to explore the importance of the metal chelating property of the aureolic acid antibiotics and their intracellular action on metalloenzymes.

The present thesis is an attempt to understand the chemical biology of the aureolic acid group of anticancer antibiotics as metal chelators, with special emphasis on the complex formation with Zn^{2+} ion, which is a physiologically important bivalent metal ion involved in various metabolic processes and intracellular activities. Then, we studied the effect of complex formation of the antibiotics with Zn^{2+} upon the structure and function of two representative Zn(II)-dependent enzymes, alcohol dehydrogenase (ADH) from yeast and alkaline phosphatase (AP) from calf intestine.

MTR and CHR forms a single 2:1 complex with the biologically relevant trace metal ion, Zn^{2+}, represented as, $[(D)_2Zn^{2+}]$, where '*D*' is the antibiotic. Results from ^{1}H-NMR spectroscopy support the complex to have a tetra-coordinated Zn^{2+}, with the carbonyl oxygen (C1-O) and the negatively charged oxygen of hydroxyl group (C9-OH) of the chromomycinone ring of two antibiotic molecules coordinating to the metal center. The formation of $[(D)_2Zn^{2+}]$ complex follows first order kinetics, the reaction being an entropy-driven process with conformational changes accompanying the association of Zn^{2+} and the antibiotics.

To further understand the biological consequences of the Zn^{2+} metal-chelating property of the antibiotics, we studied the effect of these antibiotics upon the structure and enzymatic function of two represented Zn(II)-containing enzymes, ADH and AP. Pre-incubation of the enzymes with MTR/CHR lead to alteration in the quaternary, tertiary and secondary structures of the proteins leading to monomerization of the native oligomers and culminating in enzymatic inhibition. It is also observed that these antibiotics do not interact with the apo-enzymes confirming the presence of Zn^{2+} ions in the enzyme as an essential requisite for the association of the antibiotics with ADH and AP.

We observed mixed inhibition for ADH and competitive inhibition for AP in the presence of the antibiotics. Mixed type inhibition obtained from the enzyme kinetics analysis of ADH originates from the association of the antibiotic(s) to both native enzyme and ethanol bound enzyme. Sequence of addition of the substrate and the antibiotic(s) to the enzyme does not affect the degree of inhibition. This shows that the antibiotic and the substrate (EtOH) can simultaneously bind to different sites in the enzyme instead of competing for the same binding site. These results favor binding at structural Zn^{2+} centers in the enzyme. As the structural zinc ion is located externally with less steric hindrance, the accessibility to this Zn^{2+} ion for the antibiotics is relatively higher compared to catalytic Zn^{2+} centers.

Competitive inhibition of AP by MTR/CHR with respect to the substrate pNPP (para nitrophenyl phosphate) reveals that both compete for the same active site where Zn^{2+} is present. The catalytic Zn^{2+} is located at the periphery of the catalytic pocket and is accessible from the solvent. These factors favor the association of the antibiotic(s) to the metal center in spite of this Zn^{2+} ion being a weak Lewis acid. The process of association of the antibiotics to the Zn^{2+} centers in ADH and AP could be proposed as follows. The first molecule of antibiotic binds to the Zn^{2+} ion that undergoes a change in the co-ordination from 4 to 6. Then, to accommodate the

second molecule of antibiotic, the other co-ordination bonds to Zn^{2+} from the amino acid ligands are disrupted to form $(antibiotic)_2$:Zn^{2+} complex at the zinc center, which leads to large structural changes in the proteins. This is also corroborated from the stoichiometry of two molecules per subunit and the high enthalpy and entropy values obtained on binding of the antibiotics to ADH and AP. Association of the antibiotic with the Zn^{2+} ions is able to disrupt the forces (hydrogen bonding, van der Waals forces, hydrophobic interactions and electrostatic interactions) that maintain the protein fold. The net effect is the disruption of the native structure culminating in monomerization. Results from confocal microscopy show the potential of the antibiotics to physically interact with ADH and AP. This suggests that the high affinity of MTR and CHR for Zn^{2+} makes it feasible to bind the metalloenzyme during the antibiotic's passage from the cell membrane to reach it known target, such as DNA and chromatin, in the nucleus. The inhibitory constants for these enzymes (25 μM for ADH and 40 μM for AP) are lower than the dissociation constant of the $[(MTR)_2Zn^{2+}]$ complex for chromatin (75 μM). These results emphasize the physiological relevance of our current findings and suggest that the inhibition of Zn(II)-metalloenzyme activity as a consequence of the association of the antibiotics to the Zn^{2+}-centers of the enzymes leading to structural disruption might be an additional mode of action for the drug. As MTR is a generic drug, the combined property of metal chelation and blood brain barrier permeability could be utilized to extend its therapeutic potential for the treatment of diseases related to metal dyshomeostasis and other neurodegenerative disorders.

The present work helps to understand and elevate multiple mechanisms of action of the aureolic acid anticancer antibiotics, MTR and CHR, inside the cell. Besides DNA binding property, results from this thesis work have demonstrated the ability of these antibiotics to bind and associate with Zn^{2+} ions in Zn(II)-metalloenzymes thereby disrupting their structure and function suggesting that these Zn(II)-containing proteins could be potential cytoplasmic targets for this class of antibiotics.