Zinc metalloproteins as medicinal targets

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Zinc bioinorganic chemistry has emphasized the role of the metal ion on the structure and function of the protein. There is, more recently, an increasing appreciation of the role of zinc proteins in a variety of human diseases. This *critical review*, aimed at both bioinorganic and medicinal chemists, shows how apparently widely-diverging diseases share the common mechanistic approaches of targeting the essential function of the metal ion to inhibit activity. Protein structure and function is briefly summarized in the context of its clinical relevance. The status of current and potential inhibitors is discussed along with the prospects for future developments (162 references).

I. Introduction

Zinc plays an essential role in biological systems with a variety of functions performed by zinc-binding proteins. Up to 10% of the human proteome is potentially capable of binding zinc *in vivo*, with zinc-fingers being the most abundant class of metalloproteins.[1] The zinc ion (Zn^{II}) displays suitable properties for catalytic and structural functions within proteins. Amongst these properties are: (a) a great stability towards redox reactions; (b) a d^{10} electronic configuration where the coordination geometry (4–6) is not dependent on ligand-field stabilization; (c) an intermediate polarizability or borderline hardness allowing coordination of N, S, and O donor atoms; and (d) a Lewis acid character useful to activate coordinated substrates, whilst maintaining ligand nucleophilicity.[2,3] These properties make zinc the most common transition metal observed in proteins[4] even though its total concentration in the human body (2–3 g) does not make it the most abundant.

Classification of zinc sites in proteins is broadly divided into (a) *catalytic* sites with the presence of a readily exchangeable water ligand coordinated to the zinc (*i.e.* hydrolases)[5] and (b) *structural* sites with no coordinated water and only protein residues in the coordination sphere. This "coordinative saturation" has as its purpose the creation or maintenance of an appropriate secondary/tertiary structure in the protein (*i.e.* zinc fingers).[6] A *cocatalytic* site is also recognized where a zinc ion is bridged with a second metal usually by a single protein residue; the second metal can be zinc (*i.e.* β-lactamases) or another metal (*i.e.* Cu in Cu, Zn-superoxide dismutases). Finally a fourth, *protein interface zinc* site can be defined, which influences the quaternary structure of proteins. The coordinating residues for this type of zinc site are supplied by two proteins (*i.e.* nitric oxide synthase, superantigens).[7] Additionally, roles for the zinc metal ion can be extended to regulatory (*i.e.* metallothioneins) and neuromodulation (*i.e.* presynaptic vesicles) functions, Table 1. This last topic has recently gained increasing attention due to the implied involvement of free zinc ions in neurological signaling and neurodegenerative disorders.[8–11] Zinc deficiency is detrimental in many aspects for normal function of organisms, with notable effects on growth and immune systems.[12,13]

Given the importance and the diversity of zinc-containing metalloproteins and enzymes, perturbation of zinc homeostasis, either through zinc diet deficiency or genetic alterations in zinc proteins, is correlated with the onset of many life-threatening diseases such as cancer and diabetes. These advances have resulted in the recognition of zinc enzymes and proteins in their own right as molecular targets for disease intervention. The understanding of the molecular basis of zinc biochemistry in medicine and disease has been further facilitated by

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Table 1 Representative biological functions of zinc and zinc enzymes and proteins

Zinc protein	Biological function
Alcohol dehydrogenase (ADH)	Oxidoreductase, alcohol breakdown
Farnesyl transferase (FT)	Transferase, signal transduction
Carboxypeptidase A (CPA)	Hydrolase, peptide cleavage
Carbonic anhydrase (CA)	Lyase, activation of small molecules
Phosphomannose isomerase (PMI)	Isomerase, isomerization of fructose-6-phosphate
DNA ligase III	Ligase, DNA repair
Zinc Fingers (ZF)	DNA transcription, DNA repair
Zinc $^{2+}$	Growth, neurotransmission

advances in molecular biology and spectroscopic techniques such as fluorescence, which may overcome the lack of spectroscopic properties of the metal ion and allow development of probes of zinc metabolism even under real-time conditions.[14]

This review summarizes recent information on the relevance of key zinc metalloproteins in current and important diseases for public health, ranging from cancer to antibacterial drug resistance (Table 2). The diseases are chosen for their topicality and to show the diversity of zinc relevance. The bioinorganic viewpoint emphasizes the structure of the zinc active sites; the effect of mutation or alteration of the active site on enzyme and protein mechanism and the role of the zinc protein as a medicinal target. A common theme for therapeutic intervention is zinc sequestration with concomitant inhibition of biological function. The design and current clinical status of experimental inhibitors across disease states will be contrasted. For the purposes of this review the zinc sites are broadly grouped into catalytic and structural.

II. Catalytic zinc as target

II.a Matrix metalloproteinases (MMPs)

II.a.1 Metzincin structure. Matrixins or matrix metalloproteinases (MMPs) along with astacins, serralysins, adamalysins, leishmanolysins and snapalysins are members of the metzincin superfamily, which involves >770 zinc endopeptidases. All members of this superfamily share a common zinc environment in their catalytic domain, the binding sequence motif **His**GluXX**His**XXGlyXX(**His/Asp**) (where X is any aminoacid). In the active site, the three histidines from the sequence are coordinated by the metal ion in a trigonal pyramidal coordination sphere completed by a catalytic water molecule as a fourth ligand. For the astacin and serralysin families, there is also a fifth coordinated ligand from the hydroxyl oxygen of a conserved tyrosine, which increases the coordination number around Zn^{2+} to five in a trigonal bipyramidal geometry.[15] A second *structural* zinc site exists in the catalytic domain of MMPs, in which the central zinc coordinates three histidines and one aspartic acid in a tetrahedral geometry. A detailed structural analysis for this superfamily can be found in the literature.[16]

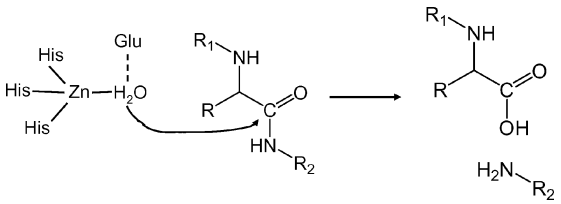
In general, peptide cleavage is achieved through polarization of the zinc-bound water molecule (by H-bonding interactions with a conserved glutamate residue) followed by attack on the scissile carbonyl group from the peptide substrate that is oriented to the catalytic site (Scheme 1). The importance of the glutamate residue for this general-base mechanism was confirmed by experiments using mutants with aspartate and alanine, where reduced and low catalytic activity was observed, respectively.[17]

MMPs are initially synthesized as a pro-enzyme or zymogen, where the catalytic water in the active site is substituted by a cysteine residue. This cysteine residue is included in a pro-peptide domain, which must be cleaved by other MMPs or proteases in order to activate the enzyme and provide access to the substrate, in what is called a ''cysteine switch'' mechanism of activation.[18] This ''cysteine switch'' is an interesting example of nature's use of both structural and catalytic zinc properties to achieve highly specific and controlled functions. The cysteine residue must be displaced to produce a catalytically active site through binding of a water ligand or oxidation. Other important domains on MMPs are the haemopexin-like C-terminal, connected to the catalytic domain by the hinge region and which can be up to 75 amino acids long (Fig. 1). Both these regions are implicated in substrate specificity and activation.

Zinc metalloproteins in the metzincin superfamily are, in general, capable of degrading all kinds of extracellular matrix proteins, in addition to being involved in cell–extracellular

Table 2 General summary of zinc metalloproteins as drug targets

Enzyme	Function	Zn coordination sphere	Medical relevance
Catalytic Zinc			
MMPs	Degradation of extracellular matrix proteins.	HisHisHis/HisHisAsp–H_2O	Cancer, diabetes, neurodegenerative disorders, arthritis, infectious diseases.
HDACs	Deacetylation of lysine residues in histone N-terminal tails.	HisAspAsp–H_2O	Cancer, neurodegenerative disorders, inflammatory related diseases, diabetes.
PTs	Prenylation of proteins involved in signal transduction.	CysHisAsp–H_2O	Cancer, rheumatoid arthritis, parasitic infections, multiple sclerosis.
SOD	Disproportionation of superoxide ion.	HisHisAspHis	Familial Amyotrophic Lateral Sclerosis (fALS).
MβLs	Hydrolysis of β-lactam ring in antibiotics.	HisHisHis/HisHisAsn (Zn1) AspCysHis/AspHisHis (Zn2)	Bacterial resistance to antibiotics.
Structural Zinc			
ZFs	Structural, DNA/RNA/protein recognition.	CysCysHisHis	Applications in human gene therapy, cancer, inflammatory conditions, antiviral therapy.
		CysCysCysHis	
		CysCysCysCys	
p53	Tumor suppressor protein	CysCysCysHis	Cancer, neurodegenerative disorders.



Scheme 1 General hydrolysis mechanism of peptide bonds by MMPs.

matrix and cell–cell interactions such as cleavage of cell surface receptors or chemokine activation/inactivation, which are correlated to several diseases. The extracellular matrix (ECM) or connective tissue is a complex structure of insoluble macromolecules consisting primarily of collagen, proteoglycans and glycoprotein molecules such as fibronectin and laminin that surrounds the cells and provides them with support. The MMP or matrixin family was one of the first metalloproteins to be recognized for therapeutic intervention as a structural target in molecular medicine.[19]

II.a.2 Matrix metalloproteinase function and role in diseases. There are 23 MMPs in humans from 24 genes due to a duplicated MMP-23 gene. These metalloproteins are tightly regulated and their expression is transcriptionally controlled by inflammatory cytokines and growth factors within the ECM, as well as hormones and cell–matrix interactions. A list of substrates cleaved and resulting biological effects produced by MMPs is given in Table 3, where their very diverse role in biological events can be appreciated. Deviations from normal MMP expression and behavior can lead to pathological disorders including cancer, neurodegenerative disorders and arthritis.

II.a.2.1 Cancer. Due to the key role of MMPs in tissue remodeling and angiogenesis, a direct link with cancer invasion and metastasis is recognized.[20] There is a general correlation between the levels of MMP expression and the stage of tumor progression.

II.a.2.2 Arthritis. MMPs as well as other metzincins such as A disintegrin metalloproteinases (ADAMs) and the disintegrin

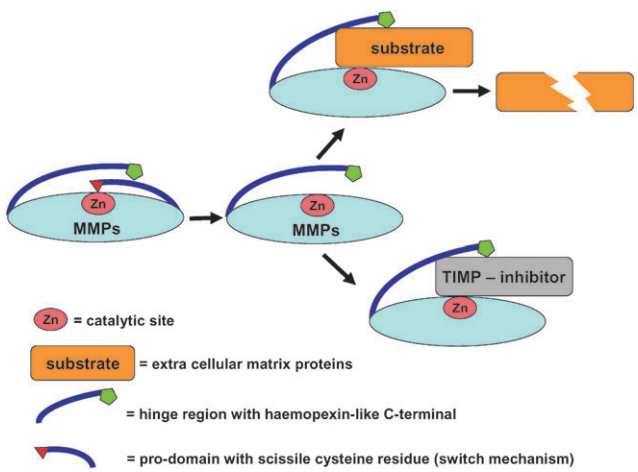


Fig. 1 Schematic representation of MMPs function (top) and inhibition (bottom).

Table 3 Representative biological activities mediated by MMP cleavage

Biological effects	MMP	Substrate
Tumor cell resistance	MMP-9	ICAM-1
Mammary epithelial cell apoptosis	MMP-3	Basement membrane
Osteoclast activation	MMP-13	Type I collagen
Adipocyte differentiation	MMP-7	Fibronectin
Cell migration	MMP-1, -2, -3	Fibronectin
Anti-inflammatory	MMP-1, -2, -9	IL-1β degradation
Disrupted cell aggregation and increased cell invasion	MMP-3, MMP-7	E-cadherin
Reduced cell adhesion and spreading	MT1-MMP, MT2-MMP, MT3-MMP	Cell surface tissue transglutaminase
PAR1 activation	MMP-1	Protease activator receptor 1
Vasoconstriction and cell growth	MMP-7	Heparin-binding EGF

metalloproteinase with thrombospondin type 1 like repeats (ADAM TSs) are generally secreted by many cell types and are involved in extracellular matrix degradation of cartilage and bone. Their role in a range of human joint pathologies including ostheoarthritis has also been identified and studied.[21]

II.a.2.3 Neurodegenerative disorders. Upregulation of MMPs is observed in all diseases of the central nervous system (CNS) including spinal cord injury, multiple sclerosis and stroke.

II.a.2.4 Infection and inflammation. MMPs play an ambivalent role in infectious diseases – beneficial roles involved in the normal immune response to infection include facilitation of leucocyte recruitment, cytokine/chemokine processing and matrix remodeling. However, the detrimental aspect of increased MMP activity following infection is correlated to onset of HIV, endotoxin shock, tuberculosis, hepatitis B and other diseases.[22]

II.a.3 MMP inhibitors (MMPIs). The appropriate regulation of ECM degradation and turnover also depends on endogenous MMPs inhibitors called TIMPs (tissue inhibitors of metalloprotease) and α_2-macroglobulin.[17] TIMPs are wedge-like proteins, 184–194 amino acids long. There are four mammalian variants (TIMP-1 to -4), each with its own profile of selectivity (MMPs, ADAMs and ADAM TSs). TIMPs interact with the substrate cleft in the MMPs by slotting its ridge composed of the first four N-terminal residues Cys1–ThrC–Val4 (amino acid abbreviation and position in the sequence) linked by a disulfide bond with Glu67–SerVal–Cys70 (Fig. 2A). In this way C1 expels the catalytic water in the active site (thus deactivating the MMP) by chelating the zinc ion through its N-teminal amino and carbonyl groups. In addition, TIMPs exhibit MMP-dependent and MMP-independent actions that complicate a complete understanding of their cell signaling process.[23] The resolution of the crystal structures of TIMP–MMP complexes has helped significantly to elucidate the types of interactions needed to design specific inhibitors that will fit in the so-called S1′ pocket of the active site, which is conserved within MMPs and highly

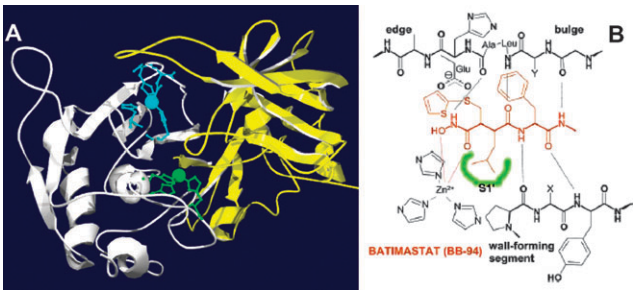


Fig. 2 (A) Crystal structure of the complex formed by the membrane type 1 matrix metalloproteinase (white) with TIMP-2 (yellow). Catalytic zinc with coordinating residues is colored green while structural zinc with coordinating residues is colored cyan (PDB ID: 1BUV).[1] (B) Schematic drawing of the non-peptide inhibitor batimastat (red) with the active site (black) is shown, substrate binding pocket S1′ is indicated in green.[24]

relevant for substrate specificity (Fig. 2B).[24] The majority of MMPIs have been developed *via* structure-based design and share three common features: (1) side chains binding to the different subpockets, (2) a peptidic backbone or a peptidomimetic scaffold, which orients the zinc-binding group (ZBG) for maximal interactions with the protein, and (3) a group binding to the catalytic zinc. Fig. 2B exemplifies this for batimastat, one of the earliest MMP inhibitors to reach clinical trials.

The matrix metalloproteinase (MMP) family has been a pharmaceutical target for over 20 years. Most of the MMPIs that have advanced into phase III clinical trials failed to increase the survival of patients and only MMP inhibitor (Periostat) has been approved by the FDA – for the treatment of periodontal disease.[25] Two main causes for this failure have been indicated as an incorrect target validation and a lack of information regarding *in vivo* MMP substrates or physiological roles.[26] Further reasons for the low success rate of MMP inhibitors in the clinic include toxic side effects caused by their lack of selectivity (inability to discriminate a specific MMP substrate), poor oral bioavailability and decreased efficacy *in vivo*. Approaches to reverse this disappointing trend have been both structural and bioinorganic. Comparison of X-ray and NMR structures obtained for MMPs indicates the flexibility of the protein backbone as one of the problems towards the development of specific inhibitors.[27]

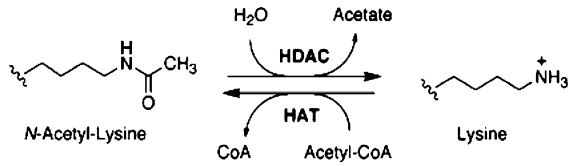
ZBGs have incorporated carboxylate, hydroxamate, phosphonate, or phosphinate as chelating groups for the active zinc ion.[28] The hydroxamate functionality appeared early on to have ideal properties as a ZBG for MMP inhibitors but recognized liabilities include lack of selectivity *versus* other physiologically important metals, sensitivity to rapid hydrolysis *in vivo*, rapid excretion and low bioavailability. In order to increase specificity, pyrimidinetrione-based compounds have been reported to discriminate between MMP-13 and -14 with high selectivity.[29] In addition, small heterocyclic pyrone-based zinc-binding groups have been found to have a broad therapeutic window and an enhanced zinc binding as measured through IC_{50} values from an MMP-3 fluorescence assay.[30,31] Novel chelating groups can contribute to overcoming observed side reactions in hydroxamate-based MMPIs, such as the production of nitric oxide. A change in MMPI design philosophy has pointed out the importance of a moderate zinc affinity in order to improve overall specificity for the compound.[32]

In the context of medicinal inorganic chemistry, it should be noted that the antimetastatic properties of NAMI-A (imidazolium *trans*-tetrachlorodimethylsulfoxideimidazoleruthenate(III)) have been linked with its interaction with MMPs, specifically MMP-2 and MMP-9 action is inhibited *in vitro* by the Ru complex.[33] Further, related organometallic ruthenium complexes such as the arene $[RuCl_2(\eta^6\text{-toluene})pta]$ (RAPTA-T, where pta = 1,35,-triaza-7-phosphoadamantane) exhibit similar antitumor behavior, and mechanistic studies to date suggest similar mechanisms of action. Platinum phosphonate compounds such as $[PtCl_2(SMP)]$ (SMP = diethyl(methylsulfinyl)methylphosphonate) are slight but specific inhibitors of MMPs 2,3,9 and 12.[34] While the anticancer activity of transition metal complexes has been naturally dominated by platinum-based agents, the demonstrated, albeit moderate, activity as MMP inhibitors suggest the possibility of extending Pt- and Ru-based chemophores beyond that of cytotoxic agents.

II.b Histone deacetylases (HDACs)

II.b.1 Structure and function. DNA organization and gene expression is tightly regulated by epigenetic processes such as DNA methylation, and histone modification.[35] Histones undergo extensive post-translational modifications affecting gene expression. Modification of histone tails, termed the histone code, by reactions such as acetylation, phosphorylation, ubiquitination, methylation, and poly-ADP-ribosylation regulates accessibility of transcription factors to DNA.[36] A major molecular epigenetic mechanism of histones is the enzymatic acetylation and deacetylation of the ε-amino groups of lysine residues controlled by histone deacetylases (HDACs) and histone acetyl transferases (HAT). Removal of the acetyl groups that nullify the positive charge of the lysine residues that maintain the histone tails attached to DNA, results in repression of transcription (Scheme 2). The reverse process is performed by histone acetyl transferases (HATs), and the mechanism for lysine acetylation generally involves the transfer of an acetyl group from acetyl-coenzyme A to the specific lysine residue. Any deviation from the delicate balance of both processes (acetylation homeostasis) results in aberrant transcriptional activity correlated with a variety of diseases including cancer, diabetes and neurodegenerative disorders. The HAT–HDAC system is also considered to modulate replication, site-specific recombination and DNA repair.

So far, 18 different HDACs have been identified and divided into four classes based on homology. Specifically for eukaryotes, 11 HDACs of classes I (HDAC-1, -2, -3 and -8) and II (HDAC-4 to -7 and -9,-10) have been identified so far. HDAC-11 is classified as the sole member of class IV and sometimes included in class II (class III is a structurally unrelated subfamily found in yeast).[37] Class I HDACs are localized exclusively in the nucleus and are ubiquitously expressed, whereas the proteins in class II are larger and are shuttled between the cytoplasm and the nucleus and display a tissue-



Scheme 2 General mechanism for acetylation/deacetylation of histone lysines.

specific expression. Currently only three-dimensional structures for HDAC-8 have been reported.[38]

The active site is thought to be essentially identical for HDACs classes I, II and IV since key catalytic residues are conserved in the overall sequence. One of the characteristic features of this family of zinc metalloproteins is the presence of a narrow hydrophobic tunnel that leads into the active site: a zinc ion coordinating one histidine (His180) and two aspartic acids (Asp178, Asp267) along with a water molecule to complete the first or inner coordination sphere. Additional key residues in the active site comprise one tyrosine (Tyr306) and two histidines (His142, His143) in the second or outer coordination sphere (*vide infra*).

The postulated catalytic mechanism of HDACs considers the water as a nucleophile, due to polarization caused by the zinc ion and a proximal histidine residue (H131). This activated water can the attack the acetyl group causing cleavage from the lysine residue (mechanism 1 in Fig. 3). This mechanism assumes a five-coordinate transition state and the key residues histidine H143 (His132 for HDLP) and H142 (H131 for HDLP) as doubly and singly protonated, respectively, acting as an acid and a base to assist in the stabilization of the bound water molecule.[39] These features are closely related to the mechanism generally accepted for zinc proteases (*i.e.* MMPs).

Theoretical studies have revealed the presence of a novel combination of catalytic motifs in histone deacetylase. One feature that differentiates the active site is the presence of two adjacent histidine–aspartate (H–D) dyads where the imidazole rings in His142 and His143 are hydrogen bonded with the carboxylate moiety of the Asp176 and Asp183 residues, respectively. The strength of the hydrogen bonding causes a modification (usually a reversal) in the pK_a for histidine and aspartate in a charge-relay mechanism.[40] A second mechanism postulates an inverse protonation for the histidines His142 and His143 in the second coordination sphere. In addition, Tyr297 is proposed to help in the stabilization of the water molecule in the first step, although the zinc ion remains as a pentacoordinated intermediate (mechanism 2, Fig. 3). A third postulated mechanism considers both singly protonated histidines stabilizing the coordinated water molecule in the first step of the reaction, and decreasing the energy for the reactant state in 20.93 kJ mol^{-1}. The zinc ion is tetracoordinated in the intermediate state and in the rest of the reaction process, with its principal role the activation of the carbonyl group of the amide (mechanism 3, Fig. 3).[41]

II.b.2 Clinical relevance. Given the importance of the acetylation process for gene regulation and the type of substrates that HDACs modulate, it is therefore straightforward to imagine the profound implications of these zinc

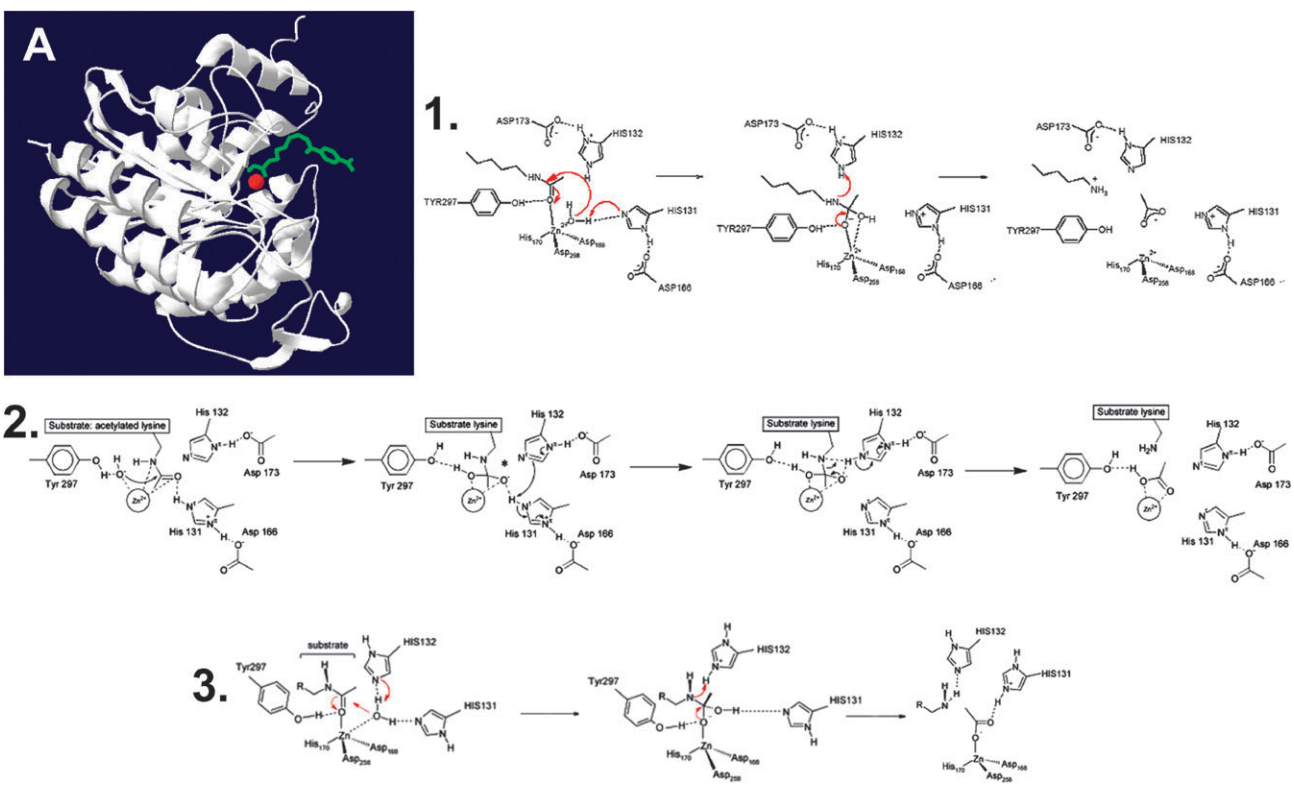


Fig. 3 HDAC-8 complexed with inhibitor MS-344 (in green), chelating the zinc ion (in red) PDB ID: 1T67 (A). Proposed mechanisms for HDAC activity (1–3).[38]

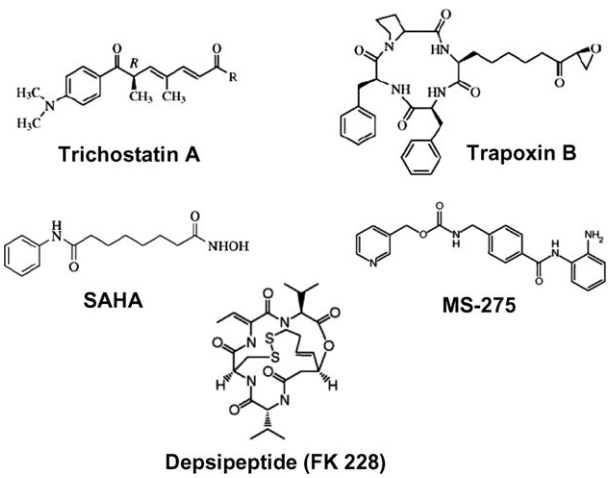


Fig. 4 Structures of different type of HDACIs.

metalloproteins in multiple diseases, notably cancer. The first disease with demonstrated HDAC involvement was acute promyelocytic leukaemia (APL), where transcriptional silencing is produced by aberrant HDAC behavior. Administration of retinoic acid, the substrate for the transcription factor retinoic acid receptor-α (RAR), along with HDAC inhibitors has proven to cause remission in transgenic models of therapy-resistant APL.[42] It is generally accepted that imbalances in epigenetic modification play a basic role in cancer development and progression, and thus efforts towards the development of HDAC inhibitors as anticancer agents have flourished in recent years (*vide infra*),[43] even though the molecular basis for their anticancer selectivity remains largely unknown.[44]

An acetylation balance is important also for neuronal vitality, and an increasing amount of evidence shows that this balance is greatly impaired during neurodegenerative conditions, in line with the fact that HDACs inhibitors prevent oxidative neuronal death and ameliorate the conditions associated with Alzheimer's, Parkinson's and Huntington's disease, multiple sclerosis and Friedreich's ataxia.[45] The potential use of HDAC inhibitors for inflammatory diseases has also been reviewed.[46]

II.b.3 HDACs inhibitors (HDACIs). The precise molecular mechanism for inhibition of the class I and II zinc-HDACs is not clearly understood, and the genes responsible for the biological response have not been identified. However, several three-dimensional structures of HDLP and HDAC-8 complexed with HDACIs (hydroxamate type) have been determined and the general features observed involve first the obstruction of the substrate pocket (rim) and hydrophobic binding tunnel that leads into the catalytic site. Secondly the chelation of the active zinc ion displaces the nucleophilic water molecule from the active site, while simultaneously saturating its coordination sphere, Fig. 3. Therefore three main characteristics are generally present in common HDACIs, beginning with a metal binding site for zinc chelation, followed by a linker or spacer that mimics the substrate and fills the hydrophobic tunnel, and lastly a hydrophobic cap that closes the entrance to the tunnel and ideally should exhibit good interaction with the outer rim.

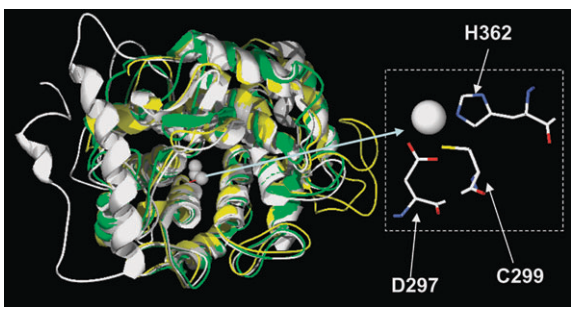


Fig. 5 Overlay of the β subunit (Cα only) in farnesyl transferase (white, PDB ID: 1FT2),[60] geranylgeranyl transferase type I (yellow, PDB ID: 1N4P)[61] and geranylgeranyl transferase type II (green, PDB ID: 1DCE).[62] First sphere of zinc coordination is shown is square, except for water (right). Zinc atoms are drawn as grey CPK balls.

The HDACI literature has been reviewed thoroughly,[47,48] and these compounds can be categorized into six different chemical classes[49] as:

II.b.3.1 Carboxylates. Including short-chain fatty acids like butyric and valproic acids, which has undergone phase II oncology trials.[50] Although generally weak inhibitors and highly unspecific, they are still a valuable (*i.e.* valproic acid) tool to study the structure and mechanism of HDACIs.

II.b.3.2 Small-molecule hydroxamates. Including early HDACIs like Trichostatin A (TSA), PXD101 and SAHA. This group exhibits an unfavorable pharmacokinetic behavior resulting from glucuronidation and sulfation, and from metabolic hydrolysis, decreasing the half-life of the hydroxamic group. The choice of hydroxamate as ZBG seems to have followed from the matrix metalloproteinase inhibitor concept but, not surprisingly, the chemical and biochemical problems remain the same. The design of more potent HDAC inhibitors would equally benefit from development of compounds with a different chelation group. The α-mercaptoketone and α-thioacetoxyketone analogs of SAHA are reported to have higher potency on *in vitro* and *in vivo* tests than the parental HDACIs.[51]

II.b.3.3 Electrophilic ketones (epoxides). Including AOE and trapoxin B, make use of the epoxy group to modify or alkylate the active site in the HDAC.

II.b.3.4 Cyclic peptides. Generally the macrocyclic peptide portion of the inhibitor is used to bind to the rim of the active site, while an aliphatic linker anchors in the hydrophobic tunnel, for example depsipeptide or FK228 is considered a pro-drug that needs intracellular reduction, is in phase III oncology trials.[47,48]

II.b.3.5 Benzamides. As represented by MS-275 and CI-944. The former is undergoing phase II clinical trials as an anticancer agent, and its use in the treatment of epigenetically induced psychiatric disorders has been suggested.[52] CI-944 on the other hand, has been used in several clinical phase I trials. Their mechanism of action remains uncertain.

II.b.3.6 Other hybrid compounds. Where recent examples include short-chain fatty acids with Zn^{2+}-chelating binding motifs[53] and a cyclic tetrapeptide (chlamydocin) with an

epoxyketone surrogate,[54] which combines the inhibition mechanisms of both cyclic peptides and epoxides and are sometimes active at the nanomolar level.

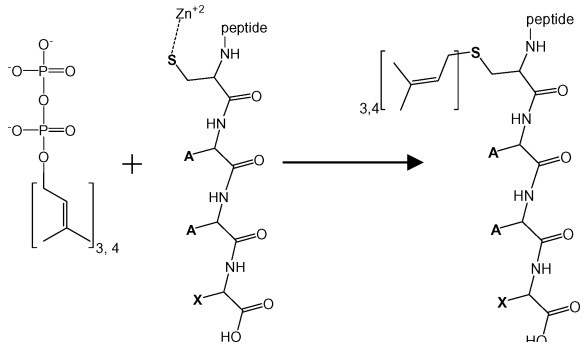
Although there is some evidence that there is certain degree of specificity among HDACi, (*i.e.* drugs capable of discrimination between HDACs classes I and II) and recent results from clinical trials are encouraging, a greater specificity even among members within a class is preferable to increase the therapeutic window of HDACIs, because key cellular functions could be disrupted by its indiscriminate use.

The success of HDACIs as anticancer agents relies on an apparent selectivity in apoptosis induction for cancer cell lines, compared to normal cells. Attempts to explain this fact point to a preferential up regulation of pro-death genes or down regulation of pro-survival genes at the transcriptional level, or a different cascade of events produced in normal and cancer cells. HDACIs can also increase reactive oxygen species preferentially in transformed cells, which would explain the selectivity towards cancer cells.[55] The need to understand in more depth the mechanism of action (anticancer, anti-inflammatory, diabetes) of these compounds is critical since non-histone substrates might be playing a more important role in the therapeutic activity, and the almost random up/down regulation of genes remains far from being controlled.

II.c Protein prenyl transferases

II.c.1 Structure and function. Protein prenyltransferases (PTs) catalyze a variety of biochemical reactions involving the isoprenyl group including chain elongation of allylic pyrophosphate groups; transfer of an isoprenyl pyrophosphate (*e.g.* farnesyl pyrophosphate) to a peptide and the cyclization of isoprenyl pyrophosphates.[56] This post-translational modification is necessary not only for subcellular localization but also plays a direct role in protein–protein and protein–membrane interactions. Transfer of the farnesyl pyrophosphate involves the covalent attachment of the polyisoprene group, from an appropriate prenyldonor *i.e.* farnesyldiphosphate (C_{15}) or geranylgeranyldiphosphate (C_{20}), to a cysteine at or near the carboxyl termini of proteins involved in signal-transduction pathways and cell growth (Scheme 3). Table 4 summarizes the three different PTs in humans. Farnesyl transferase (FT) and geranylgeranyl transferase type I (GGT-I) recognize a similar motif at the C-termini of the substrate proteins, while geranylgeranyl tranferase type II (GGT-II) does not require a specific motif as long as there is one or two cysteines available. However, it does require an escort protein called Rab escort protein (REP) to transfer two geranylgeranyl moieties to a flexible motif with two cysteines in the sequence.[57]

Ras FTAse is a Zn^{2+}-dependent enzyme that catalyzes the farnesylation on a C-terminal CysAlaAlaX motif of the Ras protein. The active zinc ion is coordinated by three conserved residues: aspartate, cysteine and histidine. There is one tightly bound water ligand, as befits the role of a catalytic zinc. The aspartate is considered to be possibly bidentate – producing an overall penta-coordinated rather than tetra-coordinated zinc. Results from computational studies have pointed out the close energetic proximity between both possibilities, although favoring the second alternative at least in the case of FT.[58] Replacement of the essential Cys reduces Zn^{2+} affinity and abolishes enzyme activity. Confirmation of the role of zinc in these metalloproteins as purely catalytic is also found from studies with zinc-depleted FT where the overall structure was shown to be identical to the original, with binding of the farnesyldiphosphate substrate possible also in the absence of zinc.



Scheme 3 General mechanism for protein prenylation.

Kinetic analysis of the enzymatic prenylation reaction revealed a relatively fast chemical step (approx. 0.8–12 s^{-1}) followed by rate-limiting product release. Scheme 3 shows the general mechanism with substrate binding resulting in the essential CAAX moiety making extensive van der Waals contacts with all the isoprene moieties but the first, and the zinc-coordinated cysteine sulfur producing an initially five-coordinated intermediate. The exact nature of the cysteine before coordination (thiol *versus* thiolate) is another matter of debate since the pK_a of the thiol depends on several factors such as the nature of residues surrounding the cysteine, presence of substrate, *etc*. Upon rotation of the first isoprenoid unit to facilitate approach of the activated cysteine to the C1 carbon in the prenyl diphosphate, an Sn_2 mechanism is indicated with the thioether formation proceeding with configuration inversion of C1. Pyrophosphate leaving then ensues and the developing charge in the diphosphate group is stabilized with the assistance of a Mg^{2+} ion in the case of FT. Inclusion of Mg^{2+} greatly enhances enzyme activity.

It can also be seen from Table 4 that there is some overlap of the protein substrates for these enzymes but particular features on each one contribute to further selectivity. Regarding the catalytic mechanism for prenylation, an important distinction between the FT and GGT-I mechanism on one side and GGT-II on the other can be made due to specific structural differences. In the former case, the binding of the prenyl diphosphate to the hydrophobic α–α barrel from the β subunit in FT or GGT-I constitutes the first step of the catalytic cycle. The depth of the cavity in the α–α barrel is an important factor to modulate selectivity between FT and GGT substrates. A second factor for substrate specificity, in addition to the depth of the cavity in the α–α barrel, is given by the last AlaX pair of residues in the recognition motif (CysAlaAlaX) of the protein terminus. Since the cysteine is coordinated to the zinc and the next A residue is basically open to the solvent, the interaction of the last two residues with the tranferase enzyme has been found to play an important role according to three-

Table 4 Comparison among PTs in terms of recognition motif and protein substrate

Enzyme	Protein substrate	Recognition motif
Farnesyl transferase	Ras (H-, N-, K-); prelamin A, HDJ2, PTP- PRL tyrosine phosphatases, Rho-B, Rheb, CENP-E, -F	CysAlaAlaX, X = Ser, Met, Gln, Ala. CysAlaAlaX/
Geranylgeranyl transferase type I	Rac1,-2; RalA, Rap1A,-1B; Rho-A,-B,-C; Rab 8, Cdc42,	CysAlaAlaX, X = Leu, Phe
Geranylgeranyl transferase type II	Rab	–CysCys, –CysXCys, –CysCysX, –CysCysXX/REP

dimensional structures obtained for models of the recognition motif. Notably the X-residue binding pocket in FT was found to be more polar than GGT-I, explaining the differences in affinity observed for the X residue among both enzymes (Table 4).

II.c.2 Clinical relevance. The clinical relevance of PTs stems from the fact that proteins involved in cell proliferation, signal transduction and malignant transformation, need to be prenylated in order to exert their vital functions. Small GTPases in Ras, Rho and Rab families in addition to nuclear lamins, cGMP phosphodiesterase are amongst the substrates for PTs. The involvement of mutated forms of ras genes and farnesylated Ras proteins in human tumors has been observed for 30–40% of cases. The mutated Ras have suppressed GTPase activity and remain active (GTP bound) independently of upstream activation, therefore relaying a signal for tumor growth. There are three ras proto-oncogenes that encode for four proteins H-Ras, K-Ras (two splice variants) and N-Ras. H-Ras mutations are rare, however they have been observed in bladder cancers (15–20%), mutated K-Ras are prevalent in some adenocarcinomas including pancreatic (>90%), colorectal (50%) and lung cancer (30%), while mutated N-Ras occur in melanoma (10–20%) and some hematologic malignancies. A correlation between the kind of cancer developed with mutations in a specific Ras protein has also been suggested.[59]

II.c.3 Prenyltransferase inhibitors. The elucidation of structural and mechanistic aspects of PTs has provided essential information for the development of inhibitors with diverse potential therapeutic applications. To summarize, the farnesyl transferase inhibitors (FTIs) as anticancer agents are designed to block the post-translational attachment of the prenyl moiety to C-terminal cysteine residue of Ras and thus inactivate it. Because Ras plays an important role in tumour progression and the ras mutation is one of the most frequent aberrations in cancer, this strategy represents an appealing approach for non-cytotoxic anticancer drug development. FTase has two binding sites; one contains the recognition site for farnesyl pyrophosphate and the other for the CysAlaAlaX box of the protein. While the role of the zinc ion in the catalytic mechanism appears well defined, inhibitor design strategy does not seem to have focused on inhibition of the catalytic activity *per se* – rather drug development has followed rational design strategies as well as screening of combination libraries to produce compounds that may be competitive with farnesylpyrophosphate and or compounds that compete with the CysAlaAlaX binding motif of the protein. At least six FTIs have been tested in human clinical trials, (Fig. 6).[63] All these compounds display a high selectivity with IC_{50} for FT inhibition in the nanomolar range although inhibition of other prenyl transferases cannot be ruled out. A well-defined proof of concept in preclinical and clinical studies, especially as single-agent anticancer drugs in solid cancers, has not unfortunately been achieved. The activity in clinical trials has been disappointing. The exact mechanism of action of this class of agents is currently equivocal and increasing lines of evidence indicate that the cytotoxic actions of FTIs are not due to the inhibition of Ras proteins exclusively, but to the modulation of other protein targets.[64] The inherent pharmacokinetic problems of peptidomimetic compounds (rapid intracellular degradation and deficient cellular uptake), are also factors to be taken into consideration. The quest for a specific target protein is complicated by the fact that there are more than 30 proteins that are known to be farnesylated emphasizing the difficulty of substrate specificity. This lack of knowledge in terms of molecular pharmacology for FTIs has been pointed out as one of the factors contributing to the poor clinical trial results. Finally, activation of K-ras geranylgeranylation may, in fact, suppress the effect of FTIs. The clinical and pre-clinical situation has been reviewed thoroughly in recent publications.[63,65]

A very promising efficacy has been found for FTIs as antiparasitic drugs and the development of a proper pharmacokinetic profile seems to be the next step towards a parasitic chemotherapy. Interestingly, there appears to be an inherent cytotoxic selectivity for pathogenic protozoa compared to normal mammalian cells, which could be due to a lack of an analogue of GGT-I or a higher sensitivity for farnesylation inhibition in the parasite.[66]

II.d Metallo-β-lactamases (mβLs)

II.d.1 Structure and function. β-Lactams are the oldest, least expensive and most used family of antibiotics, these include penicillins, cephalosporins and carbapenems (Scheme 4B). These antibiotics act by interfering with cell wall peptidoglycan biosynthesis, using their four membered β-lactam moiety to deactivate the key enzyme transpeptidase. This chemotherapeutic approach, although very selective, is hampered by a special class of enzymes that hydrolyzes the important cyclic amide C–N bond within the β-lactam ring, hence the name β-lactamases. These enzymes represent the most common mechanism of resistance to antibiotics[68] and they are mainly classified as serine-β-lactamases (classes A, C and D) or metallo-β-lactamases, mβLs, (class B) depending on the sequence homology (Ambler). An alternative classification based on substrate specificity also exists, which places mβLs under group 3 (Bush–Jacoby–Medeiros).[69] Depending on the catalytic mechanism employed, serine-β-lactamases use an activated serine nucleophile to attack the carbonyl carbon atom in the β-lactam ring, while mβLs employ a water (hydroxide) molecule coordinated to one or two zinc ions to achieve the same chemical effect (Scheme 4A).

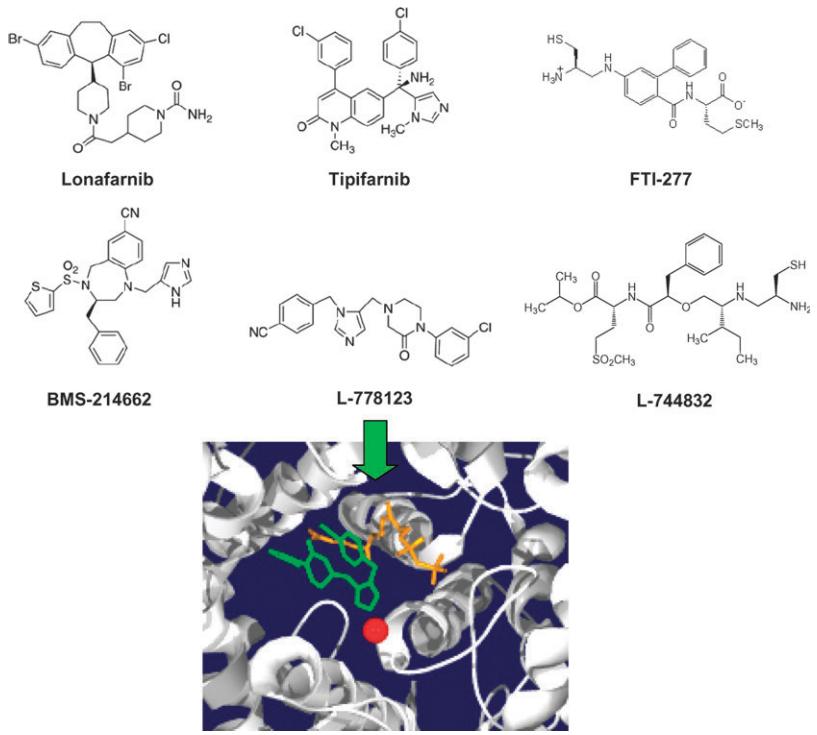
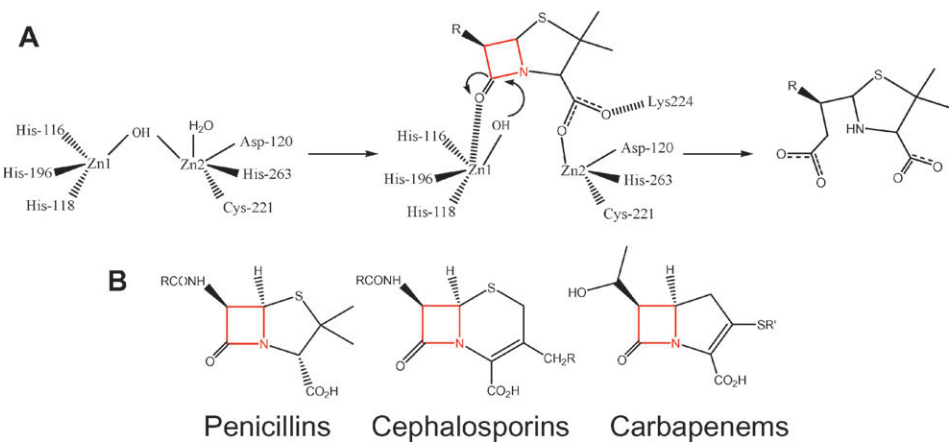


Fig. 6 Representative structures of farnesyl tranferase inhibitors (top). Image of the active site of human FT (white ribbon) showing the imidazole moiety of L-778123 (green) coordinated by the zinc ion (red). The farnesyl diphosphate molecule can also be seen (orange). PDB ID: 1S63.[67]

New zinc β-lactamases continue to be described and several pathogens are recognized to synthesize these enzymes. Metallo-β-lactamases are of special concern due to their broad activity especially against the carbepenems, the potential for horizontal transference and the current absence of clinically useful inhibitors.[70]

Based on amino acid sequence and substrate affinities mβLs are further divided in three subclasses (B1–B3, Fig. 7) but general features in the active site are somewhat conserved, that is one of the zinc ions (Zn1) has a tetrahedral geometry and the other (Zn2) is trigonal bipyramidal. Both zinc ions are usually within 3.6 Å and bridged by a hydroxide group, which is thought to act as the nucleophile in the hydrolytic reaction, although some results points to Zn2 as intimately involved in the hydrolytic reaction.[71] The nature of the coordinated residues for the zinc ions vary with the subclass. Accordingly Zn1 will coordinate three His residues for B1 and B3 subclasses, while two His and an Asn will be the coordinating residues for B2. Zn2 on the other hand coordinates an Asp, Cys and His triad in B1, B2 while the Cys is substituted by another His in B3; the coordination is completed by the bridging OH^- and a water molecule in Zn2. The affinities for zinc in the two coordinating sites are different, and it is accepted that B1 and B3 mβLs are active through Zn1 while the presence of Zn2 typically enhances their activity. On the contrary, for B2 mβLs the presence of Zn1 causes an inhibition in function and usually the active position is Zn2.[72]



Scheme 4 (A) Hydrolysis of β-lactam ring (red) in a penicillin derivative by a dinuclear zinc site (subclasses B1 and B3). (B) Basic structure in a β-lactam antibiotic.

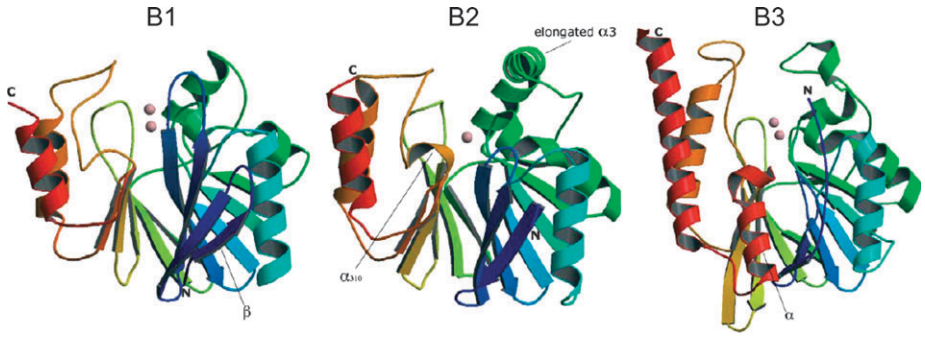


Fig. 7 Structural comparison among the three subclasses of metallo-β-lactamases from different features within each sub-class are highlighted.[73]

In terms of the mechanism employed by these enzymes it is accepted that the zinc ions are primarily responsible for substrate binding and catalysis, leaving to the surrounding ligands (with a few exceptions) the main role of maintaining the zinc ions in position. For mβLs sub-classes B1 and B3 with two zinc ions in the active site it is generally accepted that Zn2 and a conserved lysine residue Lys224 help to stabilize the substrate by interaction with the carboxylate moiety in the ring adjacent to the β-lactam, while Zn1 coordinates the carbonyl in the β-lactam ring and assists in the nucleophilic attack by the hydroxide ligand. For sub-class B2 with only one zinc, it has been proposed that the residue Asp120 acts as a general base that activates a water nucleophile for its attack to the β-lactam ring. Further steps in the cycle involve proton transfer and intramolecular arrangements to regenerate the active site.

II.d.2 Clinical relevance. β-Lactam antibiotics are the main front line against opportunistic Gram-negative bacterial pathogens that can affect severely the life expectancy in patients with a compromised immune system, *i.e.* HIV, chemotherapy, and advanced age. For this reason, and the important public health problem associated with resistant bacteria, β-lactamases are an important target for chemotherapy. mβLs in particular, despite representing a small group of β-lactamases, display a very broad substrate spectrum hydrolyzing almost all β-lactam antibiotics and especially carbapenem-derivatives, which are the newest and most powerful generation of β-lactams. In addition, there are no clinically useful inhibitors for mβLs, in contrast to serine-β-lactamases where inhibitors such as clavulanic acid or sulbactum can be used effectively. Indeed, all known inhibitors of serine β-lactamases are inefficient toward the metallo-β-lactamase class. The continued production of mβLs by major pathogens in the near future is also a very likely event due to the current abuse of antibiotics in clinical and agricultural purposes.[74] Worldwide surveillance programs such as the SENTRY, MYSTIC have confirmed an escalated rate of occurrence of mβL-mediated resistance for different gram-negative bacilli like the *P. aeruginosa* since 2000.[75] mβLs can be either chromosomally mediated or encoded by transferable genes, the most recognized among the latter are IMP-1, VIM-1, SPM-1 and GIM-1, and especially the first two types are the most frequent. The fact that genes encoding for these four mβLs can be associated with integrons and other genetic elements such as transposons or plasmids means a higher probability for worldwide dissemination.

II.d.3 Metallo-β-lactamases inhibitors (mβLIs). A variety of chemical classes have been tested as mβLIs (B1–B3), among them trifluoromethyl alcohols and ketones, biphenyl tetrazoles, succinic acids, peptides, cephamycins and several thiol compounds.[76] The most potent known inhibitors so far are 2,3-disubstituted succinic acids and mercapto-carboxylic acids, with inhibition constants in the 3–90 nM range (Fig. 8). One of the key steps in the mechanism of action for mβLIs is the displacement of the bridging hydroxyl ligand or water by an appropriate sulfur or oxygen atom in the thiol or carboxylate moiety, respectively. Formation of disulfides with the Cys ligand in Zn2 has also been observed leading to irreversible inhibition. The approach to design of an ideal mβLI, however, is by no means straightforward, since the wide distribution of dinuclear active sites in enzymes (*i.e.* human glioxalase II) could lead to the issue of a high toxicity due to inhibition of essential enzymes in the host. Also it has been shown that the affinity of any given mβLI can vary vastly among mβLs due to important differences in sequence diversity – the mutation of the highly conserved Lys224 to a Tyr224 in the important VIM-2 is a clear example of this affirmation. However, the role of two key amino acids has been highlighted in terms of design of more specific mβLIs, namely Lys224 and Asp120. The former residue and specifically the N_ε has been implicated by mutagenic and structural data as contributing to binding and orientation of substrates by hydrogen bonding to the carboxylate group usually present adjacent to the β-lactam ring (Scheme 4). A novel class of inhibitors takes into account

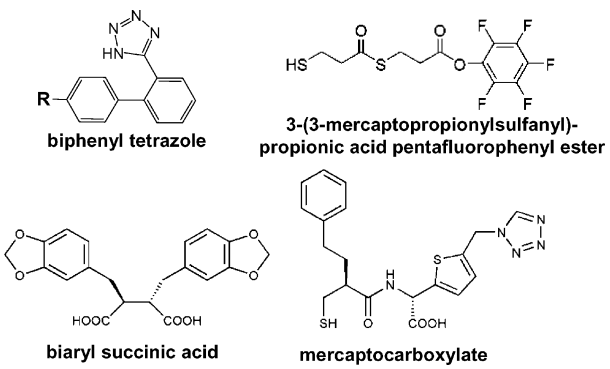


Fig. 8 Structures of representative metallo-β-lactamase inhibitors MBLIs.

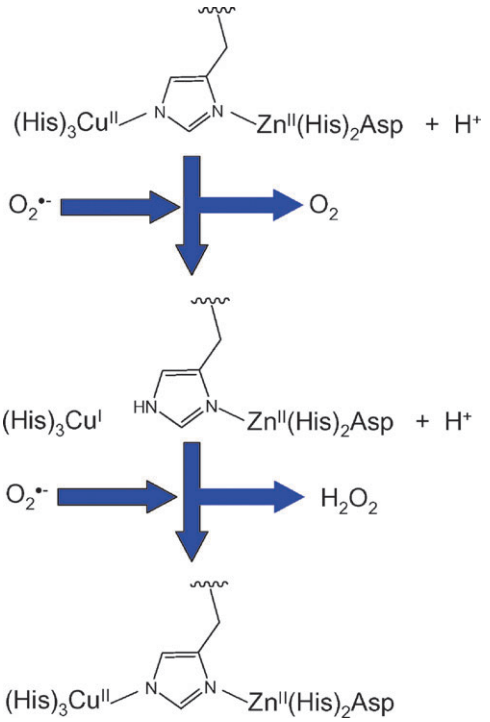
interactions with this residue in order to target the active site in IMP-1, a B1 mβL.[77] Another potentially relevant residue in the active site is Trp64 present in the flexible loop of mβLs that could be involved in hydrophobic/non-covalent interactions with the substrate. Additional residues in the active site could play a determinant role as recognition motifs and modulators of mβLI efficacy. Therefore its consideration should be taken into account in the design of novel inhibitors.[78] Up to now, as stated, none of the mβLIs reported have been developed into drugs.

II.e Zinc and neurochemistry

A growing area of considerable importance to bioinorganic chemistry is the role of endogenous metal ions (Zn, Cu, Fe) in the pathogenesis of various neurodegenerative diseases.[9,79] The role of metal ions in "metalloneurochemistry" or "metalloneurobiology" encompasses the study of metal ion homestasis and transport across membranes, the role of Zn^{2+} in synaptic transmission and in memory formation as well as the causes and treatment of neurological diseases. With specific respect to the role of Zn^{2+} ion in neurochemistry, there has been a concomitant increase in development of fluorescent sensors capable of specifically imaging the metal ion at biologically relevant concentrations. Besides the study of the "free" ions, a number of metalloproteins and their metal ion status have been implicated with the advance of some neurological disorders. This review will briefly discuss two of the most studied – superoxide dismutase and its relevance to familial ALS and the role of Zn and redox metal ions in Alzheimer's disease.

II.e.1 Cu, Zn-superoxide dismutase (SOD-1)

Structure and function. Up to 10% of the oxygen utilized by tissues may be converted by metabolism to its reactive intermediates, which impair the functioning of cells and tissues. These reactive oxygen species, such as superoxide radicals, are thought to underly the pathogenesis of various diseases. Superoxide dismutase (SOD) catalyzes the dismutation or disproportionation of the superoxide anion ($O_2^{\bullet -}$) into hydrogen peroxide and oxygen (Scheme 5). The reaction is performed in two steps, both first-order with respect to $O_2^{\bullet -}$ and is extremely efficient, being basically limited by substrate diffusion thus shortening the lifetime of the superoxide radical by a factor of $\approx 10^{10}$. There are several classes of SOD that differ in their metal-binding ability, distribution in different cell compartments, and sensitivity to various reagents. Among these, Cu, Zn superoxide dismutase (SOD1) is widely distributed and comprises 90% of the total SOD. The SOD-1 protein found in the cytosol is a homodimeric protein with 32 kDa molecular mass. Two other forms, SOD-2 (Mn) and SOD-3 (Cu, Zn), are found in the mitochondria and outside the cell, respectively forming tetramers. The structural details of Cu, Zn SOD have been well studied. Indeed, this is an example where the basic knowledge accrued from studying the enzyme from the "traditional" viewpoint of bioinorganic chemistry – structure and function related to the metal active site – was extremely helpful in the early 1990's once the role of the enzyme in neurodegenerative diseases was appreciated. Briefly, each subunit in SOD-1 contains 158 amino acids folding as a flattened eight-stranded β-barrel containing one catalytic copper and one structural zinc ion in the active site. The residues coordinating the metal ions are strictly conserved: His44, His46 and His118 for copper and His69, His78 and Asp81 for zinc. Residue His61 acts as a bridging ligand between the two metal centers that are approximately 6 Å apart, completing the tetrahedral coordination sphere for zinc and the square pyramidal coordination sphere for copper, together with a solvent molecule that is not involved in the catalytic cycle (Fig. 9). The metal ions, together with an important intramolecular disulfide bridge (Cys57–Cys146), are post-translational modifications that greatly stabilize the metalloprotein. The stability of wild type SOD-1 is remarkable, being active even after being subjected to harsh conditions such as 4% SDS, 10M urea or 80 °C. Several studies have highlighted the importance of the disulfide presence for dimer stability and further relevance in pathogenic aggregation.[80]



Scheme 5 Dismutation of two molecules of superoxide anion ($O_2^{\bullet -}$) by the Cu, Zn-SOD. The scheme shows successive breaking and formation of the inter-metallic bridge formed by His61, upon changes in copper oxidation state (Cu^{II}–Cu^{I}).

SOD-1 presents a funnel-like cavity or cone with a 24 Å diameter at the surface of the metalloprotein ending in a narrow 4 Å channel that leads to the partial exposure to solvent of the active copper ion. The zinc ion is indirectly involved in the accepted catalytic mechanism with an important role for electronic polarization and electrostatic stabilization due to the His61 coordination. The two steps in the catalytic cycle for SOD-1 correspond to a first half-reaction where the reduction of copper (Cu^{II}–Cu^{I}, inner sphere mechanism), upon binding of a first superoxide anion, is coupled to protonation of H61 and simultaneous rupture of the Cu–Zn bridge. The resulting cuprous ion with a trigonal planar coordination now releases dioxygen as the first product, diffusing out of the electrostatic cone thanks to its neutral

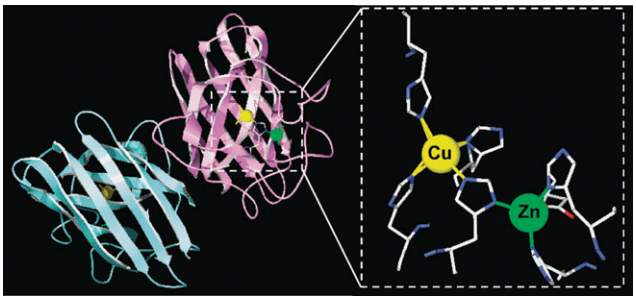


Fig. 9 Ribbon structure of Cu, Zn-SOD homodimer, showing the metal ions Cu^{2+} in yellow and Zn^{2+} in green (left). Image of the active site showing the coordinating residues (right).

charge. In the second half-reaction, a subsequent superoxide anion enters the cone and oxidizes the Cu^{I} by an outer-sphere mechanism to its initial state, with protonation of the substrate to form and release hydrogen peroxide re-establishing the imidazolate bridge through His61.

II.e.2 Clinical relevance. This ubiquitous enzyme has great physiological significance and therapeutic potential from a number of perspectives.[81] SODs are implicated in cancer and several neurological disorders. The lack of success in the development of SOD enzyme as a therapeutic agent can be attributed in part to a lack of an accurate method to quantitate SOD activity, lack of oral bioavailability and inability of the enzyme to enter cells. Small molecule mimics of SOD such as Mn-cyclams could overcome these deficiencies – again an interesting example of metal-based targeted drugs.[82] In spite of the important biological function of SOD-1 in depleting the reactive superoxide anion, a selective targeting of this metalloprotein could also be important for anticancer applications. Due to the exacerbated metabolism in cancer cells the production of reactive oxygen species in general is increased and cancer cells could exhibit a higher dependence on SOD activity. It has therefore been suggested that inhibition of this metalloprotein could have potential therapeutic interest as an antitumor strategy.[83]

Recent interest in the medical relevance of SOD-1 derives from the presence of mutants associated with Amyotrophic Lateral Sclerosis (ALS) or Lou Gehrig's disease, a neurodegenerative disorder characterized by the selective death of motor neurons in the spinal cord, brain stem and brain. This disease is one of the most common neurodegenerative disorders, with a prevalence of 4–6 per 100 000 and affecting more than 35 000 people in the USA alone. It is frequently diagnosed between the ages of 40 and 70, being 20% more common in men than women. Specifically, point mutations in SOD-1 have been linked to a sub-set of the familial form of ALS or fALS, which constitutes approximately 1/5 of the cases. Up to 130 point mutations in SOD-1 (almost 1/3 of the protein sequence!) have been identified so far. They are evenly distributed throughout the metalloprotein and have been classified mainly in two groups based on their position and metal content (see http://www.alsod.org). The first group comprises the so-called "wild-type-like mutants" with an almost intact metal content compared to the wtSOD-1. The second group comprises "metal-binding region mutants" that exhibit mutations in the metal-binding region involving coordinating residues or close neighbors, thus generating mutants with aberrant metal content. Since some mutant forms of SOD-1 retain the dismutase activity of the wild type, the toxicity arising from mutations is rather ascribed to a gain of function. Several hypotheses have been suggested in this regard but, so far, two main views are predominant in the literature, although these are not necessarily mutually exclusive. The "perplexing" role of SOD-1 in fALS has been summarized.[84] One general hypothesis states that mutant SOD-1 toxicity is due to an enhanced oxidative activity, which contributes to oxidative damage, supported by evidence of the high level of oxidative-stress in presence of SOD-1 mutants and stimulation of oxidative damage in the presence of physiological bicarbonate.[85] However, although an increased peroxidase and thiol oxidase activity has been found for zinc-deficient SOD-1,[86] and there is a certain correlation between wild-type-like mutants and this hypothesis;[87] the majority of SOD-1 mutants do not exhibit such an enhanced activity compared to the wild type further complicating support for this hypothesis.

A second hypothesis has gained considerable support and involves essentially the misfolding and aggregation of mutant SOD-1, a common point also for other neurodegenerative diseases such as Alzheimer's disease and transmissible spongiform encephalopathies. A multi-step process has been suggested for the mechanism of aggregation, involving sequential dimer dissociation, metal loss from the monomer and oligomerization of the apo-monomers.[88] This contrasts with the sequence of post-translational modifications involved for activation of the metalloprotein *i.e.* copper and zinc binding, disulfide bridge formation and dimerization. Several results support this hypothesis. Recently the equilibrium between the native dimer, monomer intermediate and the unfolded monomer was observed and studied for various mutants, with favorable conditions for the last two monomer states.[89] Different conformations in living cells have been also observed for SOD-1 mutants compared to wild-type metalloprotein, using a fluorescent assay with green fluorescent protein.[90] Determination of melting temperature in fALS relevant SOD-1 mutants by differential scanning calorimetry has been extensively used to compare relative stabilities in the apo-protein and various reports evidenced a net destabilization for the mutants compared to the wtSOD-1.[91] However, another recent study using a larger range of mutants (20) found that some of them can actually exhibit higher stability compared to the wtSOD-1 compromising to some extent the general agreement, the results were confirmed by hydrogen–deuterium exchange.[92] Clarification of this point is important since the nascent SOD-1 apo-monomer is regarded as the main fALS relevant toxic species.

As mentioned before, the metal ions are important for kinetic stability in SOD-1, and in this regard it has been determined that copper contribution is greater than Zn.[93] The other important stabilizing key feature is the sulfide bridge, where it has been shown that mutants are more susceptible to reduction on this sulfide bridge and recent *in vivo* experiments points to an incorrect disulfide cross-linking in the mutant SOD-1 as a cause for aggregation.

Aberrant intra- to inter-molecular reactivity from these immature of disulfide-reduced forms in mutants can lead to insoluble high molecular mass species containing even wild-type SOD-1.[94] Overall evidence points to stability issues generated in the SOD-1 homodimer by the mutants, and this is a biologically relevant point in the mechanism. In this regard, molecular dynamics has contributed with significant findings, first by identifying disruptions in the inter-monomer dynamics in all mutants tested. An additional study involving kinetic measurements of folding behaviour suggest that misfolding issues in the SOD-1 apo-monomer could arise from β-strands 1–3.[95]

The aggregation hypothesis is thus the most actively studied regarding mutant SOD-1 toxicity, although it is worth mentioning alternative hypotheses such as an aberrant copper chemistry producing mutations that can lead to catalytic nitration of tyrosine residues close to the copper ion.

II.e.3 SOD inhibitors. The wide variability in SOD-1 mutations linked to fALS makes a structure-based drug-design approach to inhibitors extremely difficult. Nevertheless, the screening of small molecules that can enhance SOD-1 dimer stability and prevent SOD-1 aggregation might also prevent amyeloid formation and the onset of ALS.[96] In general, results obtained for animal models of SOD1-linked fALS have helped in the development of novel chemotherapeutic approaches for ALS.[97] Current approved treatment for ALS is riluzole (rilutek) – 2-amino-6-(trifluromethoxy)benzothiazole – which has an inhibitory effect on glutamate release and ability to interfere with intracellular events that follow transmitter binding at excitatory amino acid receptors.[98] This drug prolongs survival by only three months and improvement on quality of life or muscle strength is not clear. Advances in drug development are hopefully increasing the number of potential therapeutic agents. Valproic acid has also been implicated in ALS suppression in mice models.[99] This agent has found use previously as an HDAC inhibitor (See section II.b), and by analogy, a zinc ion interaction may also exist in this case.

II.e.4 Zinc, redox metal ions and Alzheimer's disease. The role of Zn^{2+} ion in the brain cannot at this time be separated from that of the redox active Fe and Cu ions. The maintenance of metal ion homeostasis in the brain is of paramount importance to normal function. There is evidence that endogenous metal ion dyshomeostasis contributes to the neuropathology of Alzheimer's disease (AD).[100] This progressive degenerative disease destroys the mental health of millions of people worldwide, with the subsequent loss of independence producing devastating social effects on family. Four features characterize the AD brain: (i) the presence of extracellular amyloid plaques comprised mainly of aggregated, insoluble amyloid-β (Aβ) peptide; (ii) the presence of neurofibrillary tangles (NFTs) containing hyperphosphorylated tau; (iii) increased oxidative damage to lipids, proteins and nucleic acids; and (iv) loss of endogenous metal ion homeostasis (dyshomeostasis).

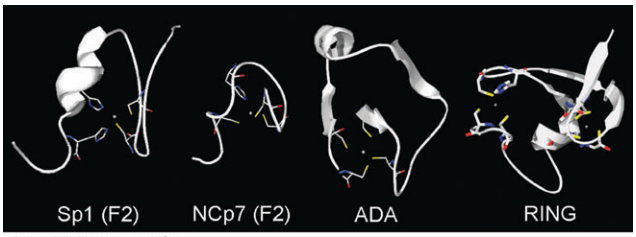
The difficulties in separating cause from consequence in delineating the role of metal ions in AD is well appreciated. Nevertheless, the evidence of neural metal ion concentration and distribution deserves examination. Iron is unevenly distributed in the brain and is enriched in areas such as the substantia nigra. Likewise, the largest labile pool of Zn^{2+} in the brain is found in the synaptic region of the hippocampus. It has been pointed out that the cortical glutamatergic synapse, where amyloid pathology is first manifested in AD, contains high concentrations of Cu and Zn, which are released during neurotransmission. All three ions interact with Aβ protein and the observation that the chelator EDTA could prevent Cu, Zn-induced Aβ aggregation suggested a therapeutic approach involving modulation of metal bioavailability. Certainly, in the absence of effective therapies, modulation of dyshomeostasis through metal ion chelation is an interesting approach, despite the difficulties of selectivity, passage through the blood brain barrier and metal-ion specificity.[101] The iron chelator desferrioxamine has been used to reverse or retard the pathology of AD. The hydroxyquinoline based chelator clioquinol has also received limited clinical trials, spurring the search for a newer generation of metal–ligand based therapeutics.

III. Structural zinc

III.a Zinc fingers (ZFs)

III.a.1 Structure and function. The zinc finger (ZF) motif, first described in the transcription factor TFIIIA from the clawed toad *Xenopus laevis*,[102] exhibits a notably diverse array of structure and functions, the latter involving important cellular processes such as transcription, DNA repair, cellular signaling, metabolism and apoptosis. Typically the term ZF implies a definite number of amino acid residues within the protein, usually 30 to 40, with suitable metal-binding sites composed of cysteines (Cys) and histidines (His). A key component of this system is the zinc ion (Zn^{2+}), which binds to the residues in a tetrahedral environment providing essential elements of structure. Release or substitution of the central zinc ion, as well as mutation of coordinating residues can result in a loss or impairment of the biological function. In ZFs the Zn^{2+} ion is thermodynamically preferred to other metal ions such as Co^{2+}, Ni^{2+} or Fe^{2+}, attributed to contributions from ligand field stabilization energy and other entropic factors.[103] Formation and dissociation of ZFs has been postulated to be a multi-step process, and the presence of an equilibrium between a tri- and tetra-coordinate zinc could exist.[104] This fact is in agreement with the reported non-equal contribution from the coordinating residues to the formation of ZFs.[105]

Typically ZFs can be classified according to the type of residues coordinated by the metal ion, *i.e.* Cys_2His_2, Cys_3His or Cys_4 (Fig. 10), although this classification might be extended to include more complex domains that need more than one zinc ion for structure stabilization. Zinc fingers are estimated to represent 3% of the human genome – a total of 4500 Cys_2His_2 zinc finger domains from 564 proteins are recognized, whereas only 17 domains from 9 proteins contain the CysCysHisCys zinc knuckle motif.[106] Double ZFs like the estrogen receptor (Cys_8) require eight binding residues, that



Sp1 (F2) = Cys_2His_2
NCp7 (F2) = Cys_3His
ADA = Cys_4
RING = Cys_4

Fig. 10 3D structures of different ZFs showing coordinating residues. PDB ID: 1VA2 (Sp1),[109] 1ESK (NCp7),[110] 1ADN (ADA),[111] an intertwined dinuclear domain is also shown PDB ID: 2D8T (RING).[112] Sequences for the domains shown are at the bottom with coordinated residues in red (coordinating residues for the second zinc in RING are underlined).

are predominantly cysteines, and the mode of Zn coordination can vary between a simple one, with the first four residues coordinating Zn1 and the second four Zn2, to an intertwined or an interleafed coordination, and in addition bridging cysteines can be found for Cys_6-ZFs.[6,107] Within this review we will use the term zinc finger for functional and independent zinc sites with at least two cysteines as binding residues, but it is useful to keep in mind that the versatility of zinc–protein complexes for modulation of biological functions depends largely on the type of secondary structure or fold formed by the ZFs. More than 10 different topologies for the ZF motif have been reported, although in general it features a Cys_2His_2-like, treble clef or zinc ribbon structure.[2,108]

The classical role of Cys_2His_2-ZFs as transcription factors has focused attention on the mode for DNA recognition, which mainly involves hydrogen-bond interactions between side chains of α-helical residues (frequently at positions −1, +2, +3 and +6) and base pairs in the DNA major groove (preferences for arginine/guanosine; aspartic acid/adenosine, cytidine; leucine/thymidine). Whereas other DNA-binding proteins generally make use of the 2-fold symmetry of the double helix, zinc fingers do not and so can be linked linearly in tandem to recognize DNA sequences of different lengths, with high fidelity. Every ZF recognizes 3 or 4 consecutive base pairs and some of the ZF units in a larger array can be used as spacers. This is true for Sp1 and Zif268 (both $3 \times Cys_2His_2$) but a slightly different DNA recognition pattern can be found for other Cys_2His_2-ZFs, *i.e.* GLI ($5 \times Cys_2His_2$). This modular design and the large number of combinatorial possibilities within the ZF motif offer an attractive approach to the design of DNA-binding proteins for the specific control of gene expression. Fusion of zinc finger peptides to repression or activation domains can selectively target genes for switching off and on. Cys_2His_2-ZFs have been methodically designed and used in an array of ZF modules ($n \times Cys_2Hys_2$) to further increase the sequence-specific recognition of DNA.[113,114]

The interaction of Cys_2His_2-ZFs with RNA on the other hand, has gained increased interest thanks to crystal structures of TFIIIA in complex with a core 5S rRNA, where two different modes of interaction are observed depending on the substrate DNA/RNA. It was found that fingers used as spacers for DNA interaction, ZF4 and ZF6, are strongly involved in the interaction with RNA. The former by interactions of a histidine residue with two guanines (His119-Gua75, Gua99) in the internal loop region E, and the latter by stacking of a tryptophan residue into an adenine (Trp177-Ade11) in loop A. ZF5 only interacts with backbone atoms in helix V.[115] This latter type of interaction involving stacking of aromatic residues in the zinc finger, mostly tryptophan, with nucleobases has been reported for the HIV Nucleocapsid 7 protein (NCp7).[116] In the particular case of an Cys_3His-ZF, exposed guanine bases bind to hydrophobic clefts in both ZFs of the protein, and additionally the ZFs form hydrogen bonds to groups in the RNA that normally engage in Watson–Crick hydrogen bonding in A helices.[117] Cys_4-ZFs are another motif for DNA recognition as exemplified in the typical DNA-binding domain (DBD) for nuclear receptors like the human estrogen related receptors (hERR1–hERR3) or the glucocorticoid receptor, both involving two sets of Cys_4-ZFs in tandem ($2 \times C_4$). In contrast to Cys_2His_2-ZFs which bind DNA as monomers, Cys_4-ZFs have been found in many instances to bind as homodimers or heterodimers, in the former case they exhibit two-fold rotational symmetry

III.a.2 Clinical relevance. One of the interesting features regarding ZFs is the high specificity and affinity in substrate binding that they can achieve, and as stated they have been proposed as key building blocks in the design of proteins towards human gene therapy.[118] One of the potential applications is to use ZFs in combination with endonuclease proteins to selectively produce DNA double strand breaks, because the presence of these lesions is reported to increase the frequency for homology recombination events thus favoring gene targeting to a specific mutation. This is a very promising area of research with recent examples of viability in targeting specific genes. In addition, several libraries of ZFs have been developed to discriminate DNA sequences, and are available for the design of novel polydactyl ZFs.[119,120]

Alternatively, the sequence specificity of ZFs can also be coupled to a transcription activation or repression domain to regulate gene expression. An example is the use of a designed zinc finger to inhibit gene expression in the virus of herpes simplex 1 using a six-finger peptide to partially repress the replication cycle in the virus.[121] This strategy has also been applied to inhibit transcription and replication of HIV-1.[122] Moreover the use of designed ZFs to regulate endogenous genes promotes expression of the natural splice variants of that gene, in contrast to standard gene therapy where only a single variant of the gene is expressed.

Cys_2His_2-ZFs belonging to the Sp (specificity protein) and KLF (Krüppel-like factors)[123] are recognized as targets for development of new anticancer drugs. Their involvement in tumorigenesis stems from a variety of reasons including its interaction with oncogenes and tumor suppressors. Sp1 alone is thought to regulate several hundreds of genes and is the most characterized transcriptional activator in mammalian cells. The importance of this family of transcription factors in gene regulation for tumor development, growth and metastasis has been reviewed.[124] The LIM superfamily of transcription factors briefly mentioned before features a double ZF

motif involved mostly in protein–protein interactions, related to cellular architecture, intracellular signaling and transcriptional processes. Their involvement in multiple human diseases has been addressed.[125] Another important superfamily exhibiting double ZF motifs ($2 \times Cys_4$) in their DNA-binding domain is the nuclear receptor. Its protein members are activated as transcription factor after binding of small lipophilic molecules such as lipids, metabolites or steroid hormones. Specifically, the steroid hormone glucocorticoid produces glucocorticoid receptor (GCR) proteins, whose clinical importance stems from the anti-inflammatory/immunosupresive effects exhibited by glucocorticoid hormones.

III.a.3 Zinc fingers inhibitors (ZFIs). In contrast to catalytic zinc, where inhibition is usually through blocking of an active site, inhibition of structural zinc must involve chemical modification of the coordinating residues (oxidation/alkylation) with metal-ion removal. Inhibition of ZFs, as in the case of MMPs and HDACs, is a double-edged sword because, although beneficial responses can be achieved, the potential risk of impairing or disturbing essential cellular functions is also high. In fact the damage of zinc fingers in DNA repair proteins such as the xeroderma pigmentosum group A (XPA), one of the proteins involved in the nucleotide excision repair pathway, by oxidizing agents or redox-active metals has been regarded as a novel mechanism of carcinogenesis.[126] In this regard it has been shown that human DNA polymerase-α is inhibited by *cis*-diamminedichloroplatinum(II), by covalent interaction with the cysteine residues on its C_4-ZF motif.[127] Other important zinc metalloproteins involved in DNA/RNA repair could be potentially inhibited, as in the case of the bacterial sacrificial protein Ada whose Cys_4-ZF motif located in the *N*-terminal domain repairs the methyl phosphotriester lesion in DNA. In this mechanism an alkyl group is transferred from the damaged DNA to an activated cysteine (Cys38) residue in the ZF.[128,129] In addition, the interaction from the DNA/ZF interaction in Sp1 and TFIIA has been reported to be disrupted by selenite ions.[126]

However, there are cases in which structural differences between the target substrate to be inhibited and alternative substrates can be exploited, as in the case of key ZFs within retroviruses and arenoviruses.[130] In addition the possibility to extend this strategy to other viruses is feasible given the importance of zinc domains for virus stability. The nucleocapsid protein NCp7 (HIV-1) in particular, has been the subject of an in-depth study in the last years including characterization and substrate interaction.[131]

This small protein with two Cys_3His-ZFs, plays many essential roles along the viral life cycle (such as RNA packaging, reverse transcription and integration). In addition it is remarkably mutation intolerant, therefore making it an attractive target for the development of novel and complementary HIV chemotherapeutic agents. Several molecules with electrophilic functional groups have been employed to oxidize or methylate cysteine residues within the ZFs causing inhibition of normal NCp7 functions. Specifically thiosulfonate and azodicarboxamide (ADA) have been shown to promote zinc ejection from NCp7 at concentrations that do not impact other important human ZFs such as Sp1, PARP or GATA-1; this fact shows that key differences in terms of ZF reactivity could lead to an appropriate discrimination of inhibition targets (*i.e.* Cys_3His in NCp7 *vs.* Cys_2His_2 or Cys_4, which are more common in human ZFs).[132] Additional non-covalent motifs can also be exploited for an additional increased selectivity towards NCp7; recently platinum–nucleobase complexes explored this area by the use of nucleobase recognition from the C-terminal ZF, reporting zinc displacement and loss of tertiary structure (Fig. 11).[133]

III.b p53

III.b.1 Structure and function. The tumor suppressor gene Tp53 codes for the p53 protein with a molecular mass of 53 kDa (hence its name), corresponding to 393 amino acids in four domains: an N-terminal transactivation domain, a central DNA-binding core domain, an oligomerization (tetramerization) domain and a C-terminal regulatory domain. The central DNA-binding core domain (DBD) with a molecular mass of 25 kDa comprises residues 94–312 and contains the essential structural zinc ion. The zinc ion coordinates Cys176, Cys238, Cys242 and His179 in a pseudo-tetrahedral coordination geometry (Fig. 12), that connects loops L2 and L3 in the domain. The Zn^{2+} is required for structural reasons, since the apo-protein exhibits a DBD significantly different with reduced DNA-binding specificity and prone for aggregation. The role of zinc in p53 function extends beyond this domain and has been shown to coordinate the movements of different structural elements in the protein required for DNA binding.[134]

The p53 metalloprotein has a central role in one of the major signal-transduction pathways in the cell, which regulates its response to environmental and internal cues. At the same time this pathway is intimately linked to others and their inter-coordinaton has been proposed to be regulated by loops, which involves proteins such as P38, COP1, PIRH2, AKT, *etc*.[137] In addition, p53 has been called the guardian of the genome due not only to its role as a tumor suppressor but also as a regulator of more than 160 genes in response to various types of stress – in fact microarray experiments have suggested 500 up-regulated and 260 down-regulated p53 target genes! In the presence of diverse stress signals that can be classified as genotoxic (DNA adducts or breaks), oncogenic (activation of proto-oncogens) or non-genotoxic (ribonucleotide depletion or oxygen oversupply), the p53 protein is activated mainly by post-translational modifications including phosphorylation, acetylation, methylation and others. These changes increase p53 concentration in the cell by several ways (p53 modifier–partner relationship) including MDM2 degradation. Subsequently, the modified p53 can bind to specific DNA sequences, tetramerize and enhance the transcription rate of required genes (Fig. 8). The details of such p53–DNA interaction involving the tetramer were characterized recently for different DNA sequences, a correlation between differential binding affinities for the sequences studied and protein–DNA contact geometry was demonstrated.[136]

III.b.2 Clinical relevance. The evidence showing an involvement of p53 in human tumors is overwhelming. Mutations of the gene encoding for this metalloprotein are found in

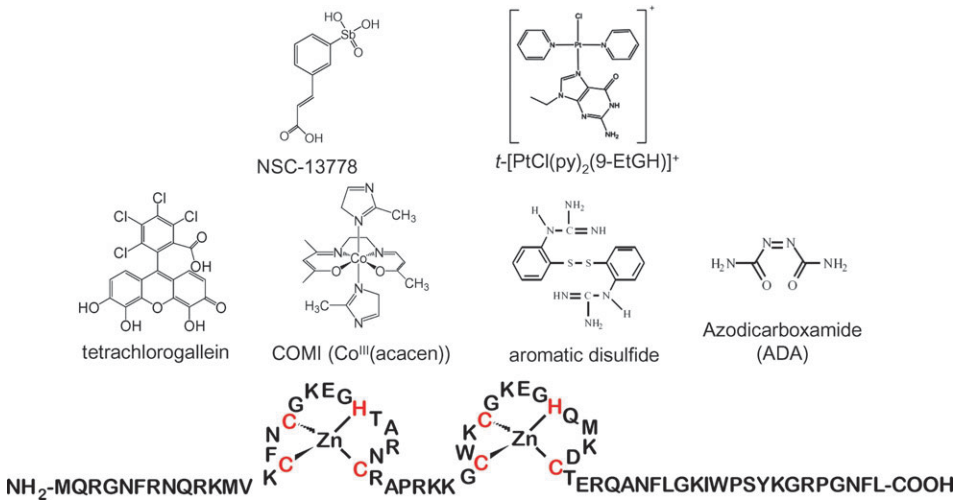


Fig. 11 Structure of representative organic and metal compounds used to target the nucleocapsid protein (NCp7, top). Sequence of NCp7 showing coordinating residues in red (bottom).

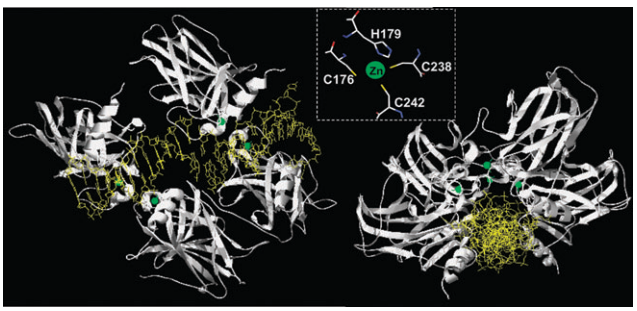


Fig. 12 Two different views of a p53 tetramer (white ribbon) with DNA (yellow), the structure was determined using the DNA-binding site only. Zinc ions are represented in green. First coordination sphere in p53-zinc site is shown inside square. PDB ID: 2FEJ, 2AC0.[135,136]

approximately 50% of tumor cases, causing either a loss of activity or a gain in function producing p53 capable of cellular transformation.[138] In the latter case two types of pathways are differentiated, the first corresponding to aberrant interactions with DNA as shown for p53 mutants interacting with different DNA regions compared to wild type p53 (wt-p53). The other type of aberrant interaction observed for mutants is with diverse cellular proteins such as with p73, an homologue protein that does not interact with wt-p53 but its reactivity with mutated p53 has shown otherwise. Moreover this interaction correlated with resistance to anticancer agents.[139] p53 is at the junction of several interrelated pathways in the cell. A wide variety of stress signals activates p53 resulting in cellular responses such as apoptosis, cell-cycle arrest, senescence and DNA repair.[140] There is evidence that p53 can play a part in determining which response is induced through differential expression of target-gene expression. The importance of p53-induced response to tumor suppression is clear but previously unanticipated contributions of these responses are being uncovered. An integrated view of p53 has lately suggested that not all of its functions are beneficial.[141]

III.b.3 p53 Inhibitors (p53Is) and the bioinorganic chemistry of apoptosis. Therapeutic strategies based around the p53 pathway include both inhibition and activation of the protein. The frequency of p53 mutations in human tumors and the presumed dominant gain-of-function effect of p53 mutations makes mutant p53 a prime target for pharmacological therapeutic intervention in cancer. Gene therapy with wtp53, mimicking downstream genes, and pharmacological rescue of mutant p53 have all been explored to reactivate mutant p53. In the case of wtp53, activation of the p53 response, inhibition of Mdm2 function and expression may all be categorized as therapeutic strategies. Novel strategies aiming to restore or control p53 function in cancer patients have also made use of viruses to either destroy wt-p53 deficient cells or in a gene-therapy approach to replace wt-p53.[142]

There are three aspects to mutations in p53, not all of which are shared by all mutants found in human cancer: loss of function (common to all), dominant negative activity (present in about one third), and gain of function (present in some).[143] Tumor cell selectivity may be achieved because the target is often expressed at high levels in tumor, but not normal, cells and tissues. Selectivity could also be expected from p53-activating post-translational modifications in tumor cells. Pharmacological reactivation of mutant p53 should efficiently eliminate tumor cells through induction of apoptosis with minor unwanted side effects. Different classes of mutants require different rescue strategies. Two broad structural classes of mutant are defined as (a) involving residues that physically contact the DNA, including the two mutational hot spots Arg248 and Arg273 and (b) those residues destabilizing tertiary structure essential for DNA binding. The first group may be further subdivided into DNA-contact mutations with little effect on folding or stability (Arg273His) and those that cause a local distortion in proximity to the DNA-binding site (Arg249Ser). DNA contact mutants need the introduction of functional groups that will establish new contacts with the DNA, compensating for the missing contacts. Globally unfolded mutants could be rescued by stabilizing agents that will lead to refolding of the mutant.

Small molecules and peptides can restore DNA-binding ability to mutant p53.[144] Some of these molecules, such as

ellipticine and CP-31398, do not bind directly to the protein but may be involved in the protein–DNA complex or in other protein-related cellular events. On the other hand, the low molecular weight compound PRIMA-1 is capable of inducing apoptosis in human tumor cells through restoration of the transcriptional transactivation function to mutant p53. The molecule restored sequence-specific DNA binding and the active conformation to mutant p53 proteins *in vitro* and in living cells and rescued both DNA contact and structural p53 mutants. *In vivo* studies in mice revealed an antitumor effect with no apparent toxicity. The molecular mechanisms of mutant p53 reactivation are largely unknown and may well be diverse. Nevertheless, the principle is established that small molecules may restore DNA-binding capacity to mutant p53s.[145]

Pifithrin is the most representative of p53Is and acts at the post-transcriptional level; it was discovered in 1999 in a screening study for compounds that block the transcriptional activity of p53. This compound can rescue wt-p53 cells from apoptosis induced by irradiation and cytotoxic drugs, therefore has been widely used as a protector against secondary effects from chemotherapy and radiotherapy. However, a careful balance between the treatment and the dose of pifithrin needs to be maintained to avoid mutagenesis. The design of improved P53Is based on pifithrin's moiety has been published recently, an additional *in vivo* cyclization from the precursors is suggested to increase the activity in this novel compounds.[146] To date, no P53Is has been approved for use; so far the mechanism of action of pifithrin and derivatives remains largely unknown, although there is strong evidence suggesting that its antiapoptotic action is p53 dependent and does not affect the ubiquitin pathway. In this regard the consideration of p53-independent therapeutic effects should also be taken into account for pifithrin and analogue compounds.

On the other hand, disruption of the MDM2–p53 interaction has been achieved by the use of *cis*-imidazoline analogues called nutlins or benzodiazepinediones (Fig. 13), these inhibitors need to basically inhibit the formation of the MDM2–p53 adduct by mimicking three key residues from p53: Leu26, Trp23 and Phe19 (Fig. 14), which are in close proximity at the same end of a helix and bind in a hydrophobic cleft of MDM2. Efforts towards the development of enhanced affinity derivatives are currently under way.[147] It has been reported that even with peptide sequences as small as nine residues, it is possible to bind and stabilize p53 mutants with subsequent restoration of DNA-binding activity.[148] Other inhibitors for MDM2 are either small hydrophobic molecules or oligomeric molecules that can reproduce the natural substrate site in p53, although novel peptoids where the side chain is directly attached to the backbone nitrogen atom rather than to the α-carbon have also been tried recently.[149]

Zn and p53 therapeutic strategies. Analysis of the role of Zn effects in p53 structure and function interestingly reveals a growing emergence of the appreciation of the role of redox-metal ions in p53 function and indeed in apoptosis. Modulation of p53 protein conformation and DNA-binding may be achieved through intracellular zinc chelation.[151] Likewise, zinc may mediate the renaturation of p53 after exposure to metal

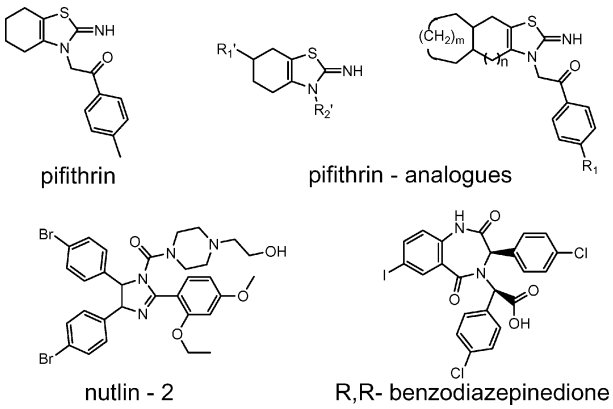


Fig. 13 p53 inhibitors (top) and mdm2–p53 inhibitors (bottom).

chelators *in vitro* and in intact cells. Copper ions are also critical for p53 protein conformation and DNA-binding activity, implying that copper ions may participate in the physiological control of p53 function.[152] In this case, unlike Zn, a role for redox activity is possible.

Thus, metal binding and oxidation–reduction may affect p53 activity *in vivo*. There is a possible involvement of thioredoxin, Ref-1 (redox factor 1), and metallothionein in the control of p53 protein conformation and activity. The data indicate that p53 lies at the center of a network of complex redox interactions. In this network, p53 can control the timely production of reactive oxygen intermediates (*e.g.*, to initiate apoptosis), but this activity is itself under the control of changes in metal levels and in cellular redox status. This redox sensitivity may be one of the biochemical mechanisms by which p53 acts as a "sensor" of multiple forms of stress.

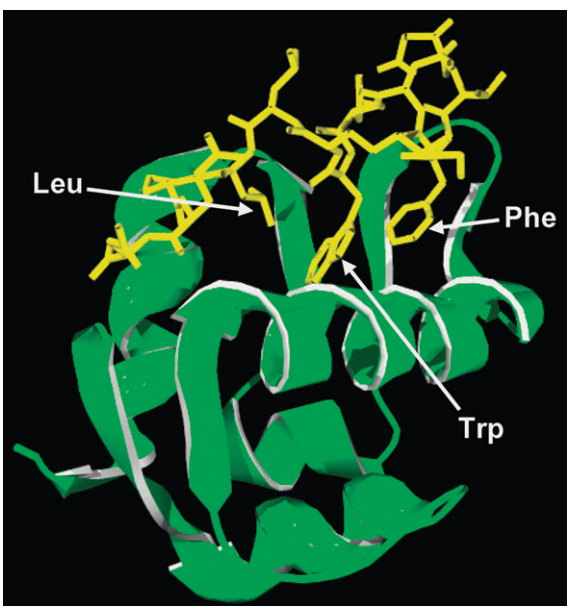


Fig. 14 Crystal structure of the 109-residue amino-terminal domain of MDM2 (green) bound to a 15-residue transactivation domain of p53 (yellow). Potential inhibitors of the p53–MDM2 interaction generally mimic the interaction of the key residues Phe19, Trp23 and Leu26 indicated with the arrows. PDB ID: 1YCR.[150]

Metal chelators as well as well-defined metal Cu and Zn compounds may affect p53 expression and apoptotic pathways in cells. Tachpyridine (*N*,*N*′,*N*″-tris(2-pyridylmethyl)-*cis*,*cis*-1,3,5-triaminocyclohexane; tachpyr) is a potent hexadentate iron chelator under pre-clinical investigation as a potential anticancer agent. Tachpyridine induces apoptosis in cultured cancer cells by triggering a mitochondrial pathway of cell death that is p53-independent.[153] Major species identified in cells treated with tachpyr were tachpyr itself, [Zn(tachpyr)](2+), and iron coordinated to two partially oxidized species of tachpyridine, [Fe(tachpyr-ox-2)](2+), and [Fe(tachpyr-ox-4)](2+). [Zn(tachpyr)](2+), [Fe(tachpyr-ox-2)](2+), and free tachpyr accounted for virtually all of the tachpyr added, indicating that iron and zinc are the principal metals targeted by tachpyridine in cells. Consistent with these findings, activation of the apoptotic caspases 9 and 3 was blocked in cells pre-treated with either iron or zinc. Pre-treatment with either of these metals also completely protected cells from the cytotoxic effects of tachpyridine.

The pro-apoptotic activity of two new synthesized isatin-Schiff base copper(II) complexes, obtained from isatin and 1,3-diaminopropane or 2-(2-aminoethyl)pyridine: (Cu(isapn)) and ($Cu(isaepy)_2$), respectively has been reported.[154] These compounds trigger apoptosis *via* the mitochondrial pathway. The extent of apoptosis mirrors the kinetics of intracellular copper uptake. Particularly, $Cu(isaepy)_2$ enters the cells more efficiently and specifically damages nuclei and mitochondria, as evidenced by atomic absorption analysis of copper content and by the extent of nuclear and mitochondrial integrity. Conversely, Cu(isapn), although less permeable, induces a wide-spread oxidative stress, as demonstrated by analyses of reactive oxygen species concentration, and oxidation of proteins and lipids. The increase of the antioxidant defense, through the overexpression of Cu, Zn-SOD, partially counteracts cell death; On the contrary, apoptosis significantly decreased when the analogous zinc complex was used or when $Cu(isaepy)_2$ was incubated in the presence of a copper chelator. Altogether, the data provide evidence for a dual role of these copper(II) complexes: they are able to vehicle copper into the cell, thus producing reactive oxygen species, and could behave as delocalized lipophilic cation-like molecules, thus specifically targeting organelles.[154]

Decreased intracellular zinc concentration has also been shown to precede early markers of apoptosis, with alterations in mitochondrial transmembrane potential preceding the loss of polarity in the cell membrane.[155] Dansylamidoethylcyclen, as a biomimetic Zn^{2+}-selective fluorophore, has been demonstrated to be a good detector of the apoptosis (induced by an anticancer agent, etoposide, and H_2O_2 in cancer cells such as HeLa and HL60 cells.[156] The macrocyclic Zn^{2+} ligand (mostly as a deprotonated form) is cell-permeable and shows weak fluorescence, but forms a strongly fluorescent 1 : 1 Zn^{2+} complex when Zn^{2+} is incorporated into the cells by a zinc(II) ionophore pyrithione.

IV Zinc as enzyme inhibitor

An interesting aspect of zinc in biology and medicine is that, in addition to its catalytic and structural roles, zinc is a known inhibitor of enzymes in general.[157] As early as 1960 a non-active zinc binding site was found in carboxypeptidase – subsequent studies confirmed this to be an inhibitory site with zinc bound in a monodentate fashion to glutamate.[158] Enzymes that have been shown to be inhibited include glyceraldehyde 3-phosphate dehydrogenase, aldehyde dehydrogenase, tyrosine phosphatase, fructose 1,6-diphosphatase and enolase.[157,159] Interestingly caspase-3 is also inhibited by zinc at nanomolar concentrations (see also above).[160] Inhibitory zinc-binding sites have also been recognized in neurotransmitter receptors of the central nervous system.[157] The presence of a catalytic cysteine in many of the enzymes inhibited by zinc suggests an important role for this residue – however, inhibition is selective as glutathione peroxidase (catalytic selenocysteine) or glutathione reductase from yeast (catalytic vicinal cysteines) are not inhibited in the presence of nanomolar concentrations of zinc.[157] Thionein, generated *in situ* by removal of zinc from metallothionein, can reverse zinc inhibition by sequestration of the metal. The chemical and biochemical features involved in the inhibitory use of zinc are not clear – as stated, the presence of a cysteine residue at the catalytic site is not sufficient by itself to provide an inhibitory site. The thionein–metallothionein balance and its role in controlling Zn(II) availability has been examined from this point of view.[161] Zinc transporters, zinc sensors and metallothioneins all contribute to controlling cellular and subcellular zinc trafficking and homeostasis. The availability of Zn(II) must be regulated tightly, because an increase of free cellular Zn^{2+} has many biological consequences from enzyme inhibition, affecting gene expression occuring when cells proliferate, differentiate, or undergo programmed cell death (apoptosis) and, at high concentrations for longer periods of time, may lead to cytotoxicity.

V. Concluding remarks

This review provides a basic outlook on key zinc metalloproteins with proven relevance in the medicinal area, for which a great amount of effort has been made in the structural characterization of active sites and design of inhibitors. Other zinc metalloproteins are expected to join this selective group in the future, given the central role played by zinc in the proteome. In this review, zinc metalloproteins were presented separately for topic simplification, however it is important to underscore that, as commonly found in living systems, there is a close interrelationship in their actual function *in vivo i.e.* p53 can be a substrate of HDACs while MDM2 (a zinc finger containing protein) regulates p53 levels inside the cell. In addition, zinc finger proteins can recruit HDACs to modulate nuclear receptor transactivation. In this regard, the importance of zinc, as a trace element, for living systems can be correlated to a large extent to the functions exerted by the enzymes and structural proteins that need it as a cofactor. Indeed, there is significant overlap in cancer, for example, where the influence of several zinc metalloproteins appears to play a decisive role.

Zinc displays a remarkable diversity of coordination spheres whose distinct chemical properties may lend themselves to systematic targeting resulting in selective inhibition. Matrix

metalloproteinases and histone deacetylases appear to share common approaches whereas the development of metallo-β-lactamase inhibitors could benefit by employing a peptidomimetic approach to carry a zinc-binding group (ZBG) rather than rely on small molecules for specificity. In all cases application of bioinorganic principles can lead to more selective chelating groups for Zn^{2+}. The FDA approval of SAHA (Varinostat) for treatment of a rare cancer, cutaneous T-cell lymphoma, does represent the first example of a clinically useful hydroxamate-based drug, albeit in a relatively rare cancer. Nevertheless, the reliance on the hydroxamate moiety has not translated to a range of clinically useful drugs, in part due to lack of selectivity.[162] There is also significant opportunity for the bioinorganic chemist and medicinal inorganic chemist in design of selective metal complexes capable of targeting zinc proteins. The examples of purported Pt- and Ru-based matrix metalloproteinase inhibitors, Mn chelates as SOD mimics and the analogy between alkylation and platination in targeting reactive cysteines of structural zinc serve as interesting examples. Further, they may suggest approaches to design of targeted ''non-cytotoxic'' metal-based agents. Analysis of the role of Zn, and Cu, in p53 structure and function leads not only to the possibilities of small molecule design to affect p53 function through Cu and Zn chelation, but also to the appreciation of the dependence of cell signaling and apoptotic pathways on redox state of the cell. Finally, the examples cited here can serve as impetus for new challenges on the interface of bioinorganic chemistry and medicine providing rich and exciting areas of research of benefit to society.

Abbreviations

ADAMs	A disintegrin metalloproteinases (ADAMs)
ADAM TSs	Disintegrin metalloproteinase with thrombospondin type 1 like repeats (ADAM TSs)
ADH	Alcohol dehydrogenase
APL	Acute promyelocytic leukaemia
CA	Carbonic anhydrase
CNS	Central nervous system
CPA	Carboxypeptidase
DBD	DNA-binding domain
ECM	Extracellular matrix
fALS	Familial mmyotrophic lateral sclerosis
FT	Farnesyl transferase
GGT	Geranylgeranyl transferase
HDAC	Histone deacetylase
HAT	Histone acetyl transferase
hERR	Human estrogen related receptors
isaepy	2-(2-aminomethyl)pyridine
MMP	Matrix metalloproteinases
mβLs	Metallo-β-lactamases
NAMI-A	Imidazolium *trans*-tetrachlorodimethylsulfoxideimidazoleruthenate(III)
NFTs	Neurofibrillary tangles
PMI	Phosphomannose isomerase
PTs	Protein prenyltransferases
RAPTA-T	Dichloro(η^6-toluene) (1,35,-triaza-7-phosphoadamantane)ruthenate(II)
RAR	Retinoic acid receptor-α
REP	Rab escort protein
Riluzole (rilutek)	2-Amino-6-(trifluromethoxy)benzothiazole
SMP	Diethyl(methylsulfinyl)methylphosphonate
SOD	Superoxide dismutase
tachpyridine (tachpyr)	*N*,*N*′,*N*″-tris(2-pyridylmethyl)-*cis,cis*-1,3,5-triaminocyclohexane
TIMPs	Tissue inhibitors of metalloprotease
XPA	Xeroderma pigmentosum group A
ZBGs	Zinc-binding groups
ZFs	Zinc fingers

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