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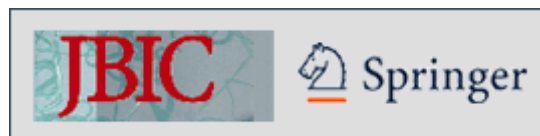
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A Role for Bioinorganic Chemistry in the Reactivation of Mutant p53 in Cancer

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April 8th, 2022

Professor Trevor Hambley
Associate Editor
Journal of Biological Inorganic Chemistry
University of Sydney, Australia

Dear Professor Hambley,

Please find enclosed our revised invited review manuscript titled "**A Role for Bioinorganic Chemistry in the Reactivation of Mutant p53 in Cancer.**" We thank the two reviewers for their positive appraisal of the manuscript and have made the requested minor changes. We hope to hear favorably from you in the near future.

Sincerely,



Tim Storr

Reviewer #1:

1. We have removed the comparison to a Zn-finger motif for the Zn-binding site of p53.
2. We have updated Figure 2 with more information on stabilization, and p21 upregulation. We are hesitant to add too much information to this Figure as the goal is to highlight where small-molecule interventions can be used.

Reviewer #2: We thank this Reviewer for their positive appraisal.

One additional change: We have added reference 113 as this provides further evidence for a possible mechanism of action of Zn metallochaperones via extracellular Cu internalization.

113. Tsvetkov, P., et al., *Science* **2022**, 375 (6586), 1254-1261.

A Role for Bioinorganic Chemistry in the Reactivation of Mutant p53 in Cancer

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Abstract:

Metal ion dysregulation has been implicated in a number of diseases from neurodegeneration to cancer. While defective metal ion transport mechanisms are known to cause specific diseases of genetic origin, the role of metal dysregulation in many diseases has yet to be elucidated due to the complicated function (both good and bad!) of metal ions in the body. A breakdown in metal ion speciation can manifest in several ways from increased reactive oxygen species (ROS) generation to an increase in protein misfolding and aggregation. In this review we will discuss the role of Zn in the proper function of the p53 protein in cancer. The p53 protein plays a critical role in the prevention of genome mutations via initiation of apoptosis, DNA repair, cell cycle arrest, anti-angiogenesis, and senescence pathways to avoid propagation of damaged cells. p53 is the most frequently mutated protein in cancer and almost all cancers exhibit malfunction along the p53 pathway. Thus, there has been considerable effort dedicated to restoring normal p53 expression and activity to mutant p53. This includes understanding the relative populations of the Zn-bound and Zn-free p53 in wild-type and mutant forms, and the development of metallochaperones to re-populate the Zn binding site to restore mutant p53 activity. Parallels will be made to the development of multifunctional metal binding agents for modulating the aggregation of the amyloid-beta peptide in Alzheimer's Disease (AD).

Introduction:

The p53 protein is known as the 'guardian of the genome' and plays a key role in the prevention of genome mutations in the body. First reported in 1979,¹ the p53 protein is a tetrameric transcription factor that regulates the expression of target genes responsible for apoptosis, DNA repair, and cell cycle arrest of damaged cells, thereby inducing an antiproliferative cellular response.²⁻⁷ Even today, the research community is still uncovering the complex roles of p53 in the cell cycle, including control of cellular metabolism. The high frequency of p53 mutation, ranging from 1 % to 85 % depending on the type of cancer,⁸ and the aggressive nature of cancers exhibiting p53 malfunction,⁹⁻¹⁰ have led to a significant research effort to restore normal p53 activity for cancer treatment.

Each p53 monomeric unit of the tetrameric transcription factor is divided into five key regulatory domains including the intrinsically-disordered N-terminal transactivation domain (TAD), a proline-rich region (Pro), the DNA-binding domain (DBD), the oligomerization domain (OD), and the intrinsically-disordered C-terminus.² Essential to the stability of the DBD is a structural Zn, which is tetrahedrally coordinated in a Zn-binding motif. In order to maintain basal p53 function, cellular levels are tightly regulated *via* posttranslational modifications (PTMs)¹¹⁻¹³ and key regulator mouse double minute 2 homolog (MDM2), which binds to the TAD region of p53 to prevent transcriptional activity and promote ubiquitin-dependent p53 degradation.¹⁴ However, under conditions of cellular stress, p53 degradation is inhibited, resulting in p53 stabilization and build-up in the nucleus.¹⁵ This in turn leads to the upregulation of target genes that initiate response mechanisms to avoid the propagation of damaged cells.¹⁶ For example, in the presence of DNA damage, p53 can either activate a reversible cell cycle arrest process that allows for DNA repair, or removal of damaged cells.¹⁷⁻¹⁸ Upregulation of the cyclin-dependent kinase inhibitor (p21) is one of the most studied p53 targets, and increased p21 expression is considered to be a canonical marker of p53 mediated cell cycle arrest.¹⁹⁻²⁰ However, mutations to p53 impairs its ability to drive gene expression and a gene signature containing several p53 target genes (e.g. *EDA2R*, *RPS27L*, *SPATA18*, *MDM2*, *DDB2*, *CENPA*, *AEN*, *BTG2*, *ZMAT3*) have been shown to discriminate tumors expressing wild-type (WT) or mutant p53 in ten different types of cancers.²¹⁻

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Given the importance of p53 in maintaining normal cell functioning, mutations resulting in malfunction can have serious consequences. This is highlighted by the fact that *over* 50% of cancers result from point mutations directly to p53.⁹⁻¹⁰ Interestingly, p53 is frequently inactivated by a single missense mutation, with 95 % of these mutations occurring in the DBD.²³ Studies have shown that the WT p53 DBD exhibits limited kinetic and thermodynamic stability,²⁴ and thus many mutations cause further instability and result in protein unfolding. p53 DBD mutations can be broadly classified as influencing DNA interactions or overall stability (**Figure 1**).²⁵⁻²⁷

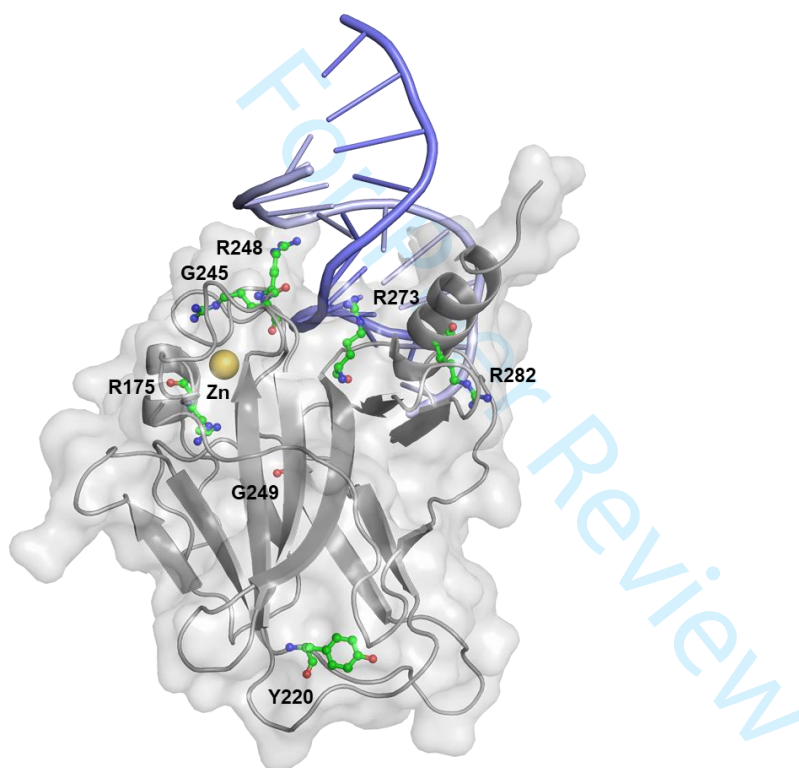


Figure 1: p53 DNA binding domain (DBD) bound to DNA (purple). Location of high-frequency carcinogenic mutations and structural Zn are shown (PDB:4AGL).

DNA-contact amino acids bind to DNA directly and mutation leads to impairment of the p53-DNA interaction, while affording little change to the overall structure and stability of the protein.²⁸ The most frequently mutated DNA-contact amino acids are R248, R273H, and R280.²⁹ Stability mutants induce conformational changes within the protein that can result in a loss of

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3 function for a variety of reasons.^{27, 30} For example, mutations that perturb Zn-binding cause
4 structural changes in neighboring loops, thereby causing a loss in DNA binding specificity.
5 Example mutants include R175H³¹⁻³² and R282Q.²⁷⁻²⁸ Other common conformational mutants that
6 impair Zn-binding and/or lower stability are G245S, R249S, and Y220C.³³ Mutations to the p53
7 DBD domain lead to a loss of function due decreased stability, protein unfolding, impaired Zn-
8 binding, aggregation and amyloid formation. In general, aggregated p53 protein exhibits
9 deleterious gain of function (GoF) effects by self-propagating and acting as seeds to accelerate the
10 aggregation process.^{23, 34-36} Cellular studies with common mutants such as R175H, R248Q, R249S,
11 and R282W show p53 aggregation as common feature, with aggregates impairing the function of
12 WTp53.^{23, 35-36} Protein misfolding and amyloid formation is also common in neurodegenerative
13 diseases (ND), with cellular and biochemical studies supporting the theory that protein aggregation
14 plays a role in Alzheimer's disease (amyloid- β), Parkinson disease (α -synuclein), amyotrophic
15 lateral sclerosis (superoxide dismutase, TDP-43), and prion disease (prion protein).³⁷⁻⁴⁰
16 Interestingly, dysregulated metal ion binding is hypothesized to play a role in peptide/protein
17 misfolding in ND,⁴¹⁻⁴³ showing some similarities to the impaired Zn binding and aggregating
18 properties of mutant p53. Indeed, targeted metal ion binding and redistribution has shown utility
19 both as an anticancer strategy⁴⁴⁻⁴⁹ and in modulating amyloidogenic protein aggregation.⁵⁰ A
20 number of innovative designs have been reported and in this review we will focus on the
21 development of small molecules to reactivate mutant p53, with a focus on agents that modulate Zn
22 levels in order to elicit a p53-dependent pharmacological effect.
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40 **p53 Drug Development:**

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43 There has been considerable interest in the development of therapeutic interventions
44 targeting the p53 pathway given that p53 is the most frequently mutated protein in cancer and
45 almost all cancers exhibit malfunction along the p53 pathway.⁵¹⁻⁵² This includes gene therapy,⁵³⁻⁵⁴
46 RNA silencing,⁵⁵ peptide vaccines,⁵⁶ and small molecules targeting p53.⁵⁷⁻⁶⁰ In terms of small
47 molecule development, **Figure 2** shows general aspects of p53 function and several intervention
48 points for small molecules along the p53 pathway.
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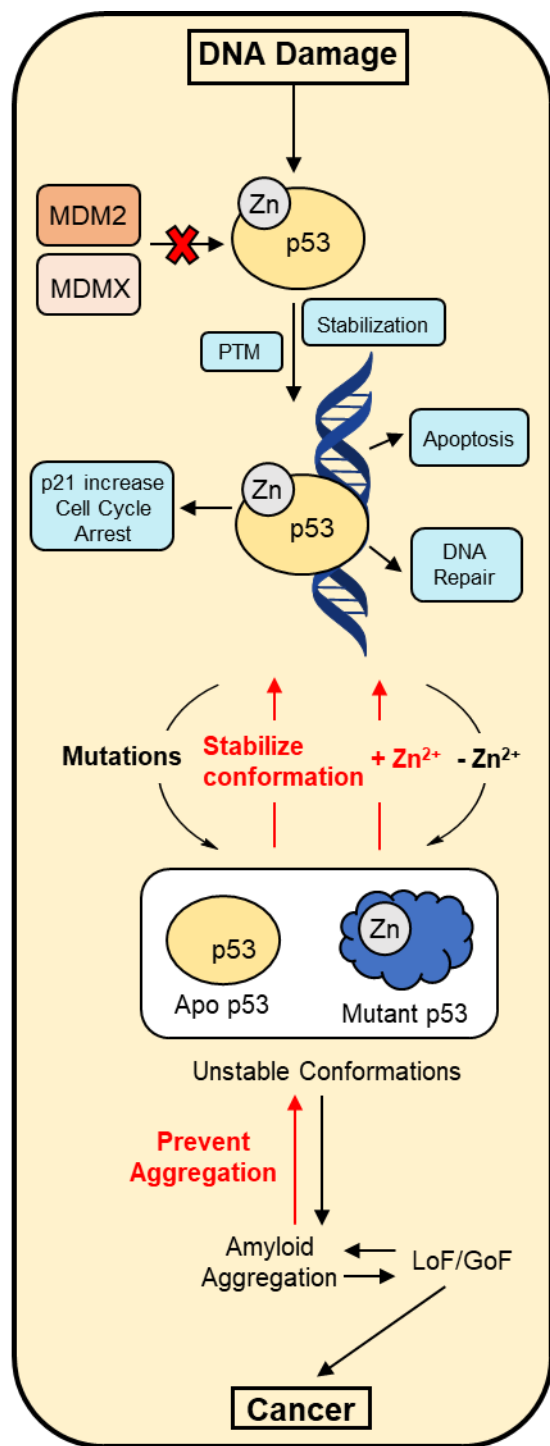


Figure 2. Overview of p53 function in response to DNA damage. MDM2/MDMX repression promotes upregulation of p53 to initiate cell repair or cell death mechanisms. Mutations resulting in zinc loss and/or unstable conformations leads to a loss of protein function and can result in protein aggregation. p53 protein aggregates are linked to both loss of function (LoF) and gain of function (GoF) effects. Possible therapeutic interventions discussed in this review are highlighted in red.

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4 One prominent example is the development of inhibitors of the p53-MDM2 interaction.⁶¹⁻
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6 ⁶³ The function of MDM2 is to regulate p53 levels via binding to the N-terminal transactivation
7 domain (TAD) and targeting p53 for ubiquitination. Many tumours increase degradation of p53
8 via increased MDM2 activity, thereby limiting p53 activity.⁶⁴ Research has therefore targeted
9 small molecules that interfere with the p53-MDM2 interaction to increase levels of functional p53.
10 An early success in this area was the discovery of Nutlins, *cis*-imidazoline small molecules that
11 mimic p53 amino acids involved in binding to MDM2.⁶⁵⁻⁶⁶ Nutlin-3a (**Figure 3**) induces
12 upregulation of p21, resulting in cell cycle arrest and p53-dependent apoptosis in a number of
13 cancer models.⁶⁷⁻⁶⁸ RITA, another promising small molecule (**Figure 3**), inhibits the p53-MDM2
14 interaction by binding to the TAD domain of p53 and inducing a conformational change.⁶⁹⁻⁷¹
15 However, RITA also displays a number of p53-independent effects, including activation of
16 mitogen-activated protein kinases (MAPK).⁷² Of interest to bioinorganic chemists is the activity
17 of protoporphyrin IX (PpIX) as a p53-MDM2 inhibitor (**Figure 3**).⁷³ While well known as an agent
18 for photodynamic therapy, PpIX also displays toxicity in the absence of light due inhibition of the
19 p53-MDM2 interaction leading to upregulation of p53 transcription factors.⁶³ PpIX has also been
20 shown to elevate ROS levels *via* thioredoxin reductase inhibition. Importantly, forced activation
21 of p53 can also cause serious side-effects as p53 is present in all cell types in the human body. For
22 instance, p53 can mediate neurotoxicity induced by activation of the amyloid precursor protein
23 (APP) pathway.⁷⁴ Hence, targeting mutant p53 instead of WT p53 represents a more selective
24 strategy by acting specifically on cancer cells instead of the whole organism. This is particularly
25 relevant in cancer types where p53 mutation is a founding event of the tumorigenesis process and
26 thus can be considered as a driver mutation, such as in gastric cancer.⁷⁵
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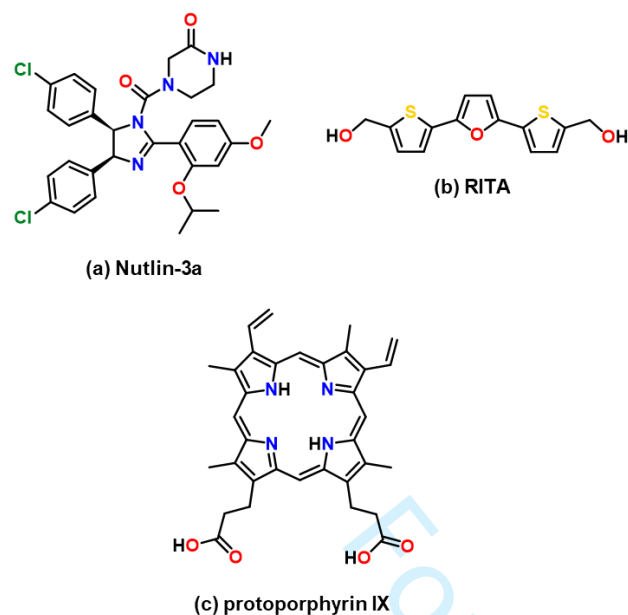


Figure 3. Selected small molecules targeting the p53-MDM2 interaction including Nutlin-3a, RITA, and protoporphyrin IX.

For this reason, the development of small molecules to stabilize the folded form of mutant p53 has also shown considerable success. For example, CP-31398 and PRIMA-1 (**Figure 4**) were shown to restore native conformation to mutant p53, and thereby inducing p53-dependent cytotoxicity.⁷⁶⁻⁷⁷ In both cases, the molecules function as Michael acceptors, implying that the mechanism of action is at least in part due to modification of p53 cysteine residues in order to promote refolding.⁷⁸⁻⁷⁹ PRIMA-1 (APR-246) was the first p53-reactivating small molecule to enter clinical trials.⁸⁰ Researchers have also developed small molecules to increase the thermal stabilization of mutant p53.⁸¹ An estimated 30 % of p53 mutations are temperature sensitive resulting in unfolded and inactive protein at physiological temperatures. As such, small molecules that bind and stabilize the folded conformation can result in increased active wild-type protein conformation.⁸¹⁻⁸² The p53-Y220C mutant is one of the most common thermally unstable mutants, in which tyrosine to cysteine substitution creates an open cleft at the surface of the protein lowering stability and causing unfolding.³³ Using *in silico* methods and fragment-based screening, a number of molecular scaffolds have been identified that bind to the mutant cavity in the low μM range and increase p53 thermal stability.^{33, 81, 83-84} These include carbazole compounds such as PhiKan083,⁸⁵ and more recently PhiKan9318,⁸¹ as well as compounds with a diiodophenol³³ core (**Figure 4**). The phenol compounds were appended with acetylene linkers to increase interactions with the

mutant cavity (PhiKan5196: 10 μ M binding) and shown to activate mutant-specific apoptosis.³³ As an example of the commercialization potential of this strategy, the US FDA recently granted Fast Track designation to PMC Pharma for PC14586 (structure yet to be disclosed), a reversible small molecule binder for the p53-Y220C mutant pocket.⁸⁶

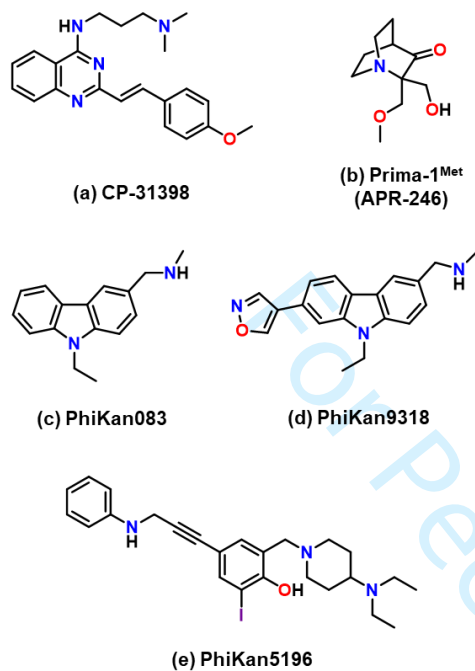


Figure 4. Selected small molecules that restore wild-type p53 function including CP-31398, PRIMA-1^{Met}, PhiKan083, PhiKan9318, and PhiKan5196.

As stated earlier, mutations to the p53 DBD domain can lead to protein unfolding, impaired Zn-binding, resulting in aggregation and amyloid formation. While p53 aggregation results in loss of function (LoF), aggregated p53 exhibits deleterious gain of function (GoF) effects by reacting with WTp53 and also the homologous proteins p63 and p73 to form amyloid oligomers and fibrils.^{23, 36, 87-88} The classification of p53 as an amyloidogenic protein is relatively recent, and amyloid aggregates are associated with more aggressive and invasive tumours.⁸⁹⁻⁹⁰ Thus, the development of small molecules that can disrupt p53 aggregation (**Figure 2**) is of considerable interest. Polyphenol compounds resveratrol and emodin (**Figure 5**) have been reported to modulate p53 aggregation, with resveratrol limiting mutant p53-R284Q aggregation in cancer cell lines and a xenograft model.⁹¹⁻⁹³ In addition, a number of PhiKan compounds (e.g. PhiKan5174, **Figure 5**) inhibit p53 aggregation via increasing thermal stability and preventing protein unfolding.⁹⁴ Finally, Eisenberg and co-workers developed a cell penetrating peptide that is a close mimic of the aggregation-prone 252-LTIITLE-258 sequence in p53, which reduced p53 aggregation, rescued

function in p53 mutants R175H and R248Q, and decreased tumour proliferation in xenograft models (**Figure 5**).⁹⁵

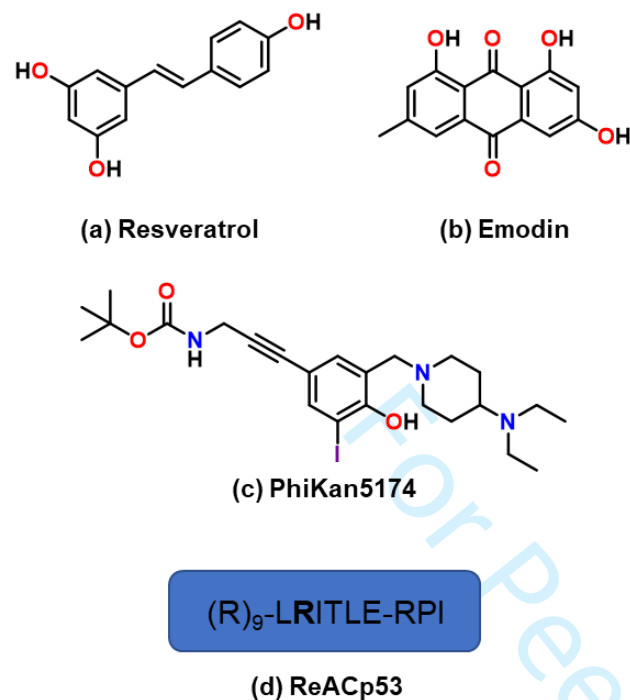
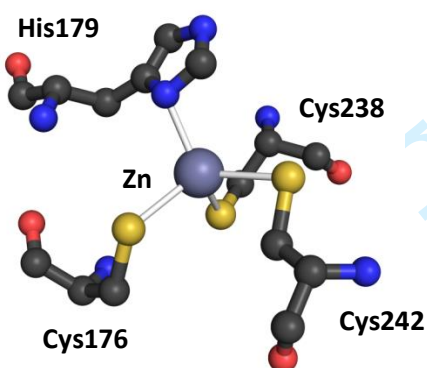


Figure 5. p53 aggregation inhibitors (a) resveratrol, (b) emodin, (c) PhiKan5174, and (d) peptide ReACp53. For the peptide (R)₉ – indicates a cell-penetrating tag and RPI indicates a three residue p53 linker. See reference 91 for further details.

The structural Zn:

Essential to the stability of the p53 protein is a structural Zn, which is tetrahedrally coordinated by a histidine (His 179), and three cysteines (Cys 176, Cys 238, Cys 242) (**Figure 6**). Interestingly, the structural Zn is not buried in a hydrophobic region as is commonly the case for Zn fingers, but bridges and stabilizes two large loops in the folded structure.⁹⁶ The p53 DBD, which contains this structural Zn, exhibits low thermodynamic and kinetic stability, and many missense mutations reduce the melting temperature (T_m) below 37 °C, leading to increased unfolding and aggregation.^{28, 97} Researchers have extensively studied the importance of the structural Zn²⁺ ion, reporting that removal of Zn²⁺ causes a loss of DNA-binding specificity and unfolding, and that Zn²⁺ binding can be influenced by overexpression of metallothioneins or addition of metal ion chelators.^{31, 98-100} While the majority of intracellular Zn²⁺ is bound to cytosolic Zn-binding proteins, the level of ‘exchangeable’ Zn²⁺ is estimated to be in the nanomolar to picomolar range.¹⁰¹ The affinity of WTp53 for Zn²⁺ is on the order of 10⁻¹² M, however, Zn²⁺

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3 binding is weaker in a number of mutants (e.g. R175H, R282Q), resulting in an increase in apo
4 (zinc-free) p53 which is primarily unfolded, aggregation-prone, and exhibits a loss in DNA binding
5 affinity.^{27, 102-103} In addition to the native Zn²⁺ binding site (K_{d1}), weaker binding to non-native
6 sites (K_{d2}) are estimated to be on the order of 10⁻⁶ M.¹⁰²⁻¹⁰³ This secondary binding site may exhibit
7 increased affinity in Tyr to Cys mutants (e.g. Y220C), in which the introduced Cys residue can
8 lead to Zn²⁺ binding in unfolded or partially-folded sites.²⁷ Loh and co-workers have elegantly
9 shown that p53 is delicately partitioned between folded and unfolded conformations in the cell,
10 and available Zn is determined to be a major factor in folding and activity.²⁷



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31 **Figure 6.** p53 structural Zn motif coordinated to His 179, Cys 176, Cys 238, and Cys 242.

32 33 34 **Modulating Zn Concentrations to Reactivate Mutant p53:**

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37 Based on the importance of Zn²⁺ binding and availability for the maintenance of p53
38 activity, modulating cellular Zn concentrations provides an attractive strategy to reactivate p53
39 mutants. Reports have shown that the addition of high affinity Zn chelators to cells leads to an
40 increase in unfolded p53 protein,¹⁰⁰ and this result can be reversed by the administration of excess
41 ZnCl₂ to cell media. Additionally, treatment of Zn-binding p53 mutant cell lines with ZnCl₂
42 restores native p53 folding and WT transcriptional activity, highlighting the utility in restoring Zn-
43 binding to mutant p53 (**Figure 2**).¹⁰⁴ D'Orazi *et al.* have studied a series of Zn-curcumin
44 complexes that increase the proper folding and activation of Zn-binding mutants R175H and
45 R273H (**Figure 7**).¹⁰⁵⁻¹⁰⁶ These studies outline the possible benefit of buffering intracellular Zn
46 concentrations *via* metallochaperones to maximize p53 folding and activity. The caveat is that high
47 concentrations of Zn can be harmful for the cell, and more specifically can lead to binding to non-
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3 native sites on p53 ($\sim K_{d2} = 10^{-6}$ M) and subsequent unfolding and aggregation.^{31, 98} Thus, the
4 development of Zn metallochaperones (ZMCs) with metal binding affinities between the native
5 site ($K_{d1} = 10^{-12}$ M) and non-native sites ($\sim K_{d2} = 10^{-6}$ M) have been pursued in order to buffer
6 appropriate Zn concentrations in the cell to promote mutant p53 refolding and activity.⁹⁹
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10 The first significant development in the design of ZMCs for p53 re-activation was the
11 discovery of a series of thiosemicarbazones that exhibited selective cytotoxicity against one of the
12 most common p53 mutations, R175H.¹⁰⁷ Carpizo *et al.* determined that ZMC1 (**Figure 7**, other
13 similar thiosemicarbazone ZMCs not shown) restored WT structure and function to p53-R175H,
14 thereby activating apoptosis in xenograft models. It was determined that ZMC1 exhibited
15 nanomolar affinity for Zn^{2+} , forming a neutral 2:1 metal complex, and facilitating Zn uptake.¹⁰⁸
16 Once internalized, Zn-redistribution to higher binding affinity ligands such as p53 can occur,
17 restoring Zn binding to mutant p53. In comparison, the A6 analogue (**Figure 7**) exhibited lower
18 affinity for Zn (μ M range), and failed to show appreciable metallochaperone activity or
19 cytotoxicity.¹⁰⁸ Interestingly, the ZMC class of compounds generated intracellular reactive oxygen
20 species (ROS).¹⁰² Thiosemicarbazones are known to exhibit high affinities for redox-active
21 transition metal ions such as Cu and Fe, which could result in ROS production *via* Fenton-like
22 chemistry. For example, the anticancer agent triapine (**Figure 7**) is known to be a strong Fe
23 chelator, and the Fe complex is hypothesized to play a role in quenching the active site tyrosyl
24 radical of ribonucleotide reductase.¹⁰⁹ However, triapine does not appear to act as a Zn
25 metallochaperone in the same manner as ZMC1.¹¹⁰ Work by Faller *et al.* has shown that in general,
26 Zn and Cu thiosemicarbazones are rapidly de-metallated by glutathione and/or metallothioneins in
27 the cytosol and nucleus, however the Fe analogues are more stable.¹¹¹⁻¹¹² Therefore, while Cu
28 binding by the ZMC compounds may not be favoured in the cytosol/nucleus, intracellular Fe
29 binding, increased internalization of extracellular Cu,¹¹³ or Cu binding in lysosomes⁴⁶ could result
30 in the observed increase in ROS. These studies serve to highlight the complexities of targeted
31 metal ion binding and redistribution in cancer therapy.
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48 While high levels of ROS can lead to off-target effects and general cytotoxicity, the
49 production of ROS for the ZMC compounds generated post-translational modifications (PTMs) to
50 the newly refolded p53 that are critical for transcriptional activity.¹⁰² While ROS generation is
51 beneficial to the p53-dependent activity, in this case the amount of ROS generated was too high to
52 observe synergism with traditional chemotherapies or radiation.¹¹⁴ Indeed, synergism could only
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be observed upon treatment with ROS scavengers such as glutathione. To further test the ROS hypothesis, a series of cell-permeable derivatives of nitriloacetic acid (NTA) were designed (**Figure 7**). These derivatives exhibited diminished Cu affinity, less ROS generation, and exhibited synergistic activity with chemotherapies without the need for the administration of additional ROS scavengers.¹¹⁴ A recent significant structure-activity relationship (SAR) study by Carpizo *et al.* identified a benzimidazole framework (**Figure 7**, C85) that exhibits a similar p53 reactivation profile to ZMC1 with lower ROS generation, which the authors ascribed to reduced Cu²⁺ affinity and lower Cu²⁺ / Zn²⁺ selectivity ratio.¹¹⁵

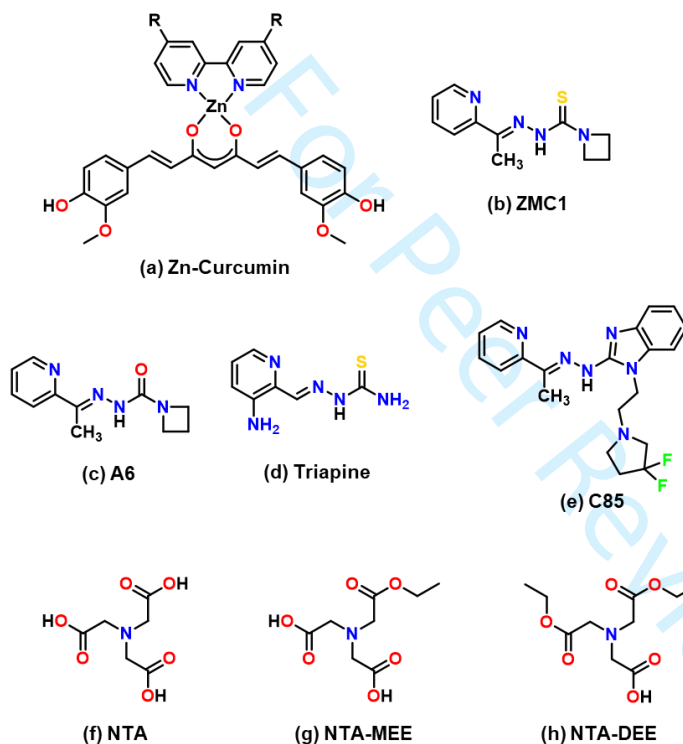
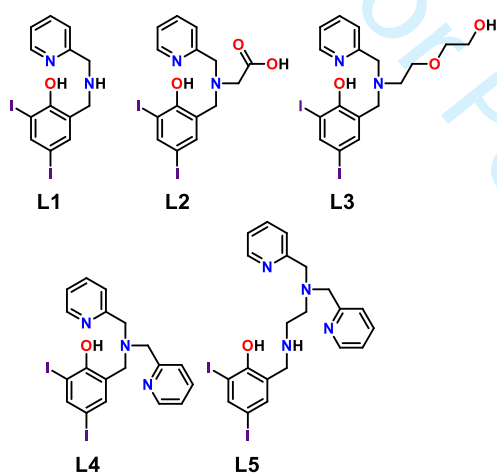


Figure 7. Metallochaperones and metal-binding molecules relevant to p53 reactivation and anticancer treatment. (a) Zn-Curcumin, (b) ZMC1, (c) A6, (d) Triapine, (e) C85, (f) NTA, (g) NTA-MEE, and (h) NTA-DEE.

In our own work we have focused on the development of multifunctional ligand frameworks designed to act as metallochaperones, while also stabilizing the mutant p53 protein by binding the exposed mutant cleft or inhibiting protein aggregation. Multifunctional compounds can offer advantages over their monofunctional counterparts by acting on multiple targets, which has been shown to enhance drug activity and lower toxic side-effects.¹¹⁶⁻¹¹⁷ We chose to investigate

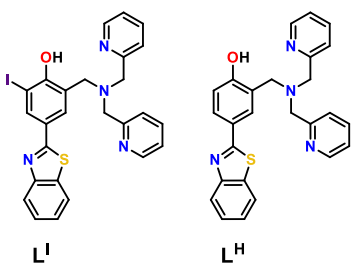
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3 the p53-Y220C mutant as it is prone to thermal destabilization, loss of Zn, and aggregation.^{27, 87,}
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5 94, 118-119 Our first-generation compounds (**Figure 8**) incorporated an iodinated phenol to bind and
6 stabilize the mutant Y220C surface cavity, while also including Zn-binding moieties for
7 metallochaperone activity.¹²⁰ While L1-L3 displayed minimal cytotoxicity in the NCI-60 cancer
8 cell line screen, L4 and L5 increased intracellular Zn, restored p53 transcriptional activity, and the
9 lead compound L5 induced mutant-selective apoptosis. However, we observed significant
10 cytotoxicity in WTp53 cell lines, showing that L5 exhibited p53 mutant-independent effects. We
11 hypothesized that significant ROS generation is responsible for the broad cytotoxicity. Design
12 improvements could include scaffolds that reduce the Cu²⁺ / Zn²⁺ selectivity ratio while
13 simultaneously increasing the binding affinity for the Y220C surface cavity.
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39
40 **Figure 8.** Multifunctional molecules L1-L5 designed to act as metallochaperones and stabilize the
41 mutant p53-Y220C cavity.
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43 Based on our initial results, we designed new multifunctional compounds capable of Zn
44 metallochaperone activity and protein aggregation disruption. Analysis of p53 aggregation has
45 shown that amyloid fibrils form, which can be monitored using Thioflavin T (ThT) fluorescence.⁸⁷
46 We therefore incorporated amyloid interacting molecules with a similar structure to ThT,¹²¹ while
47 keeping the dipyriddyamine-phenol Zn binding unit from our initial series (**Figure 9**). This ligand
48 series exhibited Zn metallochaperone activity in a p53-Y220C mutant cell line, and the iodinated
49 framework (L^I) was found to disrupt mutant p53 aggregation based on turbidity measurements in
50 solution, and *via* antibody staining in cells.¹²² Interestingly, the L^H framework did not show
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3 aggregation inhibition. Native mass spectrometry studies indicated that L^I directly interacted with
4 the p53 protein, while no interaction was observed for L^H . We hypothesized that the L^I iodine
5 substituent could favourably interact with exposed aggregation-prone, hydrophobic protein
6 segment, which inhibited p53 aggregation. The L^I derivative increased p53 transcriptional function
7 and mediated both caspase-dependent and -independent cell death pathways, and was well-
8 tolerated in a non-cancerous intestinal organoid model. Overall, these studies demonstrate the
9 cancer cell selectivity of the L^I scaffold, and *in vitro* experiments show a synergistic effect of co-
10 treatment with L^I and oxaliplatin suggesting the potential for co-administration of L^I with platinum
11 agents for tumours harbouring the p53-Y220C mutation.¹²² In general, DNA-damaging drugs such
12 as doxorubicin and platinum agents promote activation of p53-mediated cell death following
13 generation of DNA damage.¹²³⁻¹²⁵ Unfortunately, many cancers possessing mutant p53 exhibit an
14 increased resistance to such treatments and thus the development of small molecules that reactivate
15 mutant p53 may provide a widespread benefit to the field of cancer therapy.^{123, 126}



39 **Figure 9.** Multifunctional molecules L^I and L^H designed to act as metallochaperones and inhibit
40 mutant p53 protein aggregation.

41 **Outlook:**

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48 The fact that over 50 % of cancers result from point mutations directly to p53, and almost
49 all cancers exhibit malfunction along the p53 pathway has driven widespread interest in the
50 development of therapeutic strategies to reactivate mutant p53. This approach has faced many
51 challenges, including frequent off-target mechanisms. However, as our understanding of the p53
52 pathway improves, the development of selective p53 activation strategies are sure to follow. This
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review provides a snapshot of currently investigated interventions along the p53 pathway (**Figure 2**) with an emphasis on the development of Zn metallochaperones. Increasing cellular Zn concentrations has been shown to promote the re-folding and transcriptional activity of mutant p53, and while this strategy would not be appropriate for all point mutations (e.g. DNA contact mutants), a significant number of Zn-binding and general structural mutants have been shown to potentially benefit from this strategy.²⁷ A number of design criteria have been identified for Zn metallochaperones including binding constants between that of the high affinity binding site ($K_{d1} = 10^{-12}$ M) and non-native sites ($\sim K_{d2} = 10^{-6}$ M).⁵⁹ In addition, recent work by Carpizo *et al.* has identified an upper limit for Zn binding of $K_d = 2 \times 10^{-7}$ M,¹¹⁵ which will help in the design of Zn metallochaperones in the future. Another consideration is the change in cellular Zn homeostatic mechanisms such as increases in exporter ZnT1 and cytosolic Zn binding protein MT1A, and a decrease in Zn importer ZIP10, in response to metallochaperone administration.¹²⁷ This results in a transient increase in Zn levels and thus a short-term effect of drug administration. In addition, Zn homeostatic genes display differential expression levels depending on the cancer type, which may result in variable activity of the metallochaperones.¹²⁸ The requirement that metallochaperones generate ROS in order to activate folded mutant p53 transcriptional activity *via* specific post-translational modifications is intriguing, and it has been shown that the amount of ROS dictates the ability of these compounds to act synergistically with chemotherapies and radiation.¹¹⁴ Fine tuning the relative binding affinity of metallochaperones for Zn in comparison to Cu and Fe while targeting specific cellular compartments could provide important design improvements. Overall, Zn metallochaperones have shown considerable promise as mutant p53 reactivators, however many questions remain, and the bioinorganic research community has the expertise to push forward their development.

We have investigated incorporating Zn metallochaperone activity with either thermal stabilization or aggregation prevention in a multifunctional design strategy. While the compounds exhibit multifunctional effects, we observed both p53-dependent and p53-independent effects in the p53-Y220C mutant cell line and are now making design improvements to enhance p53-dependent activity. Our more recent study incorporating Zn metallochaperone activity and aggregation prevention showed that the lead compound L¹ reduced p53 aggregation, increased p53 transcriptional function, mediated both caspase-dependent and -independent cell death pathways, and was well-tolerated in a non-cancerous intestinal organoid model.¹²² There is significant scope

for the development of multifunctional metallochaperones that inhibit p53 aggregation and one needs to look no further than the work by many groups investigating small molecule inhibitors of protein misfolding in neurodegenerative diseases.^{50, 129} Cellular and biochemical studies show that protein aggregation occurs in Alzheimer's disease (AD), Parkinson disease, amyotrophic lateral sclerosis, and prion disease. Interestingly, metal ion dysregulation (Fe, Cu, Zn) is hypothesized to play a role in modulating protein aggregation and targeted metal ion chelation and redistribution has shown utility in modulating amyloidogenic protein aggregation.^{41-43, 130-131} For example, Mirica *et al.* have previously studied scaffolds similar to L¹ (**Figure 9**) as Cu/Zn binding compounds that modulate the aggregation of the amyloid-beta (A β) peptide in AD.¹²¹ Lim *et al.* have reported that flavonoids such as quercetin¹³² and epigallocatechin-3-gallate (EGCG)¹³³ modulate metal-A β interactions and limit A β peptide aggregation (**Figure 10**).

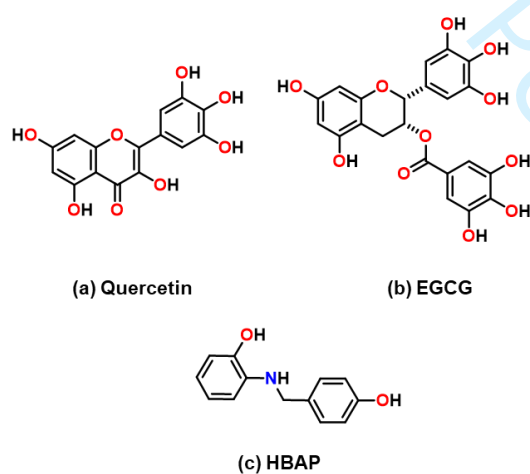


Figure 10. Example polyphenol compounds that have shown multifunctional therapeutic potential in both Alzheimer's Disease and cancer.

Work in this area has identified metal ion chelators that are capable of competing with the A β peptide for available Zn (K_d of A β for Zn *ca.* $10^{-9} - 10^{-6}$)¹³⁴ which is within the range of appropriate K_d values for p53 Zn metallochaperone activity.³¹ Extensive research has also shown that these polyphenols exhibit Zn ionophore activity and cytotoxicity in cancer models.¹³⁵⁻¹³⁶ EGCG was recently reported by Wang *et al.* to disrupt the p53-MDM2 interaction *via* binding to the intrinsically disordered N-terminal domain of p53,¹³⁷ and combined with previous studies highlighting Zn ionophore activity,¹³⁵ suggests that EGCG exhibits multifunctional properties. Finally, a recent report of an aminophenol natural product (HBAP, **Figure 10**)¹³⁸ that binds to the

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3 p53 binding domain of the R280K mutant and reactivates wild-type p53 function shows structural
4 similarity to a series of small-molecules reported by Lim *et al.* that sequester metal ions from the
5 A β peptide and modulate peptide aggregation.¹³⁹⁻¹⁴⁰ We are excited for future opportunities for the
6 reactivation of mutant p53 employing multifunctional drug design.
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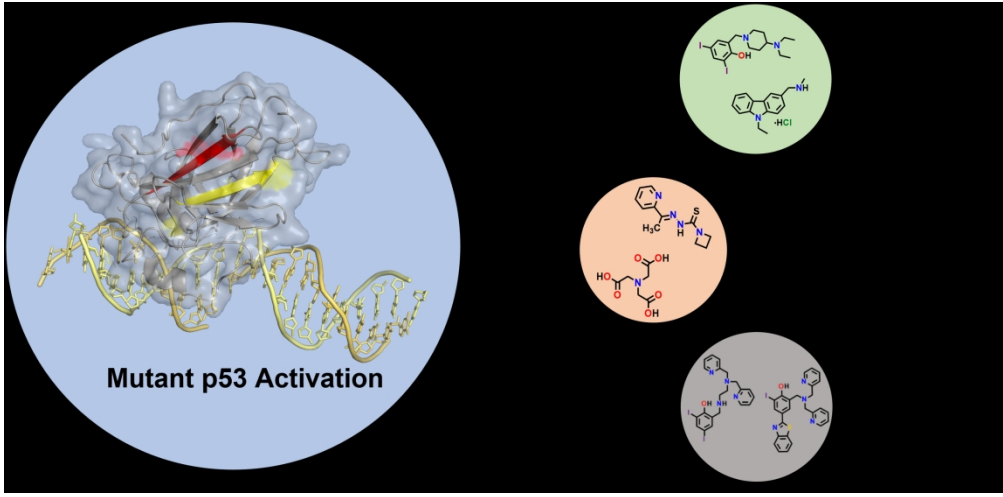
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A Role for Bioinorganic Chemistry in Mutant p53 Reactivation

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