Zinc Protoporphyrin Binding to Telomerase Complexes and Inhibition of Telomerase Activity

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Abstract

**Background:** Zinc protoporphyrin (ZnPP) is a naturally occurring metalloprotoporphyrin (MPP) that is currently under development as a chemotherapeutic agent although its mechanism is unclear. Similar to natural and synthetic porphyrins, MPPs are thought to bind DNA and stabilize secondary structures such as guanine quadruplexes (G-4) and thus potentially impact telomerase activity and DNA synthesis which are important targets for chemotherapy. Interactions of MPPs with telomerase have not been previously reported.

**Methods:** We wished to evaluate the effects of common MPPs, i.e., ZnPP, tin protoporphyrin (SnPP), and iron protoporphyrin (FePP), on cellular proliferation, apoptosis, and telomerase activity in hepatoma cells. The cytotoxicities of porphyrins were determined by *MTT* (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Native agarose gel electrophoresis was used to identify ZnPP binding of telomerase complexes. Inhibition of telomerase activity by ZnPP was assessed by conventional telomeric repeat amplification protocol (TRAP) and direct telomerase activity assays. Colocalization of ZnPP with telomerase was analyzed with immunofluorescence staining and confocal microscopic analysis.

**Results:** ZnPP was the most effective MPP for decreasing DNA synthesis and cellular proliferation, while promoting apoptosis in cultured hepatocytes. Concurrently, ZnPP down-regulated telomerase expression and was the best overall inhibitor of telomerase activity in intact cells and in vitro assays, with IC$_{50}$ and EC$_{50}$ values of ca 2.5 and 6 µM respectively. The natural fluorescence properties of ZnPP enabled direct imaging in cellular fractions using non-denaturing agarose gel electrophoresis, western blots, and confocal fluorescence microscopy. ZnPP localized to large cellular complexes (> 600kD) that contained telomerase and dyskerin as confirmed with immunocomplex mobility shift, immunoprecipitation, and immunoblot analyses. Confocal fluorescence studies showed that ZnPP co-localized with telomerase reverse transcriptase (TERT) and telomeres in the nucleus of synchronized S-phase cells. ZnPP also co-localized with TERT in the perinuclear regions of log phase cells but did not co-localize with telomeres on the ends of metaphase chromosomes, a site known to be devoid of telomerase complexes. Taken together, these results suggest that ZnPP does not bind to telomeric sequences per se, but alternatively, interacts with other structural components of the telomerase complex to inhibit telomerase enzymatic activity.

**Conclusions:** ZnPP can actively interfere with telomerase activity in neoplastic cells, thus eliciting pro-apoptotic and anti-proliferative properties. These data support further development of natural or synthetic protoporphyrins for use as chemotherapeutic agents to augment current treatment protocols for a number of neoplasms.
Telomerase is a cellular reverse transcriptase that is reactivated in about 85% of all cancers (1). The enzyme maintains adequate lengths of chromosomal 3’ DNA telomeric strand ends, which are continuous sequences of –(TTAGGG)n– that progressively shorten with each replication cycle because of the DNA polymerase 3’ end replication problem. Using an RNA template, telomerase adds complementary DNA bases to the 3’ telomere end which prevents chromosomal end damage and enables prolonged cellular proliferation, the hallmark of cancer cells. In rapidly dividing malignant cells, telomeres need constant repair to enable high replication rates (2). This activity is so crucial to malignancy that even in the 15% of neoplastic cells that do not express telomerase, telomeric ends are maintained by an alternative recombination process (3).

Considerable evidence supports the feasibility of telomerase inhibitors as chemotherapeutic agents (4, 5) (6) for a number of neoplastic diseases. As a class, planar, positively charged polyaromatic compounds such as porphyrins have been shown to have anti-telomerase activity (7). Porphyrins are known to bind and stabilize single stranded telomeric DNA sequences at guanine secondary structures known as quadruplexes (G-4) (8) and impact telomerase presumably through substrate inhibition (7, 9, 10).

Metalloprotoporphyrins (MPP) such as FePP and ZnPP, represent a subclass of important naturally occurring porphyrins that are also known to bind G-4 structures in general (11) and telomeric complexes specifically (12, 13). Additionally, ZnPP and conjugated derivatives such as ZnPP-polyethylene glycol have been widely studied in experimental rodent systems for use as chemotherapeutic agents (14, 15). Considering the widespread interest in porphyrins as telomerase inhibitors as well as work with ZnPP as a chemotherapeutic agent, it is surprising that no studies have addressed potential interactions of ZnPP with telomerase.

The aim of the present study was to determine whether common MPPs impact telomerase expression and enzymatic activity in established hepatoma cells. We show that ZnPP abruptly halts DNA synthesis and promotes apoptosis, while concomitantly depressing the expression of telomerase as well as other proliferative proteins such as cyclin D1 and β-catenin. Furthermore, ZnPP effectively inhibits telomerase activity in intact cells, crude cellular lysates, and immunoprecipitates (IP), and localizes to large protein complexes that contain telomerase. These data indicate that ZnPP and perhaps other natural or synthetic MPPs can be useful chemotherapeutic agents through inhibition of telomerase in hepatomas as well as other neoplasms.

**Materials And Methods**

Materials.

*Tel* DNA polymerase (*Perkin-Elmer Cetus*, Norwalk, CT), and Moloney murine leukemia virus reverse transcriptase (*Gibco/BRL Life Technologies*, Gaithersburg, MD) were used in these studies. Electrophoresis supplies were purchased from *Bio-Rad*, (CA).
All MPPs were obtained from *Frontier Scientific, Inc* (Logan, UT) and were > 97% purity. MPPs were dissolved in minimal volumes of Dimethyl sulfoxide (DMSO) and diluted into culture media or assay buffers to achieve the final concentration. Controls received an identical volume of diluted solvent only. BIBR 1532 was obtained from *CaymCn Chemical*, Ann Arbor, MI.

-α-32-P-dGTP, 6000 Ci/mmol was obtained from *Perkin-Elmer*, (#BLU514Z). 3H-thymidine (86 Ci/mM) was from *Amersham*, (England). Colcemid was from *Roche Diagnostics*, Mannheim, Germany.

Antibodies and probes.

See Table 1.
Table 1
List of antibodies, sources, and working dilutions

| Antibody                        | Source                | Manufacturer #       | Dilution          |
|---------------------------------|-----------------------|----------------------|-------------------|
| **TERT**                        | *Abcam*               | Ab32020, Clone Y182  | 1:1000 WB         |
|                                 |                       |                      | 1:100 IF          |
|                                 | *EMD Millipore*       | MABE14               | Immunoprecipitation |
| **Secondary Abs**               | *Cell Signaling Tech* | 7076                 | 1:3000 WB         |
|                                 |                       | 7074                 | 1:3000 WB         |
| **Actin**                       | *Sigma-Aldrich*       | A2066                | 1:1000 WB         |
| **Alexa Fluor 488 antibody**    | *ThermoFisher*        | A11001               | 1:1000            |
|                                 |                       | A11008               | 1:1000            |
| **Alexa Fluor 568 antibody**    | *ThermoFisher*        | A11004               | 1:1000            |
|                                 |                       | A11011               | 1:1000            |
| **TO-PRO-3**                    | *ThermoFisher*        | T3605                | 1:1000            |
| **GAPDH**                       | *Santa Cruz*          | SC 365062            | 1:2000 WB         |
| **B-Catenin**                   | *Cell Signaling Tech* | L54E2                | 1:2000            |
| **Cyclin-D1**                   | *Bo Pharmingen*       | 556470               | 1:1000            |
| **Telomere (Telc-Alexa 488)**   | *PNA Bio Inc*         | F1004                | 500nM             |
| **Protein-G Agarose**           | *ThermoFisher*        | 20398                |                   |
| **Bulk IgG (rabbit or Mouse)**  | *Santa Cruz*          | Sc-2027              | 1–10 mg/ml        |
|                                 |                       | Sc-2025              |                   |
| **Cyclin A2**                   | *Cell signaling Tech* | #4656                | 1:500 WB          |
| **Dyskerin**                    | *Santa Cruz*          | Sc-48794             | 1:1000 WB         |

**Cell lines and cell culture**

HUH7, HEK293, and the HCV permissive clonal line HUH7.5 cells were maintained in routine cultures as described (16). The human hepatoma cell line (HUH5.15) with replicating sub-genomic HCV RNA (genotype 1b) (HUH5.15NS) (17) was cultivated as described (18). Wild type HEK293 cells were obtained from University of Iowa Tissue Culture stocks and passed routinely in minimal essential medium.
containing 10% fetal bovine serum. Telomerase negative U2OS osteosarcoma cells were obtained from American Type Culture Collection and passed using recommended media conditions.

Vectors and constructs

Lipofectamine™ 2000 (ThermoFisher) was used for all transfections and closely followed the manufacturer's protocol. For TERT overexpression the catalytically active TERT plasmid pCI neo-hEST2, a gift from Dr. Robert Weinberg (Addgene plasmid # 1781), (19) was used. Telomerase RNA component (TERC) plasmid (pBS U3-hTR-500) was also obtained from Addgene (plasmid # 28170), a gift from Dr. Kathleen Collins (20). Construction and characterization of Flag-labelled human TERT and wild type human TERT plasmids were as described (21).

DNA synthesis, apoptosis, and cellular proliferation.

DNA Synthesis

Semi-quantitative differences in DNA synthesis were determined by measuring $^3$H-thymidine uptake into whole cells in log phase growth. At indicated time points, cell cultures were washed with serum-free, thymidine deficient medium, then incubated with fresh medium containing 1 µCi/ml of $^3$H-thymidine for two hours at 37°C. Cultures were washed twice with PBS while attached to dishes, then lysed with 0.5 M NaOH and the relative amount of $^3$H-thymidine quantified by scintillation counting.

Cell Proliferation: MPPs were tested for effects on cell proliferation and viability using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye conversion assay (Cell Titer 96, Promega) as we described (22) with some modifications. Cells were plated into 96 well plates and allowed to attach overnight. MPPs were added to the cultures 24hr later and then incubated for the times indicated. At assay time, MTT reagent was added and absorbance measured at 570 nm. Controls included buffer blanks containing MPP because of background absorbances reported for MPPs by us (22) and others (23). The formula used for determination of viable cells relative to controls was

$$\text{% viable cells} = \frac{\text{abs}_{\text{sample}} - \text{abs}_{\text{blank}}}{\text{abs}_{\text{control}} - \text{abs}_{\text{blank}}} \times 100.$$  

Cell viability was also directly determined with trypan blue staining as previously described and closely correlated with MTT assay (24).

Apoptosis: This was measured by Annexin V FITC assay with cell sorting as described by BD Biosciences. Exposure of cultures with MPP was as indicated above and the assay was conducted as recommended by the manufacturer.

Quantification of telomerase activity.

Telomerase reverse transcriptase (TERT) enzymatic activity was determined using Telomeric Repeat Amplification Protocol (TRAP) with Real Time quantification as described previously (25), or measured
directly using $\alpha$-$^{32}$P-dGTP incorporation as described (26) with modifications as below.

For TRAP assay, a Quantitative Telomerase Detection Kit (US Biomax, Inc) was used to assay cellular lysates or TERT immunoprecipitates (IP) according to manufacturer's directions. Relative telomerase activity was derived from a standard curve of reference samples and data were analyzed using relative fluorescence units as compared to controls. In some cases, telomerase reaction products were visualized and quantified with TRAPeze system, (EMD/Millipore). After separation of the DNA products using 10% non-denaturing PAGE and SYBR fluorescence labelling, [1x SYBR safe DNA gel stain (Invitrogen)], gel bands were imaged with iBright 1500 (Invitrogen). The intensity of the sample's TRAP ladder and internal control was first measured using GelAnalyzer (GelAnalyzer 19.1 (www.gelanalyzer.com) as recommended. Then, the relative telomerase activity was determined by the ratio of the intensity of the sample's TRAP ladder (telomerase products, TP) to that of the internal control (IC) band.

**Direct telomerase activity assay**

For direct telomerase activity assay, a modified procedure of Tomlinson et al was used (26). In brief, human telomerase protein (Cell Pellet from $10^7$ cells overexpressing TERT, TERC, and dyskerin) was obtained from Abbexa Ltd (UK. Abx069991). 10µl of pellet lysate was mixed with different concentrations of MPP and incubated on ice for 2hr. Then the pellet-MPP complex was added to the extension reaction mixture composed of 1x telomerase buffer, 300mM KCl, 1µM Bio-L-18GGG, 1mM dATP, 1mM dTTP, 10µM dGTP, 20µCi $\alpha$-$^{32}$P-dGTP, 10mM DTT, 5’ biotinylated DNA substrate (5’-CTAGACCTGTCATCA(TTAGGG)$_3$-3’) and 10% glycerol. The reaction was conducted at 37°C for 2hr. The purification of telomerase extension products employed Dynabeads M-280 streptavidin. 5ul of purified products were loaded on 6% sequencing gel (TBE-UREA denaturing gel) with Model S2 Sequencing Gel Electrophoresis Apparatus (LabRepCo, Horsham, PA). The gel was dried at 80°C for 30 min and exposed to phosphorimaging screen for 2 hr. This screen was then scanned using a STORM phosphorimager (Molecular Dynamics), and the bands were quantified using GelAnalyzer as described above.

**Non-denaturing agarose gel electrophoresis.**

0.8% Agarose gels were run in Tris-Borate-EDTA buffer using standard slab gels as described for high molecular weight complexes (27). Gels were loaded with cellular lysates or IP previously labelled with ZnPP. Free ZnPP and ZnPP bound to large complexes was visualized using a wide-wavelength UV lightbox or with red fluorescence using excitation (EX) 608–632 nm and emission (EM) 675–720 nm. Thyroglobulin (660 kD), a heavy MW marker, was run in parallel lanes and visualized with Coomassie Blue or fluorescence staining to size the complexes. In some cases, cell lysates or IP were incubated with RNase A (Life Technologies, NY); or DNase 1, (Qiagen, CA) 100 µg/ml for 1hr at 4C prior to electrophoresis.

**Diffusion blotting of 0.8% agarose native gels.** After electrophoresis, protein complexes were transferred to nitrocellulose (NC) membranes using direct capillary action overnight at RT in the presence of 1x Tris-
buffered saline (TBS). Then the NC membranes were washed with fresh TBS and immunodetection of complexes performed as described below for Western blot assays.

**TERT overexpression and Immunoprecipitation.**

Plasmid pCl neo-hEST2 together with TERC (pBS U3-hTR-500) were transfected into log phase HEK293 cells and non-denaturing cellular lysates were prepared 48 hr later in lysis buffer (Cell Signaling Technology, Beverly, MA). Immunoprecipitation was performed as described previously (21). Briefly, transfected cells were harvested, washed in PBS, lysed in cell lysis buffer, and clarified by cold centrifugation (14,000xg for 10 min). An aliquot of supernatant containing 500 µg protein was incubated with 2 µg anti-FLAG or anti-hTERT antibody MABE14 (EMD Millipore, MA) at 4°C overnight with gentle mixing. Then, 20 µl of recombinant Protein G Agarose (Invitrogen, CA) was added and incubated at 4°C for 3hr. IPs were collected by centrifugation at 3,000 rpm for 30s at 4°C, washed three times with ice-cold PBS, aliquoted, and stored at -80°C until use. For denaturing gel electrophoresis, aliquots were dissolved in 2X Laemmli electrophoresis sample buffer (Bio-Rad, CA) and assayed by western blot (WB). Normal rabbit or mouse IgG was always used as control (Santa Cruz, CA). Aliquots of the IP were also assayed in triplicate by TRAP assay and quantified using realtime PCR as described above. In some cases, aliquots were electrophoresed on non-denaturing agarose gels after treatment with MPP and/or nucleases.

**SDS-Polyacrylamide gel electrophoresis (PAGE) and Western blot assays.**

For SDS-PAGE, cellular lysates and protein preparations such as IP were dissolved in Laemmli buffer, boiled for 1 minute and separated on denaturing SDS gels as described (28). After electrophoretic transfer of separated proteins to nitrocellulose sheets, western blot immunoassays employed enhanced chemiluminescence for signal detection (ECL™ Prime, Amersham) (28).

Cellular fluorescence labelling. Cells were grown to semi-confluence while attached to coverslips, then washed in PBS, fixed in absolute methanol, re-washed in PBS, then incubated with the fluorescent telomeric probe TelC-Alexa488 F1004 (PNA Bio) or anti-TERT antibodies with or without 10µM ZnPP for 1hr at RT. Cells were washed in PBS, then incubated with secondary antibodies conjugated to the fluorochromes Alexa Fluor 488 (green) or Alexa Fluor 568 (red). Slides were mounted with VECTASHIELD H-1000 (Vector Labs, Burlington, Ontario) and counterstained with To-pro™-3 Iodide (ThermoFisher Scientific) to visualize nuclei. Confocal microscopy was performed on a Zeiss LSM710 confocal fluorescence microscope. Alexa Fluor 488 (green) or Alexa Fluor 568 (red) fluorochromes were used to visualize telomeres or ZnPP respectively.

**Preparation of Metaphase chromosomes**

Huh7 cells were cultured in regular DMEM with colcemid (0.1 µg/mL) for 2hr at 37°C. Cells were harvested, pelleted by centrifugation, supernatant removed, then re-suspended in a solution of warm (37°C) 0.075 M potassium chloride, and incubated for 20 min in a 37°C water bath. The cells were pre-fixed by adding fixative (3:1 ethanol/acetic acid) and centrifuged for 5 min at 1000 RPM at room temperature. The supernatant was removed, and the cells suspended in the fixative solution.
metaphase chromosomes were then spread on slides and reacted with antibodies for immunofluorescence or labelled with ZnPP or telomere probe as described above.

**S-phase synchronized cells**

To synchronize cells in S phase, we performed double thymidine block essentially as described (29) with modifications. Briefly, Huh7 cells were seeded onto coverslips and then treated with 2mM thymidine (Sigma, T9250) for 18hr, released for 9hr, again treated with 2mM thymidine for 18hr, then released 2hr before use. To determine the optimal timepoint for collecting cells in S phase, we assayed cultures with flow cytometry and Western blot for cyclin A expression (Fig. S4). The cells were fixed with 4% paraformaldehyde and washed with PBS. Telomere FISH and ZnPP staining was then conducted as described below.

**Fluorescence in situ hybridization (FISH)**

We performed telomere FISH (Zhu et al 2010) using a peptide nucleic acid (PNA) probe specific to telomeres, and labeled with Alexa488, TelC-Alexa488 F1004 (PNA Bio, Newbury Park, CA,) as per the manufacturer’s instructions with modifications. Briefly, 0.2ul of PNA probe was added to 20ul of hybridization solution which was used to cover cells attached to slides. Hybridization was at 80C for 10 min, then 37C overnight. After washing, slides were incubated with 10 µM ZnPP 2hr at room temperature, washed twice in PBS, and mounted with VECTASHIELD. Confocal fluorescence microscopy used a Zeiss LSM710 confocal fluorescence microscope using 63x oil objective.

**Statistical Determinations**

All mean values for enzymatic and proliferation assays were determined using 3–6 replicates per point. Data are plotted as the mean value of each point +/- SEM. A completely randomized design with multiple treatment groups was used for analysis of variance for each experiment and variances then pooled among experiments using appropriate degrees of freedom for among and within group comparisons. IC50 and EC50 values were determined by regression assuming sigmoid or “best fit” inhibition curves and tested either pairwise using Fisher’s Least Significant Difference test or among treatment groups using F statistic. All experiments were repeated at least twice. Graphpad Prism or Excel software was used for least squares regression and calculation of all variances; however, final multiple group statistical comparisons were set up conventionally and performed by hand using standard tables of t or F values.

**Results**

We first compared the effects of various MPPs [FePP, ZnPP, SnPP and cobalt protoporphyrin (CoPP)] on cellular proliferation and DNA synthesis, (Fig. 1A-C) in Huh7 hepatoma and HEK293 embryonic kidney cells known to express telomerase. In contrast to other MPPs, ZnPP severely attenuated DNA synthesis (Fig. 1A) and depressed cellular proliferation greater than 50% at 48 hr treatment in both cell lines (Fig. 1B-C). Interestingly, ZnPP had only minor effects on proliferation in U20S cells, a line known to be telomerase negative (30) (Fig. 1D). As a positive control, BIBR 1532, a known mixed-type non-competitive
inhibitor of telomerase (31) was tested in the same cell lines. BIBR showed greater propensity to decrease proliferation in telomerase-expressing rather than a telomerase negative line in accordance with earlier reports (Fig. 1D right chart), (32).

Consistent with effects on DNA synthesis and proliferation, ZnPP was the most effective MPP at inducing apoptosis (ca.: 50% at 10 uM) in TERT positive Huh7 or HEK293 cells in contrast to other MPPs evaluated (Fig. 1E). In support of the proliferation findings, ZnPP failed to have an increased effect on apoptosis in telomerase negative U2OS cells (Fig. 1E right panel).

Because of ZnPP actions on DNA synthesis, proliferation, and apoptosis in TERT positive cells, we determined ZnPP effects on TERT expression with western blots (WB) (Fig. 2A). As positive controls, we also evaluated other pro-proliferative proteins, β-catenin and cyclin D1 which have been closely linked to TERT expression and signaling (33, 34). ZnPP reduced expression of all three proteins by 8-24hrs and by 48hr expression was nearly eliminated (Fig. 2A). Cells treated with various concentrations of ZnPP for 48hr (Fig. 2A, left middle panel) further confirmed these findings. In contrast, CoPP, FePP, or SnPP failed to significantly alter TERT, β-catenin, or cyclin D1 expression (Fig. 2A, right middle and bottom panels) consistent with their minimal effects on DNA synthesis and apoptosis (Fig. 1). The effects of ZnPP on TERT expression were apparent in different Huh 7 constructs and, interestingly, in a NS 5.15 HCV replicon, ZnPP promoted disappearance of both 120 KD telomerase monomer as well as the 45 KD C-terminal TERT fragment that we previously reported to be specific for HCV infected cells (Fig. 2B), (21).

We next evaluated the effects of MPPs on telomerase activity in cultured cells (Fig. 3A) as well as non-denatured cell lysates (Fig. 3B-E). In cultured cells incubated with ZnPP, telomerase activity was reduced in a dose-dependent fashion, (EC$_{50}$ = 5.6–5.8 uM, upper and middle panels respectively) while SnPP or FePP, had none to mild effects (EC$_{50}$ > 10 uM, either cell line) (Fig. 3A). The loss of telomerase activity with time of ZnPP treatment in the NS 5.15 HCV replicon (Fig. 3A, lower panel) reflected the disappearance of TERT seen in the WB (Fig. 2B).

The possibility that MPPs can directly inhibit telomerase activity in cellular extracts, similar to porphyrin quadruplex ligands, (6) was addressed next. Because of concerns that some quadruplex ligands inhibit Taq DNA polymerase in addition to telomerase, we assayed MPP inhibition at both steps of the TRAP procedure with a strategy similar to that of others (35). Using equivalent extracts but separate assays, MPP was either included in the telomerase RT extension step or the extension step was conducted without MPP and then MPP added only for the amplification steps with Taq DNA polymerase. To avoid further potential errors introduced by Realtime quantification, TRAP products were labeled with SYBR green, visualized on denaturing gels, and each lane quantified by absorbance measurements as described in the Methods. The latter step also ruled out the possibility that decreases in activity were artifactual due to fluorescence signal quenching by some MPPs (36). ZnPP was significantly more active (IC$_{50}$ = 2.5 µM) than FePP and SnPP (both IC$_{50}$ > 10.0 µM), (Figs. 3B-D respectively). All three MPPs had minimal effects on Taq polymerase during telomerase product extension and the slight inhibition of Taq polymerase seen for ZnPP was not directly concentration dependent. However, at least one MPP, CoPP,
clearly inhibited Taq polymerase and could not be reliably assayed via TRAP assay (see Supplemental data, Fig. S1).

To confirm that ZnPP specifically inhibited telomerase as seen in the TRAP assays, direct telomere extension assays were conducted in the presence of α-32P-GTP. An IC_{50} of 2.7 µM for ZnPP obtained by direct extension assay was quite similar to the IC_{50} obtained with TRAP assay (2.5 µM) (Fig. 3E). Consequently, by two different assay procedures, ZnPP was observed to directly inhibit telomerase activity in cellular extracts and the IC_{50} values are roughly within a two fold range of the EC_{50} values for intact Huh7 and HEK293 cells (5 and 6 µM respectively). IC_{50} and EC_{50} values of the three MPPs obtained by different assay procedures are summarized in Table 2.
Table 2
Inhibition of telomerase by MPPs in direct telomere extension assays.

| MPP            | IC$_{50}$ (µM) | R$^2$ | Significant difference of IC$_{50}$ [ZnPP < SnPP or FePP] |
|----------------|----------------|-------|----------------------------------------------------------|
| a. Cellular lysates [Trapeze] |                |       |                                                         |
| ZnPP           | 2.5            | .974  |                                                         |
| SnPP           | 12             | .962  | P < .001                                                 |
| FePP           | > 20           | .911  | P < .001                                                 |
| b. α$^{32}$P-dGTP direct telomerase assay |                |       |                                                         |
| ZnPP           | 2.7            | .904  |                                                         |
| SnPP           | > 16           | .645  | P < .001                                                 |
| FePP           | > 20           | .777  | P < .001                                                 |
| c. Intact Huh7 cells [TRAP] |                |       |                                                         |
| ZnPP           | 5.4            | .941  |                                                         |
| SnPP           | 10.8           | .865  | P < .01                                                  |
| FePP           | > 20           | .405  | P < .01                                                  |
| d. Intact HEK293 cells [TRAP] |                |       |                                                         |
| ZnPP           | 6.4            | .928  |                                                         |
| SnPP           | > 20           | .076  | P < .001                                                 |
| FePP           | > 20           | .085  | P < .001                                                 |
| e. TERT IP vs cellular lysate [TRAP] |                |       |                                                         |
| ZnPP (IP)      | 2.4            | .953  | NS                                                       |
| ZnPP (lysate)  | 1.8            | .963  |                                                         |

ZnPP, SnPP, and non-metal, “free” Lewis base protoporphyrins exhibit autofluorescence; (36) a property that has proven useful to study intracellular activities of MPPs such as nuclear localization and DNA or cellular adduct binding (12, 23, 37, 38). Other transition metal MPPs such as FePP or CoPP are inactive fluoroscopically because they have unfilled transition metal $d$ orbitals that quench fluorescence emission. We investigated ZnPP binding to native, non-denatured telomerase-containing complexes after
separation on large pore, (0.8%), agarose gels (27) (39). Initially, non-denaturing acrylamide gels were considered for these studies, however, we discovered that ZnPP labelled complexes would not enter the largest pore size possible, a result also noted by others (23, 37) (see Supplemental data Fig. 2).

Initially, increasing amounts of cellular extracts were incubated with varying amounts of ZnPP, then electrophoresed on large pore agarose gels. ZnPP was then visualized fluoroscopically using visible red wavelengths [608–632 nm Ex and 675–720 nm EM] or wide band UV (Fig. 4A upper and lower panels respectively). ZnPP bound to high molecular weight complexes in a concentration dependent manner and the complexes electrophoresed with a mobility just above thyroglobulin (670 kD), quite similar to sizes noted by us and others for TERT ribonuclear protein particles separated by glycerol gradient centrifugation (21, 40) and large pore agarose/acrylamide gels (39). Under these conditions, free ZnPP migrated slightly cathodal. While ZnPP binding was easily identified in cellular extracts, no binding was detectable in bulk protein incubations of BSA or IgG (Fig. 4B). ZnPP also labelled complexes in intact cells as determined by electrophoresis of extracts prepared after ZnPP incubation in culture (Fig. 4C).

To assess whether TERT is a component of the ZnPP-labelled cellular extracts we incubated lysates with anti-human TERT, β-catenin, or cyclin D1 antibodies or non-specific IgG antibodies prior to electrophoresis and looked for upward mobility shift after electrophoresis (Fig. 4D). Only anti-TERT antibody led to a significant upward mobility shift of ZnPP labeled complexes from either cell type, suggesting that TERT is indeed a component of the large complexes. No mobility shift was noted for the other antibodies tested suggesting ZnPP specifically labelled TERT complexes. Note that both cyclin D1 and β-catenin would be expected to be components of large molecular complexes in non-denatured cellular lysates (41, 42).

Further characterization of the ZnPP binding complexes as to protein and DNA composition, and investigation of SnPP binding is presented in the online supplemental data (Fig. S2).

ZnPP labelled complexes from Huh7 cells were next blotted onto nitrocellulose by capillary diffusion (conditions determined empirically, see Supplementary data, Fig. S3) and probed with specific anti-TERT or anti-dyskerin antibodies, the latter a positive control for telomerase holoenzyme (Fig. 4E). Both TERT and dyskerin were easily identified in the high molecular weight complexes binding ZnPP (Fig. 4E). Interestingly, cellular lysates showed more immunoreactive TERT when incubated with ZnPP prior to electrophoresis suggesting a protective effect of ZnPP on TERT in the extracts.

Immunoprecipitation using TERT -specific antibodies further confirmed that TERT is a component of ZnPP labelled complexes. Immunoprecipitates (IP) were evaluated on native agarose as well as denaturing SDS gels and WB (Fig. 5A, left and right panels respectively). ZnPP bound the anti-TERT IP complexes intensely and IP had increased mobility as compared to non-specific IP complexes or no antibody control (Fig. 5A, left panel). As expected, the anti-TERT IP analyzed on WB (Fig. 5A right panel) showed increased TERT as compared to IP from non-specific or no antibody controls. A weaker TERT band (relative to exposure time) was also identified in the crude cellular lysate. When assayed by TRAP assay, IP TERT complexes and unpurified enzyme showed similar IC_{50} values with ZnPP, (3.1 vs 2.2 uM
respectively), (Fig. 5B). These measurements were also close to the IC$_{50}$ observed for overexpressed enzyme, (2.7 uM), when assayed by direct α-P$_{32}$-GTP extension assay (Fig. 3E), (Table 2).

To investigate whether nucleic acids are components of the ZnPP labelled complexes; IP or crude lysates, were digested with DNase I or RNase A prior to labelling with ZnPP (Fig. 5C, upper panel). Digestion of extracts with DNase I elicited minimal changes in the mobility of ZnPP labelled complexes, however, a significant upward shift was seen when extracts were digested with RNase A. Furthermore, there was a marked increase in unbound ZnPP after nuclease, most dramatic with RNase A – treated IPs, (arrow, Fig. 5C, upper panel) suggesting that ZnPP most likely binds to a ribonuclear protein complex and the binding site is at least partially disrupted with RNase digestion. Next, nuclease digested, ZnPP labelled complexes were blotted onto nitrocellulose and reacted with anti-TERT antibodies. These experiments showed that RNase A digestion severely diminished the amount of immunoreactive TERT in the labelled complexes, most notably for IP complexes (Fig. 5C, lower panel). These findings strengthened the conclusion that ZnPP binds to high molecular weight telomerase complexes. In addition to providing a telomere template, TERC is an important structural component of telomerase holoenzyme and RNase A digestion is known to completely disrupt ribonuclear complex structure and release TERT (43).

ZnPP binding to cellular structures in situ was probed by confocal immunofluorescence microscopy. While telomere sequences exist throughout the mammalian chromosome, it is known that telomerase holoenzyme only associates with telomeres at DNA replication during S phase (44). Consequently, we compared ZnPP localization in synchronized S-phase cells as compared to metaphase chromosomes which contain prominent telomere ends without telomerase. The percentages of cells in S phase were determined temporally with flow cytometry after double thymidine block and extracts were monitored on immunoblots with Cyclin A2 staining to determine optimal times for study, (Fig. S4 Supplementary data). Telomere sites were labelled with telomere sequence specific fluorescent probe (PNA TEIC-Alexa488 F1004) and TERT was localized with specific antibodies (Fig. 6).

First, we looked at ZnPP co-localization with the telomere probe. While ZnPP clearly localized with telomeres in S phase cells, (Fig. 6A left panels), it did not label the prominent telomeres on metaphase chromosome tips, (Fig. 6A, right panels) which are devoid of holoenzyme. Next, we investigated whether ZnPP would co-localize with TERT in S phase as compared to unsynchronized Huh7 cells. In S phase cells, TERT co-localized with ZnPP in the nucleus and at some cytoplasmic sites (Fig. 6B left panels). As we reported previously, TERT is sparsely present in the nucleus of unsynchronized, log phase Huh7 cells, but is chiefly found at perinuclear sites which co-localize with mitochondria (21). Interestingly, even perinuclear TERT, likely lacking telomeric DNA, showed avid TERT- ZnPP co-localization (Fig. 6B, right panels). Collectively, these data indicate that ZnPP can bind to telomerase complexes and/or associated components. While telomeric DNA does not appear to be a primary binding site of ZnPP per se, at least at prominent telomeres on metaphase chromosomes, the specific sites of interaction in the telomerase holoenzyme remain to be determined.

**Discussion**
Development of telomerase inhibitors for use in chemotherapy began shortly after the discovery of telomerase over three decades ago. Telomerase inhibitors are cytotoxic to most tumor cells and promote telomere shortening and instability, DNA damage responses, DNA synthesis arrest, apoptosis, and other cellular senescence programs (6). Multiple telomerase inhibitors are in development and include competitive, non-competitive allosteric, and apparent DNA substrate inhibitors [see (5) for recent review].

A wide variety of planar aromatic macromolecules and porphyrins have also been shown to inhibit telomerase (45). These compounds are thought to stabilize hydrogen-bonded guanidine-tetrads (G-4 complexes) that form in telomeric DNA and inhibit cyclic realignment of the DNA substrate with TERT during the enzyme cycle, thus reducing telomerase processivity (9, 46). Functionally, porphyrins such as tetra-(N-methyl-4-pyridyl)porphyrin (TMPyP4) were shown to cause cell growth arrest and apoptosis in neoplastic cells which demonstrated that G4 telomeric binding sites are useful targets for chemotherapy (10, 47).

In contrast to porphyrins, protoporphyrins have not been investigated for anti-telomerase behavior. Nevertheless, common MPPs such as FePP and ZnPP are known to bind oligomeric G-4 sequences in vitro, (12, 48) thus suggesting that they are capable of telomerase inhibition. ZnPP and long acting conjugates such as ZnPP-polyethylene glycol have been studied as chemotherapeutic agents in rodent models for some time (14) and ZnPP was reported to be synergistic for drugs such as cisplatin, (15). Consequently, investigation of ZnPP effects and sites of action on telomerase activity are timely and important.

We first evaluated the effects of common MPPs on telomerase activity, cellular proliferation, DNA synthesis, and apoptosis. ZnPP was the most potent MPP tested and down regulated TERT expression, arrested DNA synthesis, and promoted apoptosis in Huh7 hepatoma cells. In contrast, ZnPP was only minimally active in U2OS cells which contain no telomerase (49), thus suggesting that telomerase interactions play a key role in ZnPP anti-proliferative and apoptotic behavior. Downregulation of TERT was also accompanied by reduction in cyclin D1 and β-catenin which are not only important markers of cellular proliferation and apoptosis per se (50), but they have known positive signaling relationships with telomerase (33) (34). Our findings support earlier work that documented the specific anti-proliferative actions of ZnPP (23) and now indicate that anti-telomerase behavior plays an additional, important role.

ZnPP directly inhibited telomerase enzymatic activity in cellular lysates, IP, and intact cells in culture. This was verified by both TRAP and direct [α-32P]-dGTP extension assays with similar IC$_{50}$ (Table I). To date, this is the first demonstration that ZnPP has direct anti-telomerase activity and these findings support consideration of ZnPP or related MPPs for use in chemotherapeutic programs. These studies also lay groundwork for modeling and development of improved, anti-telomerase derivatives using a ZnPP-based model design.

ZnPP is a naturally occurring MPP and it inhibited telomerase at EC$_{50}$ and IC$_{50}$ concentrations of ca 5 µM or less, close to levels that can be achieved physiologically. During normal heme synthesis in
Reticulocytes ZnPP is produced at low levels (0.5 µM), however, in times of iron deficiency it can rise to values of 5 µM or higher (51), well within the effective anti-telomerase concentrations seen here. The IC$_{50}$ of ZnPP (Table 2) is also close to the micromolar ranges of IC$_{50}$ reported for other synthetic experimental porphyrins (6, 45).

The major components of the telomerase holoenzyme complex include TERT, dyskerin, p23, Hsp90, TERC and telomerase-associated protein (52). TERT is absent from telomeres until it is assembled into telomerase holoenzyme and then recruited to selective chromosomal telomeric sites at the start of S phase (44, 53). Telomere addition is known to be coupled to DNA synthesis and the processes likely occur sequentially in neoplastic cells (44).

With the structural and functional characteristics of telomerase in mind, we investigated whether ZnPP can bind at or near holoenzyme complexes. On non-denaturing agarose gels, ZnPP specifically bound to high molecular weight complexes [> 670 kD] in cellular extracts. Binding of anti-TERT antibodies to ZnPP-labelled complexes resulted in an upward mobility shift, thus demonstrating that the complexes contained TERT. Both TERT and dyskerin were identified in ZnPP labelled complexes by native agarose gel immunoblot analysis. Consequently, ZnPP can bind native high molecular weight complexes that contain components of the telomerase holoenzyme.

As might be expected, IP showed faster mobility than complexes from crude lysates indicating a smaller, immunopurified preparation. DNase 1 (mostly selective for DS DNA) had minimal effects on ZnPP binding while RNase A digestion led to decreased complex mobility, decreased ZnPP binding, and increased free ZnPP. Moreover, RNase A digestion caused loss of TERT from the complexes, a result that might be anticipated since TERC binds TERT in the ribonuclear protein complex and RNase A treatment disrupts holoenzyme structure (40). Collectively, these data demonstrate that ZnPP binds ribonuclear protein complexes containing TERT, however, identification of the definitive binding sites requires further study.

ZnPP is known to bind a variety of G-4 structures as well as selected oligomeric telomere sequences in vitro (12), thus, considerable heterogeneity of ZnPP binding should be expected. G-rich DNA sequences likely modulate DNA structure-regulatory activities at sites such as gene promoters for enzyme systems in addition to telomerase (54). Consequently, we cannot yet account for how other ZnPP targeted G-4 sites may contribute to our results here.

Confocal immunohistochemical experiments demonstrated that ZnPP binds at or near telomere/telomerase complexes in S phase cells; which is the only time in the cell cycle when telomerase is found at telomeres (44). Surprisingly, telomere rich sites on metaphase chromosomal ends did not overtly bind ZnPP (Fig. 6A). Consequently, ZnPP interactions with telomerase complexes appear more complex than just telomere G-4 binding and may depend on S phase chromatin structure, site accessibility, and composition of the complexes. Furthermore, these data suggest there may be a primary interaction of ZnPP with TERT or closely associated proteins since there was co-localization of TERT and
ZnPP in cytoplasmic as well as nuclear locations (Fig. 6B). Interestingly, earlier work showed that ZnPP and FePP could directly bind other reverse transcriptases such as HIV (55) also supporting a direct interaction of ZnPP and TERT. More studies characterizing the site(s) of ZnPP binding and kinetics of telomerase inhibition are necessary. Overall, the ability of ZnPP to avidly bind telomere sites in S-phased cells *in situ* indicates it can impact telomerase activity as supported by the kinetic and immunoblot experiments.

Aside from telomerase, other cellular sites, both nuclear and cytoplasmic, have been proposed to be responsible for the pro-apoptotic qualities of ZnPP. Since ZnPP is both a transcriptional inducer for heme oxygenase-1 (HO-1) (56) and a competitive HO-1 inhibitor, (57), earlier studies attributed many ZnPP mechanisms to HO-1 antagonism. Presently it is not known whether ZnPP actions on the telomerase system are impacted by HO-1 antagonism and this concept requires further study. ZnPP also inhibited transcriptional promoter sites for cyclin D1 (23) and attenuated Wnt/β-catenin expression leading to increased apoptosis (58). Our proliferation studies support these findings, yet it is not yet clear whether telomerase inhibition may affect these other pathways.

**Conclusions**

In summary, our data indicate that ZnPP interacts with the telomerase enzyme system at two major regulatory points: 1) downregulation of TERT and 2) direct inhibition of telomerase enzymatic activity. Concomitantly, ZnPP attenuates DNA synthesis and cellular proliferation while promoting apoptosis. Structurally, ZnPP binds to TERT containing ribonuclear protein complexes and co-localizes with a subset of nuclear telomeres that likely contain holoenzyme complexes. Our findings support the use of ZnPP and potentially the development of other synthetic or natural protoporphyrins for use as chemotherapeutic agents in the treatment of neoplastic disease.

**Abbreviations**

ANOVA = Analysis of variances

MPP = metalloprotoporphyrin [Fe, Zn, Sn, Co].

MTT = (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

TERT = telomerase reverse transcriptase

RT-PCR = Reverse Transcriptase - Polymerase Chain Reaction

TRAP = Telomerase repeat amplification protocol

TERC = telomerase RNA component

WB = Western blot
NS = nonstructural replicon of HCV

TMPyP4 = tetra-(N-methyl-4-pyridyl) porphyrin

SYBR = $N',N'$-dimethyl-$N$-[4-[E-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-$N$-propylpropane-1,3-diamine [asymmetrical cyanine dye that binds DS DNA].

ZnPP = zinc protoporphyrin

SnPP = tin protoporphyrin

FePP = iron protoporphyrin (heme).

CoPP = cobalt protoporphyrin

**Declarations**

- *Ethics approval and consent to participate*

Not applicable.

- *Consent for publication*

Not applicable.

- *Availability of data and materials*

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

- *Competing interests*

The authors declare that they have no competing interests.

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- *Authors’ contributions*

**ZZ:** Acquisition of data, intellectual content, manuscript editing, and statistics. **HT:** acquisition of data, intellectual content, manuscript editing, figures, statistics. **MMM:** Acquisition of data and technical support. **BDF:** Acquisition of data and technical support. **JAA:** Acquisition of data and technical support. **TOM:** Acquisition of data and technical support for confocal microscope analysis. **JLM:** sequencing gel electrophoresis, gel drying and imaging analysis. **LM:** Acquisition of data and autoradiography of
isotopes, intellectual content, manuscript editing. **WNS:** Study funding, design, data interpretation, statistics, text author, and intellectual content. All authors read and approved the final manuscript.

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**References**

1. Jafri MA, Ansari SA, Alqahtani MH, Shay JW. Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. Genome Med. 2016;8(1):69.
2. Kubicka S, Rudolph KL, Hanke M, Tietze MK, Tillmann HL, Trautwein C, et al. Hepatocellular carcinoma in Germany: a retrospective epidemiological study from a low-endemic area. Liver. 2000;20(4):312–8.
3. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med. 1997;3(11):1271–4.
4. Djojosubroto MW, Chin AC, Go N, Schaetzlein S, Manns MP, Gryaznov S, et al. Telomerase antagonists GRN163 and GRN163L inhibit tumor growth and increase chemosensitivity of human hepatoma. Hepatology. 2005;42(5):1127–36.
5. Ivancich M, Schrank Z, Wojdyla L, Leviskas B, Kuckovic A, Sanjali A, et al. Treating Cancer by Targeting Telomeres and Telomerase. Antioxidants (Basel). 2017;6(1).
6. De Cian A, Lacroix L, Douarre C, Temime-Smaali N, Trentesaux C, Riou J-F, et al. Targeting telomeres and telomerase. Biochimie. 2008;90(1):131–55.
7. Shi DF, Wheelhouse RT, Sun DY, Hurley LH. Quadruplex-interactive agents as telomerase inhibitors: Synthesis of porphyrins and structure-activity relationship for the inhibition of telomerase. Journal of medicinal chemistry. 2001;44(26):4509–23.
8. Huppert JL. Four-stranded nucleic acids: structure, function and targeting of G-quadruplexes. Chem Soc Rev. 2008;37(7):1375–84.
9. Dixon IM, Lopez F, Esteve JP, Tejera AM, Blasco MA, Pratviel G, et al. Porphyrin derivatives for telomere binding and telomerase inhibition. Chembiochem. 2005;6(1):123–32.
10. Shammas MA, Shmookler Reis RJ, Akiyama M, Koley H, Chauhan D, Hideshima T, et al. Telomerase inhibition and cell growth arrest by G-quadruplex interactive agent in multiple myeloma. Mol Cancer Ther. 2003;2(9):825–33.
11. Sen D, Poon LC. RNA and DNA complexes with hemin [Fe(III) heme] are efficient peroxidases and peroxynogenases: how do they do it and what does it mean? Crit Rev Biochem Mol Biol. 2011;46(6):478–92.
12. Zhang Z, Sharon E, Freeman R, Liu X, Willner I. Fluorescence detection of DNA, adenosine-5'-triphosphate (ATP), and telomerase activity by zinc(II)-protoporphyrin IX/G-quadruplex labels. Anal...
13. Saito K, Tai H, Hemmi H, Kobayashi N, Yamamoto Y. Interaction between the heme and a G-quartet in a heme-DNA complex. Inorg Chem. 2012;51(15):8168–76.

14. Fang J, Sawa T, Akaike T, Greish K, Maeda H. Enhancement of chemotherapeutic response of tumor cells by a heme oxygenase inhibitor, pegylated zinc protoporphyrin. International journal of cancer. 2004;109(1):1–8.

15. Liu Y-S, Li H-S, Qi D-F, Zhang J, Jiang X-C, Shi K, et al. Zinc protoporphyrin IX enhances chemotherapeutic response of hepatoma cells to cisplatin. World J Gastroentero. 2014;20(26):8572–82.

16. Zhu Z, Wilson AT, Luxon BA, Brown KE, Mathahs MM, Bandyopadhyay S, et al. Biliverdin inhibits hepatitis C virus nonstructural 3/4A protease activity: mechanism for the antiviral effects of heme oxygenase? Hepatology. 2010;52(6):1897–905.

17. Lohmann V, Korner F, Koch JO, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science. 1999;285(5424):110–3.

18. Zhu Z, Wilson AT, Mathahs MM, Wen F, Brown KE, Luxon BA, et al. Heme oxygenase-1 suppresses hepatitis C virus replication and increases resistance of hepatocytes to oxidant injury. Hepatology. 2008;48(5):1430–9.

19. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, et al. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell. 1997;90(4):785–95.

20. Fu D, Collins K. Distinct biogenesis pathways for human telomerase RNA and H/ACA small nucleolar RNAs. Mol Cell. 2003;11(5):1361–72.

21. Zhu Z, Tran H, Mathahs MM, Moninger TO, Schmidt WN. HCV Induces Telomerase Reverse Transcriptase, Increases Its Catalytic Activity, and Promotes Caspase Degradation in Infected Human Hepatocytes. PLoS ONE [Electronic Resource]. 2017;12(1):e0166853.

22. Hu K, Zhu Z, Mathahs MM, Tran H, Bommer J, Testa CA, et al. Metalloprotoporphyrin Inhibition of HCV NS3-4A Protease: Structure-Activity Relationships. Drug design, development and therapy. 2020;14:757 – 71.

23. La P, Fernando AP, Wang Z, Salahudeen A, Yang G, Lin Q, et al. Zinc protoporphyrin regulates cyclin D1 expression independent of heme oxygenase inhibition. J Biol Chem. 2009;284(52):36302–11.

24. Zhu Z, Mathahs MM, Schmidt WN. Restoration of type I interferon expression by heme and related tetrapyrroles through inhibition of NS3/4A protease. The Journal of infectious diseases. 2013;208(10):1653–63.

25. Zhu ZW, Wilson AT, Gopalakrishna K, Brown KE, Luxon BA, Schmidt WN. Hepatitis C Virus Core Protein Enhances Telomerase Activity in Huh7 Cells. Journal of medical virology. 2010;82(2):239–48.

26. schmidtwtomlinson CG, Sasaki N, Jurchyluk J, Bryan TM, Cohen SB. Quantitative assays for measuring human telomerase activity and DNA binding properties. Methods. 2017;114:85–95.
27. Kim R. Native agarose gel electrophoresis of multiprotein complexes. Cold Spring Harb Protoc. 2011;2011(7):884–7.

28. Abdalla MY, Britigan BE, Wen F, Icardi M, McCormick ML, LaBrecque DR, et al. Down-regulation of heme oxygenase-1 by hepatitis C virus infection in vivo and by the in vitro expression of hepatitis C core protein. The Journal of infectious diseases. 2004;190(6):1109–18.

29. Acharya S, Kaul Z, Gocha AS, Martinez AR, Harris J, Parvin JD, et al. Association of BLM and BRCA1 during Telomere Maintenance in ALT Cells. PLoS ONE [Electronic Resource]. 2014;9(8):e103819.

30. Tilman G, Loriot A, Van Beneden A, Arnoult N, Londoño-Vallejo JA, De Smet C, et al. Subtelomeric DNA hypomethylation is not required for telomeric sister chromatid exchanges in ALT cells. Oncogene. 2009;28(14):1682–93.

31. Pascolo E, Wenz C, Lingner J, Hauel N, Priepke H, Kauffmann I, et al. Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate. J Biol Chem. 2002;277(18):15566–72.

32. Damm K, Hemmann U, Garin-Chesa P, Hauel N, Kauffmann I, Priepke H, et al. A highly selective telomerase inhibitor limiting human cancer cell proliferation. Embo J. 2001;20(24):6958–68.

33. Li J, Huang X, Xie X, Wang J, Duan M. Human telomerase reverse transcriptase regulates cyclin D1 and G1/S phase transition in laryngeal squamous carcinoma. Acta oto-laryngologica. 2011;131(5):546–51.

34. Zhang Y, Toh L, Lau P, Wang X. Human telomerase reverse transcriptase (hTERT) is a novel target of the Wnt/beta-catenin pathway in human cancer. J Biol Chem. 2012;287(39):32494–511.

35. De Cian A, Cristofari G, Reichenbach P, De Lemos E, Monchaud D, Teulade-Fichou M-P, et al. Reevaluation of telomerase inhibition by quadruplex ligands and their mechanisms of action. Proc Natl Acad Sci USA. 2007;104(44):17347–52.

36. Lamola AA. Fluorescence methods in the diagnosis and management of diseases of tetrapyrrole metabolism. J Invest Dermatol. 1981;77(1):114–21.

37. Yang G, Nguyen X, Ou J, Rekulapelli P, Stevenson DK, Dennery PA. Unique effects of zinc protoporphyrin on HO-1 induction and apoptosis. Blood. 2001;97(5):1306–13.

38. Tong Aj, Liu L, Liu L, Li L-d, Huie CW. Solid-substrate room-temperature phosphorescence study on zinc(II) and tin(IV) protoporphyrins and their interaction with DNA. Fresenius' Journal of Analytical Chemistry. 2001;370(8):1023–8.

39. Gardano L, Holland L, Oulton R, Le Bihan T, Harrington L. Native gel electrophoresis of human telomerase distinguishes active complexes with or without dyskerin. Nucleic Acids Res. 2012;40(5):e36.

40. Venteicher AS, Meng ZJ, Mason PJ, Veenstra TD, Artandi SE. Identification of ATPases pontin and reptin as telomerase components essential for holoenzyme assembly. Cell. 2008;132(6):945–57.

41. Diehl JA, Yang W, Rimerman RA, Xiao H, Emili A. Hsc70 regulates accumulation of cyclin D1 and cyclin D1-dependent protein kinase. Mol Cell Biol. 2003;23(5):1764–74.
42. Gerlach JP, Emmink BL, Nojima H, Kranenburg O, Maurice MM. Wnt signalling induces accumulation of phosphorylated beta-catenin in two distinct cytosolic complexes. Open Biol. 2014;4(11):140120.

43. Venteicher AS, Abreu EB, Meng Z, McCann KE, Terns RM, Veenstra TD, et al. A human telomerase holoenzyme protein required for Cajal body localization and telomere synthesis. Science 2009 (New York, N Y). ;323(5914):pp. 644–8.

44. Tomlinson RL, Ziegler TD, Supakomndej T, Terns RM, Terns MP. Cell cycle-regulated trafficking of human telomerase to telomeres. Mol Biol Cell. 2006;17(2):955–65.

45. Cao Q, Li Y, Freisinger E, Qin PZ, Sigel RKo, Mao Z-W. G-quadruplex DNA targeted metal complexes acting as potential anticancer drugs. Inorganic Chemistry Frontiers. 2017;4(1):10–32.

46. Sun D, Thompson B, Cathers BE, Salazar M, Kerwin SM, Trent JO, et al. Inhibition of human telomerase by a G-quadruplex-interactive compound. Journal of medicinal chemistry. 1997;40(14):2113–6.

47. Izbicka E, Wheelhouse RT, Raymond E, Davidson KK, Lawrence RA, Sun D, et al. Effects of cationic porphyrins as G-quadruplex interactive agents in human tumor cells. Cancer research. 1999;59(3):639–44.

48. Gray LT, Puig Lombardi E, Verga D, Nicolas A, Teulade-Fichou MP, Londono-Vallejo A, et al. G-quadruplexes Sequester Free Heme in Living Cells. Cell chemical biology. 2019;26(12):1681–91.e5.

49. Alawi F, Lin P. Dyskerin is required for tumor cell growth through mechanisms that are independent of its role in telomerase and only partially related to its function in precursor rRNA processing. Molecular carcinogenesis. 2011;50(5):334–45.

50. Veeramachaneni NK, Kubokura H, Lin L, Pippin JA, Patterson GA, Drebin JA, et al. Down-regulation of beta catenin inhibits the growth of esophageal carcinoma cells. J Thorac Cardiovasc Surg. 2004;127(1):92–8.

51. Iyer JK, Shi L, Shankar AH, Sullivan DJ. Jr. Zinc protoporphyrin IX binds heme crystals to inhibit the process of crystallization in Plasmodium falciparum. Mol Med. 2003;9(5–8):175–82.

52. Wyatt HDM, West SC, Beattie TL. InTERTpreting telomerase structure and function. Nucleic Acids Res. 2010;38(17):5609–22.

53. Vogan JM, Collins K. Dynamics of Human Telomerase Holoenzyme Assembly and Subunit Exchange across the Cell Cycle. J Biol Chem. 2015;290(35):21320–35.

54. Huppert JL, Balasubramanian S. Prevalence of quadruplexes in the human genome. Nucleic Acids Res. 2005;33(9):2908–16.

55. Argyris EG, Vanderkooi JM, Paterson Y. Mutagenesis of key residues identifies the connection subdomain of HIV-1 reverse transcriptase as the site of inhibition by heme. European journal of biochemistry. 2001;268(4):925–31.

56. Kwok SCM. Zinc Protoporphyrin Upregulates Heme Oxygenase-1 in PC-3 Cells via the Stress Response Pathway. Int J Cell Biol. 2013;2013:162094.
57. Maines MD. Zinc protoporphyrin is a selective inhibitor of heme oxygenase activity in the neonatal rat. Biochim Biophys Acta. 1981;673(3):339–50.

58. Wang S, Hannafon BN, Lind SE, Ding W-Q. Zinc Protoporphyrin Suppresses beta-Catenin Protein Expression in Human Cancer Cells: The Potential Involvement of Lysosome-Mediated Degradation. PloS one. 2015;10(5):e0127413.

Figures
Figure 1

Effects of MPPs on DNA synthesis, cellular proliferation, and viability. A. Incorporation of 3H-thymidine. Early log phase cultures of Huh7 (left chart) or HEK293 (right chart) cells were treated with 10 µM of the indicated MPP. (* p < .001). B. Inhibition of proliferation. MTT assays were performed after 10 uM MPP treatment of Huh7 cells (left) or HEK293 cells (right). (*p < .01, **p < .05) C. Cell growth and viability. ZnPP (left) and FePP (right) were added to log phase cultures of Huh7 cells. The number of viable cells...
was determined daily using direct cell counting and trypan blue dye exclusion. (**p < .01, *p < .05) (A-C Statistics: Ranked t-test ZnPP < other MPPs. (Points represent mean, +/- SEM, n = 3-6). D. Effects of ZnPP in TERT negative (U2OS) as compared to TERT positive (Huh7) cell lines Upper panel: Early log phase cells were treated with the indicated concentrations of ZnPP for 48hr (upper panel) or BIBR for 72hr (lower panel) and proliferation then determined using MTT assay. (ANOVA with paired t-test U2OS vs Huh7). (*p < .01 all points, **p < .01, 10 and 20 uM). E. Apoptosis was measured with BD Biosciences Annexin V FITC assay in HEK293 (upper), Huh7 (middle) and U2OS (lower) panels cells after incubation with the indicated concentration of MPP for 48hr. (**p < .025, *p < .01 ZnPP apoptosis > other MPPs.), (ANOVA with ranked T test:)

Figure 2

MPP effects on cellular expression of proliferative proteins TERT, β-catenin, and cyclin D1. A. ZnPP decreases expression of proliferative proteins. Log phase Huh7 cells were treated with the indicated concentrations of MPPs and assayed for various times, (upper panel) or after 48hr (middle and lower panels) on WB using specific antibodies. B. Log phase cultures of different clonal Huh7 and HEK293 cell
lines were incubated with the indicated concentrations of ZnPP for 48hr. TERT was then identified on WB with specific anti-TERT antibodies [*HCV positive replicon (21)].

**Figure 3**

MPP inhibition of telomerase enzymatic activity. A. Telomerase inhibition by MPP in cultured cells. Log phase Huh7, HEK293 and Huh5.15 HCV replicon cells, (upper, middle, and lower panels respectively), were incubated with MPPs (ZnPP, SnPP, or FePP,) for 48hr; then telomerase activity was determined by TRAP assay in enzymatic lysate. (#p < .01, *p < .001, **p < .01 ranked t-test), (Points represent mean +/- SEM n = 6, ANOVA). B-D. Telomerase inhibition by MPP in enzymatic extracts. Enzymatic extracts were prepared from semi-confluent phase Huh7 cells and aliquots were assayed in triplicate using TRAP assay. MPPs were added to RT-PCR reactions either before or after the RT telomere elongation step to test whether MPPs inhibit Taq polymerase. Visualization of amplified telomeric products was on 12% denaturing gels using SYBR fluorescence labelling followed by quantification with densitometry. IC = Internal Taq polymerase control. E. MPP inhibition of telomerase activity as determined with direct α-32P-dGTP extension assays. Aliquots of 107 HEK293 cell pellet lysates from cells overexpressing hTERT, TERC, and dyskerin were incorporated into direct telomere extension assays using biotin-linked DNA substrate.
Reactions were incubated at 37°C for two hours, then biotin labelled products purified with strepavidin linked agarose beads and electrophoresed on denaturing acrylamide gels. Bands were visualized radiographically (upper panel) and relative activity was quantified with densitometry (lower panel). (*p < .01, ranked t test of ZnPP IC50 < other MPPs) (Points represent mean +/- SEM, n =3)

Figure 4

ZnPP fluorescent labelling of cellular extracts. A. Titration of complexes with ZnPP. Cellular lysates were prepared from log phase Huh7 cells and incubated 1hr at RT with various concentrations of ZnPP. Mixtures were electrophoresed on non-denaturing 0.8% agarose gels and visualized fluorescently using red excitation and emission (upper panel) or broad band UV light (lower panel). B. ZnPP labelling of extracts. ZnPP (10 μM) was added to BSA, non-specific rabbit IgG, or cellular lysates at the indicated concentrations and incubated for 1hr at RT. Aliquots were then electrophoresed on 0.8% agarose gels and visualized under UV light. C. Labelling complexes with ZnPP in intact cells. ZnPP (10 μM) was incubated with cultured cells for 24hr, then enzymatic extracts were prepared, electrophoresed on 0.8% agarose gels, and visualized under UV light. D. ZnPP labelled complexes have mobility shift with anti-TERT antibodies. Enzymatic cellular extracts (10 ug as protein) were prepared and labelled with 10 μM ZnPP for 1hr at RT.
The indicated antibodies were then added and mixtures incubated at 4C for 1hr prior to electrophoresis on 0.8% agarose gels and UV light visualization. (ab = antibody). Arrow = mobility shift of anti-TERT antibodies.

E. Immunostaining of ZnPP labelled complexes blotted to nitrocellulose. Lysates from Huh7 cells were incubated for 1hr at RT with 10 uM ZnPP or vehicle control, then electrophoresed on agarose gels and diffusion-blotted onto nitrocellulose (NC). ZnPP complexes were visualized in gels by UV and on NC blots by fluorescence. NC blots were then stained with specific anti-TERT or anti-dyskerin antibodies.

**Figure 5**

ZnPP inhibition and labelling of Immunoprecipitated TERT complexes. A. ZnPP binds TERT IP complexes. Left upper panel: Immunoprecipitation was performed on lysates from cells transfected with TERT and TERC plasmid constructs as described in the Methods. The IP were then re-suspended and incubated with ZnPP (10 uM) for 1hr at RT prior to electrophoresis on 0.8% agarose gels and visualized under UV light. Right upper panel: Identical aliquots were also denatured for SDS-PAGE and evaluated on WB. B. ZnPP inhibition of telomerase activity in cellular lysates and IP. TRAP assays were performed with IP or crude cellular lysates in the presence of various concentrations of ZnPP. No statistical difference of ZnPP IC50 of telomerase from either source. (Table 1). C. Nuclease digestion of ZnPP labelled complexes. Upper
Enzymatic extracts or IP (10 ug protein) were treated with DNase 1 or RNase A (0.1 mg/ml at 4°C for 1hr), then labelled with ZnPP (10 uM) for 1hr at RT, before electrophoresis on 0.8% agarose gels and visualization of ZnPP by fluorescence emission. Lower panel: Following electrophoresis, native proteins were transferred to NC by diffusion blotting and stained with anti-TERT antibodies. For whole cell lysates, GAPDH was used as a loading control after lysates were electrophoresed on SDS-denaturing polyacrylamide gels and stained using WB methods. (LB = lysis buffer); (IP= immunoprecipitate); (N = Native).

Figure 6

Fluorescence co-localization of MPPs with telomeres and TERT
A. Co-localization of ZnPP and telomeres. Synchronized S phase Huh7 cells (left panels) and metaphase chromosomal spreads (right panels) were prepared as described in Methods. Fixed preparations were reacted with the specific fluorescent telomere probe TelC-Alexa488 F1004 and 10 uM ZnPP. Confocal microscopy used Alexa Fluor 488 (green) or Alexa Fluor 568 (red) to visualize telomeres or ZnPP respectively and merged sites (yellow). Yellow arrows, (left panels) show co-localization of telomeres and ZnPP. White arrows, (right panels) show localization of telomeres which do not co-localize with ZnPP.

B. Co-localization of ZnPP and TERT. Synchronized S
phase (left panels) and unsynchronized log phase (right panels) Huh7 cells were incubated with 10 μM ZnPP after labelling with anti-TERT antibodies. Confocal microscopy was then conducted using Alexa Fluor 488 (green, anti-TERT) or Alexa Fluor 568 (red, ZnPP) fluorochromes to visualize TERT or ZnPP respectively and merged sites, (yellow). Arrows depict example sites of co-localization of ZnPP and TERT in the nuclei of S phase cells (left panels) or cytoplasm/perinuclear regions of unsynchronized semi-confluent cells (right panels).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.docx
- ZnPPtelomeraseSupplementalfiguresnearfinal.pptm
- ZnPPtelomeraseSupplementarynearfinalMS.docx