Discovery of the Antitumor Effects of a Porphyrazine Diol (Pz 285) in MDA-MB-231 Breast Tumor Xenograft Models in Mice

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S Supporting Information

ABSTRACT: A series of porphyrazines (Pzs) with chiral bis-acetal moieties in the β -pyrrole positions ((2*R*,3*R*)-2,3-dimethyl-2,3-dimethoxy-1,4-diox-2-ene) have been synthesized and screened as antitumor agents in MDA-MB-231 breast tumor cells *in vitro*. The lead **Pz 285** was further tested in a mouse tumor xenograft model with Td-tomato-luc2 fluorescent breast tumor cells (MDA-MB-231 LM24 Her2+) that are highly metastatic to the lungs. **Pz 285** shows marked antitumor effects *in vivo*, with treated mice exhibiting longer median survival that we attribute to smaller primary tumor regrowth after resection and less occurrence of metastasis when compared to vehicle control groups. **Pz 285** is further compared to the clinically approved chemotherapeutic doxorubicin (Dox). This report lays the groundwork for development of an understudied class of compounds for classical chemotherapy.

KEYWORDS: Porphyrazine, antitumor, MDA-MB-231 LM24 Her2+

Porphyrazines (Pzs) are tetrapyrrolic macrocycles made by a synthetic pathway that allows for heteroatom substitution (S, N, O) in the β -pyrrole positions.^{1,2} Such direct functionalization of the pyrrole ring confers distinct optical properties, prompting the study of these molecules as optical agents in biological systems. The related porphyrins, phthalocyanines, and chlorins have long been studied as photodynamic therapy (PDT) agents for their light activated production of toxic singlet oxygen,³ and several platforms are in various stages of clinical approval for treatment of cancers of the head, neck, and lungs.^{4,5} Heteroatom functionalized Pzs have been recently examined as PDT agents,^{6–10} with some N atom functionalized macrocycles exhibiting remarkably high singlet oxygen quantum yields ($\Phi_{\Delta} > 50\%$).¹¹ The O atom functionalized Pzs with bis-acetal moieties at their periphery are the youngest subgroup, discovered less than two decades ago.¹² These macrocycles are enantiomerically pure as a synthetic convenience; starting materials are derived from homochiral L-(+)-dimethyltartrate.¹³ It is unclear whether the chirality of these molecules plays a role in their biocompatibility or biodistribution, but members of this subclass of Pzs have seen success against a variety of tumor targets.

In the first example, a series of mixed benzo porphyrazines of the form MPz(A₄–B_n), where the peripheral “A” group is (2*R*,3*R*)-2,3-dimethyl-2,3-dimethoxy-1,4-diox-2-ene and “B” is β,β' -di-isopropoxybenzo, were screened as potential photo-

dynamic therapeutics in A549 lung carcinoma cells *in vitro*.¹⁴ It was noted that the symmetric H₂Pz(A₄), with four bis-acetal moieties, exhibited classical chemotherapeutic efficacy *in vitro*; Pz treatment had a drastic effect on cell viability of lung tumor cells (A549) but had no effect on the nontumorigenic reference cell line (WI38-VA13). The A₄ derivatives will be discussed in more detail below. The most efficacious member of the series for PDT, H₂Pz(*trans*-A₂B₂) henceforth denoted **Pz 247**, not only displayed marked phototoxicity, but intense intracellular fluorescence that could be used as an *in vitro* tool by confocal fluorescence microscopy, allowing for in depth study of uptake mechanism.¹⁵ **Pz 247** enters cells by low-density lipoprotein receptor (LDL-*r*) mediated endocytosis, a known tumor specific uptake mechanism,^{16,17} and exhibits preferential tumor accumulation in tumor xenografts *in vivo*. Poor solubility and synthetic yields of the structurally complex **Pz 247** ultimately precluded further preclinical advancement; derivatization to address these drawbacks is discussed herein.

A Pz platform was sought after that would combine the structural features of the promising compounds mentioned above, while possessing versatility for a wider variety of applications (Chart 1). In an effort to take advantage of the

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Chart 1. Bis-Acetal Porphyrazines of Interest

tumor specific accumulation of **Pz 247** and toxic preference of **H₂Pz(A₄)**, a structurally similar **H₂Pz(A₃B)** was developed, where “B” is β,β' -2-hydroxyethoxybenzo. Pendant alcohols were added to improve solubility, with the added benefit of providing functionality for conjugation to bioactive compounds that could be subsequently translocated into tumor cells.¹⁸ Contrary to previous strategies for tetrapyrrole development, this macrocycle, denoted **Pz 285** (Chart 1), was chosen for its poor singlet oxygen quantum yield ($\Phi_{\Delta} < 1\%$) so that phototoxicity from the vehicle would not interfere with transport. Much like **Pz 247**, **Pz 285** was found to enter cells by serum protein mediated endocytosis in A549 lung adenocarcinoma cells.

Conjugation of tetrapyrroles,¹⁹ including S atom functionalized Pzs,²⁰ with clinically approved Gd³⁺ MRI contrast agents has a balancing effect on amphiphilicity, whereby the hydrophobic macrocycle promotes cellular uptake of the hydrophilic Gd³⁺ chelate that would otherwise be restricted to the vascular space. This effect was observed for conjugates of **Pz 285** with a Gd³⁺ chelate *in vivo*,²¹ where uptake was more persistent and MRI contrast enhancement more pronounced for the conjugate over the Gd³⁺ chelate alone.

Concurrent studies examined conjugation to the clinically approved chemotherapeutic doxorubicin,¹⁸ with the expectation that doing so would reduce harmful side effects such as cardiotoxicity.²² The curious observation that forms the basis of this work is that the unconjugated **Pz 285** alone showed greater effects on cell viability *in vitro*, relative to the conjugates, despite the absence of singlet oxygen generation. Herein we report the side-by-side comparison of promising bis-acetal Pzs *in vitro* and the advancement of the lead **Pz 285** to tests of antitumor activity in a MDA-MB-231 tumor xenograft models in mice.

A group of four structurally similar Pzs were chosen for initial testing. Linstead cyclization of an excess of (2R,3R)-5,6-dimethyl-1,4-diox-2-ene-2,3-dinitrile (**1**) with 4,7-bis(2-tetrahydropyranyloxyethoxy)-1,3-diimino-iso-indoline (**2**), followed by treatment with acetic acid to remove Mg²⁺ and tetrahydropyran (THP) protecting groups, produces a mixture of **H₂Pz(A₄)** and **Pz 285** that can be isolated by chromatographic purification (Scheme 1). Of course, cyclization of (**1**) alone produces the **A₄** derivatives in higher yield.

Subsequent treatment of metal-free Pzs with zinc(II) acetate produces **ZnPz(A₄)** and **ZnPz(A₃B)** in near quantitative yield. The addition of Zn(II) to the core of the macrocycle was chosen for its changes to the shape of the molecule that can produce subtle changes to amphiphilicity and biodistribution; Zn(II) binds a solvent molecule on axis and sits 0.38 Å above the macrocycle plane,²³ giving the Pz a slight cone shape, whereas the metal-free Pz is essentially planar.²⁴ A graphical representation of this effect from the literature is demonstrated in Figure S2. The Zn(II) Pz derivatives are more polar, thereby affecting their biocompatibility in tumor cells. Screening of Zn(II) derivatives against the metal-free counterparts can

Scheme 1. Metal-Templated Synthesis of Bis-acetal Pzs^a

^aReagents and conditions: i) Mg(OPr)₂, 95 °C, 18 h; ii) AcOH, RT, 24 h; iii) Zn(OAc)₂, RT, 24 h.

provide mechanistic insight into whether the macrocycle might be embedded in a hydrophobic protein of membrane for efficacy. Zn(II) also increases the production of singlet oxygen and phototoxicity; however, these particular Pzs exhibit low Φ_{Δ} , with and without Zn(II). Mass spectrometry (ESI-HRMS) and NMR (¹H, ¹³C) is tabulated for the previously unreported **ZnPz(A₃B)** (see Supporting Information).

Although assays of cell viability have been reported for some of these compounds, the series reported here have not been screened at the same dosages against the same cell lines. The effects on cell viability were tested in MDA-MB-231 breast tumor cells. Singlet oxygen production is low for the series, but care was taken to perform MTS assays of cell viability in the absence of light to minimize phototoxicity. Initial screening showed that **Pz 285** reduced viability more effectively than the other members of the series. Upon optimization, it was found that cells treated for 48 h at a **Pz 285** dose of 25 μ M were ca. 40% viable versus untreated control, whereas the other three Pzs of the series showed no effect on viability under the same conditions (Figure 1). It should be noted that the analogous

Figure 1. MTS assay of *in vitro* cell viability.

H₂Pz(A₃B) tested in WI38-VA13 nontumor cells showed no effect on cell viability over 72 h treatment at a much higher dosage (50 μ M).¹⁴ Therefore, one can reasonably assume that similar differential toxicity to tumor over nontumor cells may be observed *in vivo*. The **Pz 285** was therefore the sole member of the series advanced to study *in vivo* in a metastatic breast tumor model in mice using MDA-MB-231 LM24 Her2+ tumor xenografts. In these particular breast tumor cells, an IC₅₀ of 15.0 $\pm$ 0.1 μ M was observed for **Pz 285** *in vitro* (Figure S3).

The MDA-MB-231 LM24 Her2+ breast tumor xenograft model is highly metastatic to the lungs, and tumor cells stably express a dual reporter, Td-tomato and Luc2, that allows for fluorescent and bioluminescent imaging. Tumor cells were

subcutaneously (SC) implanted in the mammary fat pad (MFP) of female SCID-beige mice, and the model was executed to mimic the clinical standard of care treatment for metastatic breast cancer. The primary tumor was staged to 300–500 mm³, then surgically resected; test article treatment was initiated after tumor resection when metastases were observed in the lungs by bioluminescent (lung radiance) imaging. Mice were randomized into four cohorts ($n = 10$) after detection of lung metastases by imaging. **Pz 285** was the primary test article and was compared to the FDA approved metastatic breast cancer chemotherapeutic Dox, as well as a negative vehicle control. The vehicle used was 8% dimethyl sulfoxide (DMSO) in saline to ensure solubility of all test articles. A **Pz** dose of 2.5 mg/kg was chosen alongside two doses of Dox; a maximum tolerated dose (MTD) dose (2.5 mg/kg) and an equimolar dose (1.25 mg/kg), since Dox has a molecular weight that is roughly half that of **Pz 285**. Doses were based on clinical doses²⁵ and converted to the equivalent dose in mice according to Freireich et al.,²⁶ and treatments were administered intravenously every 7 days. Drug activity was assessed using overall survival, time to primary tumor regrowth in the MFP, and the progression of metastases.

Mice were euthanized when moribund or if excessive weight loss (ca. 10–15% relative to the first day of treatment) was observed. Survival data is presented in Figure 2. The lowest

Figure 2. Kaplan–Meier survival curve.

median survival was observed for the group treated with vehicle alone (9.5 d), followed by Dox 2.5 mg/kg (14 d) and Dox 1.25 mg/kg (20 d). The group treated with **Pz 285** had the longest median survival of 22.5 d. The apparent reverse dose dependence for Dox could be attributed to toxicity at the higher dose causing animals to be euthanized earlier. The **Pz 285**-treated group showed a significant therapeutic benefit [log-rank (Mantel-Cox) test $p = 0.0005$] in overall survival, when compared to the vehicle control, which was comparable to Dox (Figure 2). In addition to survival, tumor regrowth at the site of the initial inoculation and the extent of lung metastasis measured by bioluminescence (lung radiance) were assessed as objective measures of antitumor activity and support the survival benefit observed in the **Pz 285** treated cohort.

The primary tumors in the MFP regrow after resection as it is impossible to completely remove these tumors (Figure 3). Dox treatment at both doses led to a reduction in primary tumor regrowth relative to vehicle control, with the extent of regrowth between the two groups corresponding to mean

Figure 3. Primary tumor regrowth.

survival (Figure 3). **Pz 285** had the largest effect on primary tumor regrowth leading to a significant >65% inhibition of regrowth compared to vehicle control (one-way ANOVA, $p = 0.0002$).

Finally, the progression of lung metastases was monitored by bioluminescence (lung radiance) imaging *in vivo*. The MDA-MB-231 LM24 Her2+ tumor cells express Luc2, allowing for detection of luminescence after luciferin is delivered systemically to the mice as previously described.²⁷ The extent of metastases to the lungs is proportional to the overall radiance of bioluminescence observed, and the extent of metastasis correlated with median survival for all four groups (Figure 4).

Figure 4. Lung metastases as monitored by fluorescence.

Mice treated with the higher dose of Dox and vehicle control showed the largest metastatic tumor burden after 2 weeks; ca. 2-fold higher luminescence signal than the Dox 1.25 mg/kg treated group. The least metastasis was observed for the group treated with **Pz 285**, exhibiting an average bioluminescence signal that was an order of magnitude less than vehicle control treated mice (one-way ANOVA $p < 0.001$).

Bis-acetal functionalized Pzs have growing importance in cancer diagnosis and treatment, from fluorescence imaging and MRI, to PDT and, reported here, chemotherapy. We have shown that small changes to the peripheral structure of the macrocycle can elicit different response in breast tumor cells *in vitro* and that the lead member of the series, **Pz 285**, has excellent potential as a chemotherapeutic anticancer agent. The *in vivo* activity of **Pz 285** improves survival, inhibits primary

tumor regrowth, and inhibits lung tumor metastasis compared to vehicle control animals; **Pz 285** has similar antitumor activity to the clinically active agent Dox. Synthesis of a library of analogous Pzs is underway, and their antitumor activity will be reported forthwith, with lead compounds, including **Pz 285**, being advanced into additional preclinical evaluation.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acsmmedchem-lett.7b00063](https://doi.org/10.1021/acsmmedchem-lett.7b00063).

Experimental details, synthetic characterization, and additional *in vitro* data (PDF)

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Author Contributions

The manuscript was written through contributions of all authors and all authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Pz, porphyrazine; LDL-*r*, low-density lipoprotein receptor; Dox, doxorubicin

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