Published in final edited form as:

Chembiochem. 2010 November 22; 11(17): 2374–2377. doi:10.1002/cbic.201000477.

Bioorthogonal Small Molecule Ligands for PARP1 Imaging in Living Cells

Dr. Thomas Reiner, Dr. Sarah Earley, Anna Turetsky, and Prof. Ralph Weissleder*

Center for Systems Biology Massachusetts General Hospital Richard B. Simches Research Center, 185 Cambridge Street, Suite 5.210, Boston, MA 02114

Keywords

PARP-1; cycloaddition; tetrazines; imaging agents; bioorthogonal reaction

Poly(ADP-ribose) polymerase 1 (PARP1) is an important cellular protein that senses DNA damage and initiates the base excision repair pathway.[1] DNA damage (strand breaks) occurs during each cell cycle and must be repaired for a cell to survive. In the absence of functional BRCA (another class of complementary DNA repair enzymes), cells depend primarily on the PARP repair mechanism. Thus, PARP inhibitors (PARPi) are emerging as a useful option for cancer therapy, either as single agents or in combination with other DNA-damaging molecules.[2,3,4] In view of their encouraging results in breast cancer trials, there is now considerable interest in expanding PARPi to other primary tumors. However, despite the increasing body of literature on PARP, many questions remain unanswered not only regarding its basic biology, regulation, heterogeneity and role in individual tumor types, but also regarding the efficacy of new PARPi, optimum dosing, timing and combination of treatment, among other factors.[2,5] For example, PARP1 has a number of additional roles, [2] including the restarting of stalled replication forks, the inhibition of nonhomologous end-joining repair, the regulation of transcription, the initiation of a unique cell death pathway, and the modulation of cellular bioenergetics.[2] Thus, to gain more insight into these additional roles and to better understand PARP1 regulation *in vivo*, it would be an enormous advantage to be able to image PARP1 in live cells and ultimately in whole living organisms. Whilst green fluorescent protein (GFP) fusion proteins remain a valuable tool for imaging at the cellular level, labeled affinity ligands, which are cell permeable, will ultimately be required for whole body preclinical and clinical imaging.

To date, a variety of small molecule PARPi have been developed. These include the lead group 1(2H)-phthalazinones, such as AZD2281 (**1**) and its derivatives.[2,5,6] It has been shown that the 4-NH-piperazine of AZD2281 tolerates a diverse range of capping groups without significantly decreasing PARP1 binding affinity.[6] We therefore used this anchor point to attach bioorthogonal reactive groups and/or different fluorophores to the phthalazinone. We hypothesized that these modifications would still result in low nanomolar affinity ligands and that conjugates would retain their cell permeability, particularly with the use of bioorthogonal linkers.

As a precursor for the synthesized AZD2281-inhibitors (Scheme 1), 4-[[4-Fluoro-3-(piperazine-1-carbonyl) phenyl]methyl]-2H-phthalazin-1-one (**2**) was obtained according to known literature procedures.[6] For the synthesis of the bioorthogonally reactive AZD2281 derivatives AZD2281-NOB (**6**) and AZD2281-TCO (**7**), compound **2** was first reacted with

*Fax: (+1) 617-643-6133 rweissleder@mgh.harvard.edu.

glutaric acid anhydride to produce the glutaric acid-modified 4-(5-oxopentanamide)piperazine (**3**) at 72% yield. Subsequently, an ethylene diamine spacer was attached to precursor **3**, yielding the amine-functionalized AZD2281-derivative (**4**). Norbornene-functionalized AZD2281-NOB (**6**) was obtained by amide bond formation with 5-norbornene-2-carboxylic acid in the presence of polymer-supported dicyclohexylcarbodiimide (DCC)-beads. In the case of AZD2281-trans-cyclooctene (TCO; **7**), precursor **3** was reacted with (*E*)-cyclooct-4-enyl 2,5-dioxopyrrolidin-1-yl carbonate (**5**) in the presence of triethylamine.[7] The identity of all 1(2H)-phthalazinone-based bioorthogonal probes and their precursors was confirmed using HPLC-LC/MS, high resolution MS and NMR spectroscopy.

The fast reaction kinetics of trans-cyclooctenes (TCO) and tetrazines (Tz)[7-10] make AZD2281-TCO (**7**) a potential candidate for live-cell imaging using fluorophore-tetrazine derivatives. Cycloaddition of both AZD2281-TCO (**7**) and Texas Red-Tz (**8**) was detected by mixing the two compounds (0.3 mM), agitating for several minutes, and analyzing the products by HPLC/MS (figure 1a shows the HPLC trace for the AZD2281-Texas Red (**9**), crude reaction mixture). LC/MS spectra confirmed the quantitative conversion of Texas Red-Tz (**8**). Multiple peaks were identified with a molecular mass corresponding to AZD2281-Texas Red (**9**;figure 1b, $m/z = 1536.0 [M + H^+]^+$). These were the result of different isomers formed in the tetrazine trans-cyclooctene cycloaddition.[7,9,10] The fast and selective conversion of AZD2281-TCO (**7**) to AZD2281-Texas Red (**9**) in the presence of Texas Red-Tz (**8**) indicates that these small molecules also have potential applicability to *in vivo* experiments. The inhibitory potential of AZD2281-derivatives (**6** and **7**) and of pre-reacted AZD2281-Texas Red (**9**) were tested using a PARP1 activity assay (see supplementary materials for details). Analysis of the AZD2281-derivatives **6** and **7** resulted in IC_{50} values of 10.1 ± 1.3 nM and 11.8 ± 1.4 nM, respectively. Thus, modification and conjugation of linkers and fluorophores to the 4-NH-piperazine group of AZD2281-precursor **2** appear to be tolerated by the enzyme, and allow the design of bifunctional derivatives. The IC_{50} value of pre-reacted AZD2281-Texas Red (**9**; 15.4 ± 1.2 nM) demonstrates that the trans-cyclooctene/tetrazine cycloaddition only minimally reduces binding of the 1(2H)-phthalazinone to PARP1, which confirms its possible application as an imaging probe.

Due to their fast reaction kinetics compared to AZD2281-NOB (**6**),[7] the trans-cyclooctene conjugated AZD2281 (AZD2281-TCO; **7**) and Texas Red-Tz (**8**) were tested under *in vivo* conditions in live cells. MDA-MB436 cells, a well characterized BRCA1-mutant breast cancer cell line, were incubated with Texas Red-Tz (**8**; 1 μ M in growth medium, 0.1% DMSO) for 20 minutes, before the medium containing Texas Red-Tz (**8**) was removed. AZD2281-TCO (**7**) was then added (3 μ M in growth medium, 0.1% DMSO) and the mixture was incubated for another 20 minutes. Texas Red-Tz (**8**) cleared out of the cells very quickly, allowing the identification of AZD2281-Texas Red (**9**), which stayed with its target for long periods of time. After washing, fixing and permeabilizing the MDA-MB436 cells, PARP1 was visualized using monoclonal antibodies (Mabs; figure 3b). Staining patterns for the Texas Red dye (figure 3a) showed that it not only localized to the nucleus but also accumulated in the nucleolus. Whilst anti-PARP1 Mabs showed similar nuclear localization (figure 3b), there was an absence of Mab-signal in the nucleoli. This was presumably due to steric hindrance and not to the absence of PARP in the nucleolus, as earlier reports have indicated.[11,12] The results in figure 3c confirm that there was excellent spatial correlation between the small molecules (AZD2281-TCO (**7**) / Texas Red-Tz (**8**)). We also constructed a PARP1-GFP fusion protein and expressed it in MDA-MB436 cells as an independent confirmation of probe localization. In the PARP1-GFP expressing cells, GFP expression was clearly observed, primarily in the nucleoli but also in the nucleus. This pattern was identical to that seen with the AZD2281-TCO/Texas Red-Tz pair.

Interestingly, incubation of live cells with Texas Red-Tz (**8**) without AZD2281-TCO (**7**) did not lead to nuclear localization of the dye. Instead, there was non-preferential distribution of fluorescence in the perinuclear cytoplasmic region as well as in the nucleus (ratio = 0.95:1). When incubated with different concentrations of the trans-cyclooctene probe (**7**; 3 μ M - 10 nM), the nuclear/cytoplasmic signal ratios gradually increased (figure 3d, not blocked). This concentration-dependent binding of AZD2281-TCO (**7**) to PARP1 can be inhibited with 30-fold excess of AZD2281 (**1**). This prevents nuclear localization and therefore binding of probe **7** to PARP1. With different concentrations of **7**, the nuclear/cytoplasmic signal ratio remains unchanged (figure 3d, blocked).

Like its two building blocks **7** and **8**, pre-reacted AZD2281-Texas Red (**9**) is able to penetrate the nuclear membranes in live cells, although incubation of MDA-MB436 with **9** leads to lower nuclear/cytoplasmic localization ratios (modest increase of 38 ± 2 %). The superior results of sequential treatment of MDA-MB436 cells with AZD2281-TCO (**7**) and Texas Red-Tz (**8**) demonstrates the advantages of a bioorthogonal *in vivo* reaction, since both partners (the targeting molecule **7** as well as the fluorophore **8**) are small in size (**7** and **8**; 674.76 g/mol and 889.05 g/mol respectively), and this contributes to easier permeation. Once assembled, however, penetration is less efficient leading to trapping of the conjugate and high target-to-background ratios.

In this report, we present potential small molecule-based PARP1 imaging agents and confirm their utility for cell imaging. We found that AZD2281-based bifunctional small molecules were most suited to bioorthogonal *in vivo* target assembly. All bioorthogonal and imaging probes had IC₅₀-values ranging between 10.1 nM and 15.4 nM. In particular, the TCO-modified derivative was not only successfully reacted using a bioorthogonal cycloaddition in cells but was found to co-localize with anti-PARP1 Mabs. In turn, this co-localization could be prevented by blocking the targeted fluorescent probe with AZD2281. The design strategy employed here, as well as the specific compounds investigated, should prove useful for the future study of PARP1 biology in live cells. It is also highly likely that similar strategies could be used for the design of PET imaging agents for whole body imaging of PARP1 and its inhibition by PARPi. Such agents would be particularly useful in drug development and in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Ralph Mazitschek, Neal Devaraj, Scott Hilderbrand and Claudio Vinegoni for many helpful discussions, Dr. Yvonna Fisher-Jeffes for manuscript review and Joshua Dunham for image processing. This research was supported in part by the NCI grants P50 CA86355 and NIBIB RO1 EB-010011. AT was supported by NIH grant NIGMS T32 GM008313. TR was supported by a grant from the Deutsche Akademie der Naturforscher Leopoldina.

References

1. El-Khamisy SF, Masutani M, Suzuki H, Caldecott KW. Nucleic Acids Res. 2003; 31:5526–5533. [PubMed: 14500814]
2. Rouleau M, Patel A, Hendzel MJ, Kaufmann SH, Poirier GG. Nature Rev. Cancer. 2010; 10:293–301. [PubMed: 20200537]
3. Ratnam K, Low JA. Clin. Cancer Res. 2007; 13:1383–1388. [PubMed: 17332279]
4. Comen EA, Robson M. Oncology (Williston Park). 2010; 24:55–62. [PubMed: 20187322]
5. Ferraris DV. J. Med. Chem. 2010; 53:4561–4584. [PubMed: 20364863]

6. Menear KA, Adcock C, Boulter R, Cockcroft X-I, Copsey L, Cranston A, Dillon KJ, Drzewiecki J, Garman S, Gomez S, Javaid H, Kerrigan F, Knights C, Lau A, Loh VM Jr, Matthews ITW, Moore S, O'Connor MJ, Smith GCM, Martin NMB. *J. Med. Chem.* 2008; 51:6581–6591. [PubMed: 18800822]
7. Devaraj NK, Upadhyay R, Haun JB, Hilderbrand SA, Weissleder R. *Angew. Chem.* 2009; 121:7147–7150. *Angew. Chem. Int. Ed.* 2009; 48:7013–7016.
8. Devaraj NK, Weissleder R, Hilderbrand SA. *Bioconj. Chem.* 2008; 19:2297–2299.
9. Royzen M, Yap GPA, Fox JM. *J. Am. Chem. Soc.* 2008; 130:3760–3761. [PubMed: 18321114]
10. Devaraj NK, Hilderbrand S, Upadhyay R, Mazitschek R, Weissleder R. *Angew. Chem.* 2010; 122:2931–2934. *Angew. Chem. Int. Ed.* 2010; 49:2869–2872.
11. Meder VS, Boeglin M, de Murcia G, Schreiber V. *J. Cell Sci.* 118:211–222. [PubMed: 15615785]
12. Haince J-F, McDonald D, Rodrigue A, Déry U, Masson J-Y, Hendzel MJ, Poirier GG. *J. Biol. Chem.* 2008; 283:1197–1208. [PubMed: 18025084]

Scheme 1.

A synthetic scheme for the synthesis of bifunctional 1(2H)-phthalazinone-based targeted probes. a) polystyrene-bound DCC (dicyclohexylcarbodiimide), Et₃N, DCM (dichloromethane), r.t. over night; b) Et₃N, DCM, r.t. over night; For detailed synthetic descriptions, see supplementary information.

Figure 1.

(a) An HPLC trace of AZD2281-TCO (7); Texas Red-tetrazine (8; one isolated stereoisomer shown); and AZD2281-Texas Red (9; crude reaction mixture); (b) LC/MS spectrum of AD2281-Texas Red (9; LC/MS of major product peak shown).

Figure 2.
 IC_{50} curves for the bifunctional 1(2H)-phthalazinone-based targeted probes.

Figure 3.

The reaction of AZD2281-TCO (7) and Texas Red-Tz (8) in MDA-MB436 cells. (a) AZD2281-TCO reacted with Texas Red-Tz; speckles outside the cells, presumably resulting from precipitated Texas Red-Tz (8), were removed; (b) anti-PARP1 monoclonal antibody staining; (c) a composite overlay on phase contrast (d) the ratio of nuclear/cytoplasmic signal for AZD2281-TCO/Texas Red-Tz in the absence (blue) and presence (green) of blocking reagent AZD2281. Scale Bar: 20 μ m.

Table 1

IC₅₀ values for the bifunctional 1(2H)-phthalazinone-based targeted probes and for AZD2281 -Texas Red.

Compound	Name	#	MW [g/mol]	IC ₅₀ [nM]	Reactive with
Reactive inhibitor	AZD2281-NOB	6	642.7	10.1 ± 1.3	Tz-fluorochromes
	AZD2281-TCO	7	674.8	11.8 ± 1.4	Tz-fluorochromes
Fluorescent inhibitor	AZD2281- TexasRed	9	1535.6	15.4 ± 1.2	NA