

Activity-Based Probes

Isotopologous Organotellurium Probes Reveal Dynamic Hypoxia In Vivo with Cellular Resolution

Landon J. Edgar, Ravi N. Vellanki, Trevor D. McKee, David Hedley, Bradly G. Wouters, and Mark Nitz*

Abstract: Changes in the oxygenation state of microenvironments within solid tumors are associated with the development of aggressive cancer phenotypes. Factors that influence cellular hypoxia have been characterized; however, methods for measuring the dynamics of oxygenation at a cellular level in vivo have been elusive. We report a series of telluriumcontaining isotopologous probes for cellular hypoxia compatible with mass cytometry (MC)-technology that allows for highly parametric interrogation of single cells based on atomic mass spectrometry. Sequential labeling with the isotopologous probes (SLIP) in pancreatic tumor xenograft models revealed changes in cellular oxygenation over time which correlated with the distance from vasculature, the proliferation of cell populations, and proximity to necrosis. SLIP allows for capture of spatial and temporal dynamics in vivo using enzyme activated probes.



Figure 1. Design and synthesis of a probe for monitoring hypoxia dynamics. a) Schematic of the SLIP approach to measuring dynamic cellular hypoxia in vivo. Murine tumor models are injected with temporally spaced doses of isotopologues of Telox 2 followed by sacrifice, staining of cells with metal-chelating polymer–antibody conjugates and analysis of tumor tissue via MC. The difference in the quantity of tellurium isotopes reports on the oxygenation state of a cell at a given time point. In the plot on the right, a low ¹²⁵Te signal would be expected compared to ¹²²Te due to the lower pO₂ during exposure to ¹²²Telox 2. b) Reaction conditions: i) *N*-bromosuccinimide (1.15 equiv), AgNO₃ (1.0 equiv), acetone, RT, 3 hours, 85%. ii) 3-Butyn-1-ol (1.2 equiv), CuCl (2 mol%), 30% BuNH₂(aq.), 0°C \rightarrow RT, 0.5 hours, 80%. iii) Tetrabutylammonium fluoride (TBAF) (4.0 equiv), THF, 0°C, 0.5 hours, not isolated. iv) Te⁰, sodium hydroxymethylsulfinate (rongalite) (11.0 equiv), NaOH(aq.), 70°C, 4 hours, 90%. v) PPh₃ (1.05 equiv), DIAD (1.05 equiv), azomycin (1.05 equiv), THF, 0°C \rightarrow RT, 3 hours, 90%. c) Mechanism of enzyme-mediated reduction of the 2-NI functionality to produce the electrophilic 2-nitrenium ion leading to non-specific protein labeling.^[7c-e]

Regions with limited access to oxygen are common in solid tumors from many cancers.^[1] Poor patient prognosis and an increased risk of metastasis have both been correlated with the degree of tumor hypoxia.^[2] Cells exposed to repeated cycles of hypoxia and reoxygenation have a long-term

[*] Dr. L. J. Edgar, Prof. M. Nitz Department of Chemistry, University of Toronto 80 St. George Street, Toronto, Ontario, M5S 3H6 (Canada) E-mail: mnitz@chem.utoronto.ca Dr. R. N. Vellanki, Dr. T. D. McKee, Dr. D. Hedley, Prof. B. G. Wouters Departments of Radiation Oncology, Medical Biophysics, and the STTARR Innovaton Centre, Princess Margaret Cancer Centre, University Health Network 101 College Street, Toronto, Ontario, M5G 1L7 (Canada)
Supporting information for this article can be found under:

Supporting information for this article can be found under: http://dx.doi.org/10.1002/anie.201607483. survival advantage as compared with chronically hypoxic cells.^[3] Cyclic hypoxia may be particularly important in its ability to activate pathways that promote metastasis, genomic instability, and resistance to treatment, but an understanding of temporal changes in hypoxia at the cellular level is largely lacking.^[4] The ideal method for monitoring oxygenation dynamics would allow for highly parametric spatiotemporal analysis with cellular resolution. To achieve this, we turned to mass cytometry (MC); a technology that employs bioorthogonal heavy isotopes to report on a large (>40) number of biological parameters simultaneously.^[5] MC is compatible with activity-based profiling of single cells using organotellurium mass tags, and we reasoned that this approach could be translated to spatiotemporal investigation of hypoxia using recently developed imaging MC (IMC).^[6,7]

We envisioned an approach where isotopically coded tellurium-containing probes, which form covalent conjugates in hypoxic cells, could be serially injected into a tumor xenograft-bearing mouse. Changes in cellular hypoxia would

Angew. Chem. Int. Ed. 2016, 55, 1-6

© 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Wiley Online Library



Communications





Figure 2. Evaluation of Telox 2 as a probe for cellular hypoxia in vitro and in vivo. a) PANC-1 cells in differently oxygenated atmospheres (3 h) were stained with metal-containing nucleic acid intercalators (103 Rh and/or $^{193/191}$ Ir), mixed, and analyzed via MC. Cartoons of expected MC readouts are presented at right. b) Event length vs. 130 Te signal. c) Intercalator signals from low, d) intermediate, and e) high 130 Te gates. f) PANC-1 tumor xenograft-bearing SCID mice were co-injected with Telox 2 and Pimo (60 mg kg⁻¹ each). After 3 h exposure, sacrifice, and digestion of tumor tissue, single cells were sorted via FACS and then analyzed by MC for 130 Te. Notes: All MC signals are reported as median values (arbitrary units). % of cells within drawn gates are indicated for (c)–(e).

be quantified by comparing tellurium isotope labeling by MC (Figure 1 a). This strategy of serial labeling with isotopologous probes (SLIP) has the unique advantage of removing complications that arise from using structurally different probes to report on the time-dependence of a biological phenomenon. Specifically, these isotopologous probes would have no significant differences in pharmacokinetics or metabolism that could confound interpretation of results, unlike other approaches to this type of strategy.^[8]

Our previous MC-compatible hypoxia probe exhibited favorable activity in vitro, but failed to reliably identify hypoxic cells in vivo likely due to the stability of the telluroether.^[7a] Chemical optimization of the organotellurium mass tag revealed that a more stable tellurophene would likely yield a better performing probe.^[9] Indeed, combining a 2-nitroimidazole (2-NI) with a tellurophene produced a probe (Telox 2) with excellent solution stability and low toxicity both in vitro and in vivo (Figure 1 b; Supporting Information, Figures S1, S2, and S6). We reasoned that this probe would label hypoxic cells similarly to other 2-NIbearing probes, where bioreductive metabolism converts the 2-NI to a 2-nitrenium imidazole which proceeds to nonspecifically label nucleophile-bearing proteins (Figure 1 c). $^{[7b-e]}$

The performance of Telox 2 was investigated in vitro using the human pancreatic cancer cell line PANC-1, which is known to form xenografts with a high hypoxic fraction.^[10] Cells were incubated with Telox 2 under either near-anoxia $(< 0.02\% O_2)$, hypoxia $(1\% O_2)$, or normoxia $(21\% O_2)$ (Figure 2a). The cells were then washed, fixed, and coded by staining with one of three combinations of heavy isotopecontaining nucleic acid intercalators (¹⁰³Rh and/or ¹⁹³Ir). The intercalator code allowed validation of the ¹³⁰Te signals from individual cells following mixing and analysis by MC (CyTOF2, Fluidigm). A density map of ¹³⁰Te vs. event length revealed three populations of cells (Figure 2b). ¹⁰³Rh vs. ¹⁹³Ir output from each of these three populations retrieved the original nucleic acid staining combination expected for each population demonstrating that Telox 2 was capable of labeling cells in an oxygen concentration-dependent manner (Figure 2c-e). Labeling with Telox 2 was found to be dosedependent, linear with time, and was observed over a wide range of O_2 concentrations suggesting that Telox 2 may be used as a probe for measuring hypoxia on a gradient of O_2 (Supporting Information, Figures S3 and S4). Additionally,

www.angewandte.org

© 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

These are not the final page numbers!



Figure 3. Isotopically enriched Telox 2 and imaging mass cytometry report on diffusion-limited hypoxia in vivo. a) A mouse bearing a PANC-1 tumor xenograft was injected with ¹²⁵Te-enriched Telox 2 (60 mg kg⁻¹). After 3 h the mouse was sacrificed, and the tumor cryosectioned. Tissue sections were stained with MC-compatible reagents (i.e. anti-Ki-67 antibody (¹⁶⁸Er-labeled) and ^{191/193}Ir nucleic acid intercalator) and analyzed via IMC. b) Tissue from experiment in (a) stained with H&E (top). A separate adjacent section analyzed via IMC (large panel). Red = ¹²⁵Te (¹²⁵Telox 2), green = ¹⁶⁸Er (Ki-67), blue = ¹⁹³Ir (DNA intercalator). ¹⁶⁸Er (middle) and ¹²⁵Te (bottom) channels are in grayscale on left. c) Processed image of data from (b). Segmented cells are shaded on a thermal scale that represents the intensity of the ¹²⁵Te mass channel (thermal legend on the right, arbitrary units). d) ¹²⁵Te intensity as a function of distance from the blood vessel. The vertical axis represents the average ¹²⁵Te signal for each segmented cell within 20 µm-thick circular slices of increasing distance from the blood vessel. Error bars represent the standard deviation of ¹²⁵Te for all cells within the corresponding 20 µm thick circular slice. Note: Scale bars = 100 µm. The blood vessel is outlined with a dashed oval.

cellular Te accumulation was found to be potentiated by the over-expression of cytochrome P450 oxidoreductase (POR) in HCT116 cells, and reduced following POR knockout, consistent with the mechanism of action of the 2-NI activity-based group (Figure 1c; Supporting Information, Figure S5).^[11]

Next, we confirmed the ability of Telox 2 to label hypoxic cells in vivo using the previously validated hypoxia probe pimonidazole (Pimo, Hypoxyprobe) as a reference (Figure 2 f).^[12] PANC-1 tumor xenograft-bearing mice were injected with both Pimo and Telox 2. After three hours the mice were sacrificed and the tumors excised/digested into single cells. These cells were then stained with a FITC-tagged antibody against Pimo conjugates and sorted using fluorescence-based flow cytometry (FACS) into four fractions. We reasoned that cells enriched in Pimo should also be enriched in Telox 2 since both probes were designed to label hypoxic cells using the same 2-NI-dependent mechanism. Injection of each fraction obtained through FACS into a CyTOF2 revealed Pimo-dependent enrichment of ¹³⁰Te, suggesting that the two probes labeled similar populations of cells in vivo.

We investigated parameters that would influence the ability of Telox 2 to measure dynamic changes in tumor hypoxia using the SLIP approach (Figure 1a). In order to obtain meaningful data from this type of experiment, it was necessary to understand when a second isotopologue of Telox 2 could be administered without interference from the first. Thus, we measured the circulating $t_{1/2}$ of the probe in

murine plasma using LC/MS/ MS.^[13] This experiment indicated that a second isotopologue could be administered 12–16 hours (ca. $4t_{1/2}$) after the first, as the majority of the initial isotopologue would have cleared circulation by that time (Supporting Information, Figure S7). Additionally, we measured the invitro stability of Telox 2 conjugates via MC to determine how long the tellurium mass tag would remain detectable in cells after reoxygenation (Supporting Information, Figure S8). Even after 48 hours tellurium levels were well above the limit of detection indicating that conjugates should remain detectable at the end of a SLIP experiment.

In order to evaluate dynamic cellular oxygenation in the context of underlying tissue morphology, we investigated Telox 2 labeling using IMC.^[6] We synthesized isotopically enriched variants of Telox 2 containing either

a ¹²⁵Te or ¹²²Te nucleus (>92% and >91% enrichment, respectively). Since ¹²⁵Te does not share a mass with any other stable isotope and ¹²²Te only shares its mass with a low-abundance (4.6%) isotope of tin, there should be no isotope background in the tissue.

Initially, a PANC-1 tumor xenograft-bearing mouse model was injected with ¹²⁵Telox 2 and introduced to an oxygenreduced atmosphere $(7 \% O_2)$ for 3 hours in order to promote an increase in tumor hypoxia (Figure 3a).^[10] The tumor was then harvested, cryosectioned (5 µm), and stained with an antibody against the proliferation marker Ki-67 (labeled with ¹⁶⁸Er) and the ^{191/193}Ir nucleic acid intercalator (an IMCcompatible surrogate for DAPI nuclear stain). IMC identified an example of diffusion-limited hypoxia, where the most intense ¹²⁵Te signal was detected in viable cells most distant from an apparent blood vessel, adjacent to necrosis, and antilocalized with proliferating cells as identified by H&E stain from an adjacent tissue section and the Ki-67 antigen, respectively (Figure 3b). Using a cell segmentation algorithm, the approximate size and shape of individual cells were traced and a ¹²⁵Te signal intensity assigned to each individual cell (Figure 3c). This data processing revealed that the maximum ^{125}Te signal was observed at a distance of ca. 300 μm (beyond the average diffusion distance of O₂ in tissue) from the blood vessel, and upon transition into necrotic tissue the signal decreased, likely due to decreased enzymatic activity in this region.^[14]

We next investigated hypoxia dynamics using isotopologues of Telox 2. Mice with PANC-1 tumor xenografts were

www.angewandte.org



Communications



Figure 4. The SLIP strategy reports on dynamic hypoxia in pancreatic tumors. a) Schematic of a SLIP IMC experiment. Mice bearing PANC-1 tumor xenografts were injected with ¹²⁵Telox 2 + EF5 and left to breathe 21% O₂. After 24 h, ¹²²Telox 2 was injected and animals were exposed to either 7%, 95%, or 21% O₂. Sacrifice occurred 24 h later and tissues were processed as in Figure 3 a. b) H&E-stained section, c) fluorescence microscopy image (anti-EF5 antibody = pink, DAPI = blue), and d) raw IMC image of tissue from a reduced O₂-breathing (7%) model. Red = ¹²²Te (¹²²Telox 2), green = ¹²⁵Te (¹²⁵Telox 2), blue = ¹⁶⁸Er (Ki-67). Individual mass channels on right. e) Processed image of data in (d). Segmented cells are shaded on a thermal scale (right of panel i) representing the value of ¹²²Te⁻¹²⁵Te. f) Processed image from an increased O₂-breathing (95%) model, and g) constant normoxia (21%) model. h) Enhanced view of (g). Ki-67 + (¹⁶⁸Er) cells are in the grayscale panel. Proposed region of hypoxic cell turnover and direction of cell displacement indicated by the white box and arrow, respectively. i) Processed image from a 21% O₂-breathing OCIP-51 tumor model. j) Histograms of segmented cells from panels (e), (f), (g), and Figure S13. Notes: All probes dosed at 60 mg kg⁻¹. All scale bars = 400 µm. Blue channel in processed images is ¹⁹³Ir (nuclear stain). Gray masks mark necrotic zones.

injected with a mixture of ¹²⁵Telox 2 and EF5 (60 mg kg⁻¹ each) and left to breathe normal atmosphere to establish baseline levels of hypoxia (Figure 4a). After 24 hours the mice were injected with ¹²²Telox 2 and subsequently split into four treatment groups. The first group was transferred to a sealed chamber containing 7% O₂, the second to a sealed chamber containing 95% O₂, and the third was left in normal atmosphere (21% O₂). The fourth group was injected with the vasodilator hydralazine one hour post-injection with ¹²²Telox 2 (Supporting Information, Figure S9). After an additional 24 hours the tumors were harvested and cryosectioned. Three adjacent tissue sections were stained for each

tumor. The first was stained with H&E for brightfield imaging (Figure 4b). The second was prepared for fluorescence microscopy via staining with Hoechst nuclear stain and a Cy5.5-labeled anti-EF5 antibody—a well-validated probe against hypoxia. The third adjacent tissue section was stained with reagents compatible with detection via IMC including the ^{191/193}Ir-containing nucleic acid intercalator and the ¹⁶⁸Er-labeled anti-Ki-67 antibody. Fluorescence microscopy revealed regions of the tumor section that had been labeled with EF5. We used this information to guide our IMC experiments since this technology is currently lower-throughput than optical techniques making ablation of an entire

www.angewandte.org

© 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

These are not the final page numbers!

tumor section impractical (Figure 4c). As expected, a similar localization of EF5 (Cy5.5) and Telox 2 (125Te) in adjacent sections was observed; co-localization was not expected as adjacent tissue sections were being examined and the structurally distinct probes have different pharmacokinetics (Figure 4c,d). Following IMC analysis, single cells were digitally segmented and the difference between signals for the two tellurium isotopes $(^{122}\text{Te}-^{125}\text{Te})$ was reported for each cell that exhibited significant labeling with either isotope (Figure 4e). We propose that cells exhibiting a value of ¹²²Te– 125 Te > 0 became increasingly hypoxic following administration of the second isotopologue whereas those with a value < 0 became less hypoxic (reoxygenated). As expected, tumors from mice subjected to reduced O₂-breathing following injection of the second isotopologue exhibited a markedly increased number of cells with $^{122}\text{Te}-^{125}\text{Te} > 0$ as compared to mice subjected to increased O2-breathing, where a high proportion of cells exhibited negative ¹²²Te-¹²⁵Te values (Figure 4e,f,j). Samples from the group treated with hydralazine after dosing with the second isotopologue also exhibited exacerbated hypoxia, consistent with reduction of blood flow through tumor vasculature as a result of lowered blood pressure (Figure 4j; Supporting Information, Figure S16).^[15] In all cases, hypoxic regions were generally anti-correlated with cell proliferation as reported by the presence of Ki-67 (Figure 4d; Supporting Information, Figures S12, S21, S30, S39, and S48).

Having demonstrated that the SLIP approach could reveal controlled changes in oxygenation in vivo, we used these tools to measure hypoxia dynamics in a tumor model that was a closer analogue of a clinical situation. The fourth group of tumors, having not been subjected to artificial O2 manipulation, were analyzed for regions of increased and decreased hypoxia taking place over 48 hours. Indeed, regions were detected that presented with either higher ¹²⁵Te, ¹²²Te, or near-equal quantities of both isotopes suggesting reoxygenation, reduced oxygenation, or no change during the second isotopologue-exposure phase, respectively (Figure 4g). Of particular interest was our observation of a region bordering necrosis that appeared to experience a dramatic increase in the number of hypoxic cells (Figure 4h). We speculate that proliferating cells displace cells close to the necrotic region, forcing them into an O2-deficient microenvironment, thereby inducing hypoxia as reported by the ¹²²Telox 2 isotopologue. Our data suggests that without artificial O₂-manipulation nearly equal quantities of cells become newly hypoxic and newly normoxic over the time frame investigated (Figure 4j, green histogram). Dynamic changes in oxygenation were also observed in a patient-derived pancreatic cancer xenograft model demonstrating the general applicability of this approach to the study of different tumor types (Figure 4i).

Taken together, these data indicate that isotopologous probes compatible with IMC can provide a general approach for investigating dynamic biological phenomena with spatiotemporal resolution without confounding effects from differential pharmacology of distinguishable probes. Telox 2 shows promise for translation to the clinic to capture dynamic oxygenation as a biomarker for better understanding cancer biology and treatment.

Acknowledgements

The authors acknowledge Fluidigm for providing access to instrumentation, reagents, and expertise in collecting MC and IMC data. Specifically, we would like to thank D. Bandura, V. Baranov, T. Closson, O. Loboda, O. Ornatsky, and J. Watson. We also acknowledge Q. Chang for productive discussions and G. McKeown for assistance processing IMC data. Additionally, we recognize W. Wilson for providing access to the mutant HCT116 cell lines. The authors would like to acknowledge the Spatio-Temporal Targeting and Amplification of Radiation Response (STTARR) program and its affiliated funding agencies. Finally, the authors acknowledge funding from the Canadian Cancer Society, Natural Sciences and Engineering Research Council of Canada, and Fluidigm Canada.

Keywords: activity-based probes · cellular hypoxia · dynamic biology · mass spectrometry · tellurium

- [1] W. R. Wilson, M. P. Hay, Nat. Rev. Cancer 2011, 11, 393-410.
- [2] N. Rohwer, T. Cramer, Drug Resist. Updates 2011, 14, 191-201.
- [3] P. Martinive, F. Defresne, C. Bouzin, J. Saliez, F. Lair, V. Grégoire, C. Michiels, C. Dessy, O. Feron, *Cancer Res.* 2006, 66, 11736–11744.
- [4] M. W. Dewhirst, Y. Cao, B. Moeller, Nat. Rev. Cancer 2008, 8, 425–437.
- [5] O. Ornatsky, D. Bandura, V. Baranov, M. Nitz, M. A. Winnik, S. Tanner, J. Immunol. Methods 2010, 361, 1–20.
- [6] C. Giesen, H. A. O. Wang, D. Schapiro, N. Zivanovic, A. Jacobs, B. Hattendorf, P. J. Schüffler, D. Grolimund, J. M. Buhmann, S. Brandt, Z. Varga, P. J. Wild, D. Günther, B. Bodenmiller, *Nat. Methods* **2014**, *11*, 417–422.
- [7] a) L. J. Edgar, R. N. Vellanki, A. Halupa, D. Hedley, B. G. Wouters, M. Nitz, Angew. Chem. Int. Ed. 2014, 53, 11473-11477; Angew. Chem. 2014, 126, 11657-11661; b) K. Tanabe, Z. Zhang, T. Ito, H. Hatta, S. Nishimoto, Org. Biomol. Chem. 2007, 5, 3745-3757; c) R. A. McClelland, R. Panicucci, A. M. Rauth, J. Am. Chem. Soc. 1985, 107, 1762-1763; d) J. L. Bolton, R. A. McClelland, J. Am. Chem. Soc. 1989, 111, 8172-8181; e) R. J. Hodgkiss, Anti-Cancer Drug Des. 1998, 13, 687-702.
- [8] J. Russell, S. Carlin, S. A. Burke, B. Wen, K. M. Yang, C. C. Ling, *Int. J. Radiat. Oncol. Biol. Phys.* 2009, 73(4), 1177–1186.
- [9] H. Park, L. J. Edgar, M. A. Lumba, L. M. Willis, M. Nitz, Org. Biomol. Chem. 2015, 13, 7027–7033.
- [10] N. Pham, J. M. M. M. Magalhaes, T. Do, J. Schwock, N. Dhani, P. Cao, R. P. Hill, D. W. Hedley, *Int. J. Cancer* **2009**, *124*, 280–286.
- [11] J. Su, Y. Gu, F. B. Pruijn, J. B. Smaill, A. V. Patterson, C. P. Guise, W. R. Wilson, J. Biol. Chem. 2013, 288, 37138–37153.
- [12] K. L. Bennewith, J. A. Raleigh, R. E. Durand, *Cancer Res.* 2002, 62, 6827–6830.
- [13] C. J. Koch, Methods Enzymol. 2002, 352, 3-31.
- [14] R. H. Thomlinson, L. H. Grey, Br. J. Cancer 1955, 9, 539-549.
- [15] M. R. Horsman, M. Nordsmark, M. Hoyer, J. Overgaard, Br. J. Cancer 1995, 72, 1474–1478.

Received: August 2, 2016 Published online: ■■ ■■, ■■■

© 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

These are not the final page numbers!

www.angewandte.org

Angew. Chem. Int. Ed. 2016, 55, 1-6

Communications



Communications

Activity-Based Probes

L. J. Edgar, R. N. Vellanki, T. D. McKee, D. Hedley, B. G. Wouters, M. Nitz* _

Isotopologous Organotellurium Probes Reveal Dynamic Hypoxia In Vivo with Cellular Resolution



Isotopologous Probes

Monitoring oxygenation : Incorporation of isotopically enriched tellurium into a biocompatible tellurophene enabled construction of a series of analytically distinguishable, but pharmacologically identical activity-based probes sensitive





towards cellular hypoxia. Serial injection of these isotopologous probes into human tumor-bearing mice revealed cellular hypoxia dynamics with spatiotemporal resolution as reported by imaging mass cytometry.

6 www.angewandte.org © 2016 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Angew. Chem. Int. Ed. 2016, 55, 1-6

These are not the final page numbers!