Journal Pre-proofs

Synthesis and Evaluation of New Dinitrobenzamide Mustards in Human Prostate Cancer

Alireza Basiri, Wenting Zhang, Jered Garrison

PII: S0960-894X(20)30808-8
DOI: https://doi.org/10.1016/j.bmcl.2020.127697
Reference: BMCL 127697

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 2 September 2020
Revised Date: 11 November 2020
Accepted Date: 13 November 2020

Please cite this article as: Basiri, A., Zhang, W., Garrison, J., Synthesis and Evaluation of New Dinitrobenzamide Mustards in Human Prostate Cancer, *Bioorganic & Medicinal Chemistry Letters* (2020), doi: https://doi.org/10.1016/j.bmcl.2020.127697

Synthesis and Evaluation of New Dinitrobenzamide Mustards in Human Prostate Cancer

Alireza Basiri†,‖, Wenting Zhang†,‖, Jered Garrison†,§, ‖,¦, *

†. Department of Pharmaceutical Sciences, College of Pharmacy, University of Nebraska Medical Center, 985830 Nebraska Medical Center, Omaha, NE 68198, United States

§. Department of Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, 985870 Nebraska Medical Center, Omaha, NE 68198, United States

‖. Center for Drug Delivery and Nanomedicine, University of Nebraska Medical Center, 985830 Nebraska Medical Center, Omaha, NE 68198, United States

¦. Eppley Cancer Center, University of Nebraska Medical Center, 985950 Nebraska Medical Center, Omaha, NE 68198, United States

ABSTRACT: Tumor hypoxia has been widely explored over the years as a diagnostic and therapeutic marker. Herein, we have reported the design and synthesis of a series of dinitrobenzamide mustards (DNBM) based on the PR-104A hypoxia-selective prodrug. Specifically, we explore the impact of various leaving groups and the introduction of a carboxylic acid group on the biological performance of the DNBM constructs. Once in hand, the Log D values, cytotoxicity in PC-3 and DU-145 human prostate cancer cells lines and the hypoxia selectivities of the DNBM analogs were examined. Overall, the DNBM constructs were found to be tolerant to modification with none of the explored modifications substantially degrading the cytotoxic potential of the constructs.

Keywords: DNBM, prodrug, prostate cancer, hypoxia

Corresponding Author

* Phone: +1-402-559-3453. Fax: 001-402-559-9365. E-mail: jcgarrison@unmc.edu.

The prevalence of hypoxic tissue, areas with oxygen partial pressure (pO_2) values significantly lower than normal, is a characteristic of many solid tumors. It has been shown that up to 50-60% of tumors in cancers of the breast, head and neck, prostate, pancreas and soft tissue sarcomas exhibit significant amounts of hypoxia and/or anoxia.[1-3] Also, hypoxia has been linked as a major driving force behind the aggressiveness and treatment resistance of many human cancers. This has provided the impetus to the development of therapeutic and diagnostic agents that target tumor hypoxia.[4] Several strategies for exploiting tumor hypoxia are now in preclinical or clinical development, with the main focus on prodrugs that are activated by metabolic reduction under hypoxic conditions to form cytotoxins.[5]

Dinitrobenzamide mustards (DNBMs) represent a class of hypoxia-activated prodrugs.[6] This class of mustards contains a latent nitrogen mustard moiety, which can be activated upon reduction of the molecule under hypoxic conditions. PR-104, a DNBM, is a phosphate ester "pre-prodrug" currently in clinical trials. [7, 8] When administered, this prodrug is dephosphorylated to its alcohol analog PR-104A, see **Fig. 1**. The nitro groups are subsequently reduced under hypoxic conditions via PR-104N to the corresponding hydroxylamine or amine (i.e., PR-104H and PR-104M).[9] This reduction-activated "electronic switch" triggers a substantial increase in the alkylating power of the nitrogen mustard.[10] These activated mustards, then induce the formation of DNA interstrand cross-links, which is generally considered the therapeutic action of this class of chemotherapeutics.[11, 12]

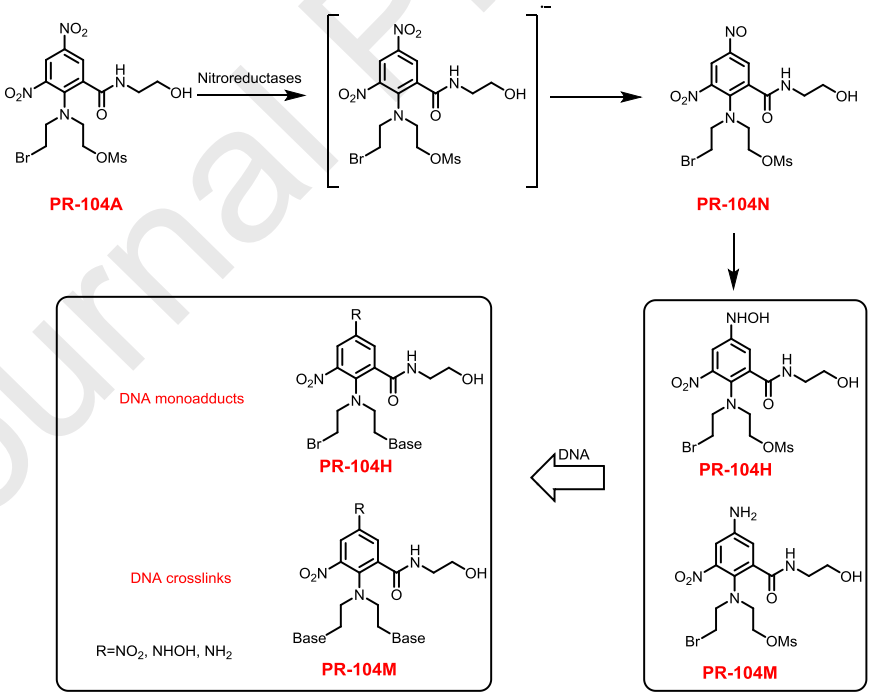


Fig. 1: Mechanism of metabolic activation of PR-104A, catalyzed by one-electron reduction under hypoxia, modified from [13]

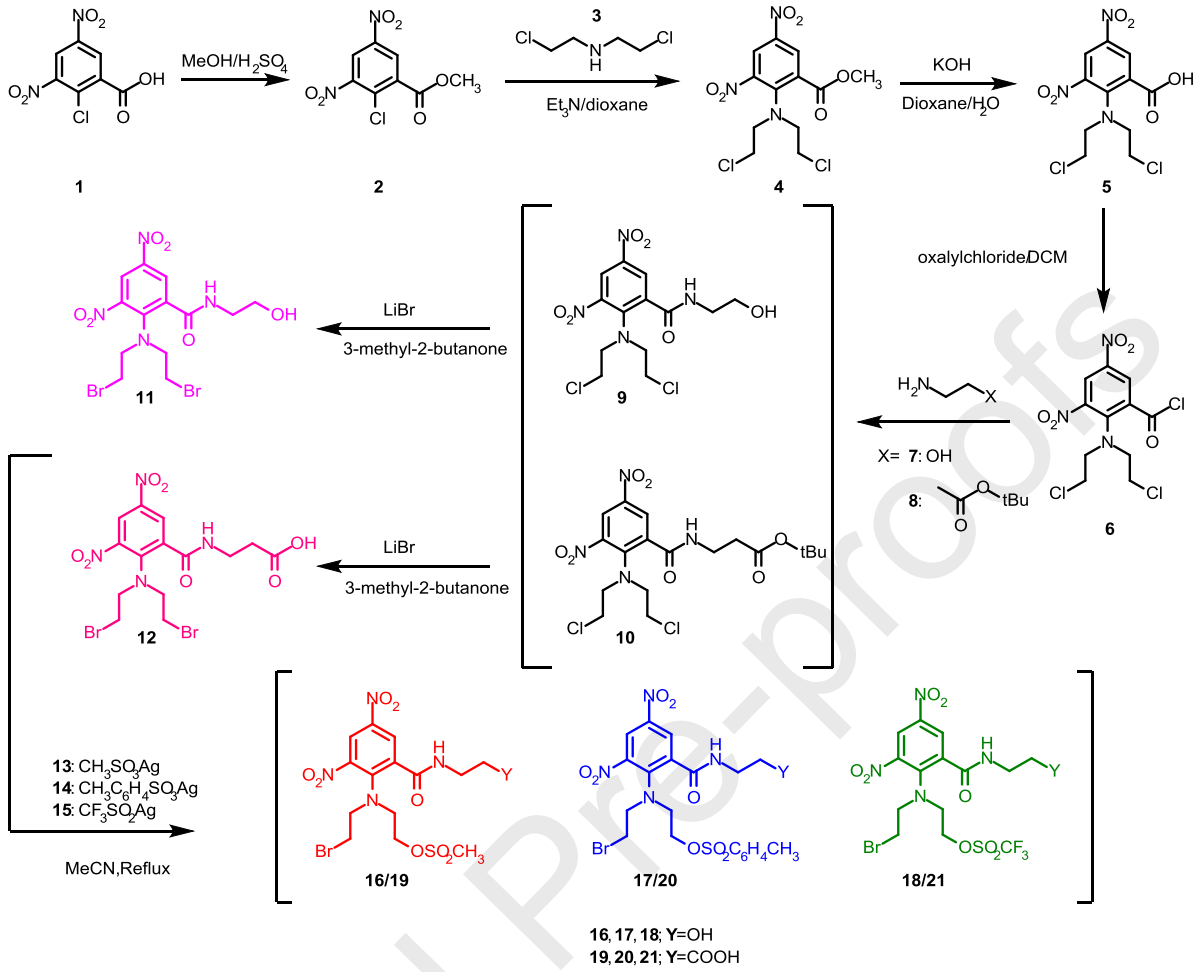
In the following, we describe the synthesis and *in vitro* investigation of seven DNBM prodrugs resembling the PR-104A paradigm. The design of these mustard prodrugs, depicted in **Scheme 1**, followed two major aims. Firstly, we hypothesized that by increasing the leaving group potential (i.e., Br < mesylate ~ tosylate << triflate) of the mustard moiety, an increase in cytotoxicity would be observed. To that end, we synthesized compounds **11**, **16** (PR-104A), **17** and **18**. Previously, we have utilized hypoxia-selective prodrugs and other adduct-forming constructs as trapping agents to increase the long term residualization of receptor-targeted constructs in tumors.[14, 15] In the same context, our secondary goal was to include a chemical handle on the prodrugs, to allow future conjugations to fluorophores, radioisotopes and/or receptor-targeted agents. With this purpose in mind, the alcohol functional group of the above-mentioned analogs was replaced with a carboxylic acid, yielding compounds **12**, **19**, **20** and **21**. With these compounds in hand, we examined what impact these structural modifications had on the physiochemical properties, hypoxia selectivity and biological activities in PC-3 and DU-145 human prostate cancer cell lines.

We initiated the synthesis by the acid-catalyzed esterification of 2-chloro-3,5-dinitrobenzoic acid, **1**, in methanol. The protected dinitrobenzoate, **2**, was subsequently reacted with bis(chloroethyl)amine, **3**, to furnish compound **4**, which was isolated through flash column chromatography in 86% yield. The structure of **4**, depicted in **Scheme 1**, was confirmed by single-crystal x-ray diffraction analysis. In the next step, compound **4** was reacted with an aqueous solution of KOH (3N) in dioxane to remove the protecting methyl group furnishing the free benzoic acid product, **5**. Compound **5** was subsequently converted to the acid chloride, **6**, using oxalyl chloride under mild conditions. In situ, the acid chloride, **6**, was reacted with ethanolamine (**7**), or β-Alanine t-butyl ester (**8**), to yield the DNBMs **9** and **10**, respectively, in reasonable yields after flash column chromatography.

Halide exchange was carried out on compounds **9** and **10** using LiBr in 3-methyl-2-butanone to provide the corresponding crude dibromo analogs **11** and **12**. The crude material was further purified using silica gel chromatography to yield pure forms of **11** and **12** in 72 and 82% yields, respectively. Specifically, for compound **12**, the bromide exchange reaction led to the removal of the *t*-Bu protecting group from the side chain. Compound **16** (PR-104A) was prepared by refluxing **11** and silver mesylate (**13**) in acetonitrile. Unreported sulfonate esters **17-21**, were prepared by refluxing **11** or **12** with the corresponding sulfonate silver salts (i.e., silver mesylate, **13**; silver tosylate, **14**; and silver triflate, **15**) in acetonitrile and isolated through flash column chromatography. For compounds **19-21**, due to the presence of the acid moiety on the side chains, 0.1% formic acid was used as a modifier for better separation during column chromatography. The structures of PR-104A, as well as the newly synthesized prodrug analogs were confirmed by mass spectrometry, ^{1}H and ^{13}C NMR spectral analyses (supporting information).

Partition or distribution coefficients (Log P or Log D) are known to impact the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of drugs. These values are widely used in drug discovery for quantitative structure-activity relationships and quantitative structure-property relationships.[16] Generally, compounds with moderate lipophilicity (log D = 0-3) are thought to provide a good balance between solubility and cellular permeability, which is needed to access intracellular targets.[17] To examine the impact of the structural modifications on the distribution properties, log $D_{7.4}$ values of the synthesized prodrugs were measured using the shake-flask method (**Table 1**).[18]

Owing to the presence of the ionizable carboxylic acids present in **12**, **19**, **20** and **21**, these compounds were expected to have lower distribution coefficient values at physiological pH, compared to their alcohol side chain counterparts **11**, **16** (PR-104A), **17** and **18**. The distribution coefficients indicated a more dichotomic result. For compounds **11** and **16**, replacement of the alcohol with a carboxylic acid resulted in an order of magnitude increase in hydrophilicity for the resulting **12** and **19**. However, this same alteration for **17** and **18** had a negligible impact on hydrophilicity for the resulting **20** and **21**. Replacement of the bromide in **11** and **12** with a methyl sulfonate furnished analogs **16** and **19**, which displayed lower distribution coefficients in aqueous media. For instance, log $D_{7.4}$ values of **11** shifted from 1.7 ± 0.2 to 0.80 ± 0.12 for analog **16**. Interestingly, when the side chain is an alcohol, the tosylate (**17**) and triflate (**18**) leaving groups have substantially higher hydrophilicities than the bromide (**11**). Conversely, when the side chain is a carboxylic acid, the bromide (**12**) yields higher hydrophilicity compared to the tosylate (**20**) and nearly analogous hydrophilicity relative to the triflate (**21**) derivative.



Scheme 1. Synthesis of mustard DNBM prodrugs **11-21**

The activation and therapeutic mechanism of hypoxia-activated DNBM prodrugs has been well-established.[19, 20] Under hypoxic conditions and in the presence of nitroreductase enzymes, the electron-withdrawing 4-nitro group transforms into an electron-donating 4-hydroxylamine or amine moiety (see **Fig. 1**). This "electronic switch" triggers the formation of the highly reactive aziridinium species via an intramolecular SN_2 reaction.[21] These aziridinium groups can subsequently alkylate DNA to yield their cytotoxic/ therapeutic effect.[22]

To examine the cytotoxicities and hypoxia-selectivities of the prodrugs **11**, **12,** and **16-21**, these compounds were evaluated in PC-3 and DU-145 human prostate cancer cell lines, under hypoxic and normoxic conditions using a cell viability assay. In brief, the cells were exposed to the mustard agents, at a range of concentrations for two hours under hypoxic or normoxic conditions, thereafter, washed with fresh media and incubated under normoxic conditions for three days. The determined cytotoxicity potential of these agents was based on cell viability assays using almarBlue® and reported as half-

maximal inhibitory concentration (IC_{50}) values. The results of these studies are summarized in **Table 1**. The established prodrug PR-104A (**16**) was used as a positive control for comparison against the newly synthesized analogs.

Table 1. Measured log $D_{7.4,}$ IC_{50} and hypoxia selectivity ratio values of the synthesized prodrugs **11,12,16-21**

Compd.	Log $D_{7.4}$†	IC_{50}					
		PC-3 cells		DU-145 cells		Hypoxia selectivity	
		N_2	Air	N_2	Air	PC-3	DU-145
11	1.7± 0.2	26±2	193±13	80±4	177±6	7.4	2.2
12	0.42 ± 0.09	75±4	490±30	47±6	413±6	6.5	8.8
16 (PR104A)	0.80 ± 0.12	48±4	320±30	78±5	210±20	6.7	2.7
17	0.95± 0.10	37±3	146±11	28±2	108±5	3.9	3.9
18	0.33 ± 0.04	42±4	245±15	51±4	470±30	5.8	9.2
19	-0.71 ± 0.12	41±3	417±18	56±3	326±14	10.	5.8
20	1.03 ± 0.07	42±3	460±30	54±4	363±19	11	6.7
21	0.15 ± 0.14	90.±3	350±30	68±2	480±30	3.9	7.0

† Log $D_{7.4}$ (mean ± SEM) are from two or more independent experiments carried out in triplicate; ‡IC_{50} (mean ± SEM); hypoxia selectivity = IC_{50} (air)/ IC_{50} (N_2)

In PC-3 cells, the positive control PR-104A (**16**), as expected, demonstrated higher cytotoxicity under hypoxic relative to normoxic conditions, with the IC_{50} values of 48 ± 4 and 320 ± 30 μM for hypoxia and normoxia, respectively, and a hypoxia selectivity ratio of 6.7. Patterson *et. al.* has reported the IC_{50} value for PR-104A in PC-3 cells was 7.3 μM under anoxia (< 10 ppm oxygen in gas phase) and a hypoxia-selectivity ratio of approximately 15. The discrepancy between these values almost certainly stems from the residual oxygen concentrations used for the hypoxic/anoxic conditions. Tissue hypoxia is generally defined as a pO_2 pressure less than 15 mmHg (19,800 ppm), although this cut-off value is somewhat subjective when considering the range of physiologically relevant oxygen levels in normal tissues.[23, 24] We opted to examine our compounds under a 0.1% oxygen environment (1,000 ppm) due to it being more in-line with possible clinical hypoxia conditions. The higher residual oxygen concentrations for our

experiments likely resulted in less efficient hypoxia-activation of the prodrugs leading to higher IC_{50} values and a lower hypoxia selectivity ratio, compared to that reported under anoxic conditions.

As expected, all the compounds demonstrated greater cytotoxicities under hypoxic relative to normoxic conditions. In PC-3 cells under our hypoxic conditions, the compounds demonstrated cytotoxicities that ranged from 26 ± 2 to 90 ± 3 µM. All of the analogs displayed similar or higher cytotoxicities compared to PR-104A (**16**) under hypoxic conditions, except for prodrugs **12** and **21**. Compound **11** demonstrated the highest cytotoxicity with an IC_{50} value of 26 ± 2 µM, followed by compound **17** with an IC_{50} value of 37 ± 3 µM (**Fig. 2**). Hypoxia selectivity ratios for the constructs ranged from 3.9 to 11 in PC-3 cells. Only **11**, **19** and **20** exhibited better hypoxia selectivity ratios than PR-104A (**16**), see **Fig. 4**.

Conversely, in the DU-145 cell line under identical conditions, the cytotoxicities under hypoxia ranged slightly narrower from 28 ± 2 to 80 ± 4 µM. In this case, PR-104A (78 ± 5 µM) exhibited cytotoxicity that was statistically analogous to **11** but was significantly lower than all other constructs investigated. Construct **17** (28 ± 2 µM) gave the lowest IC_{50} value followed by **12** with 47 ± 6 µM (**Fig. 3**). In DU-145 cells the hypoxia selectivity ratios ranged from 2.2 to 9.2. With exception to compound **11**, all investigated constructs had better hypoxia selectivity ratios than PR-104A (**16**), see **Fig. 4**.

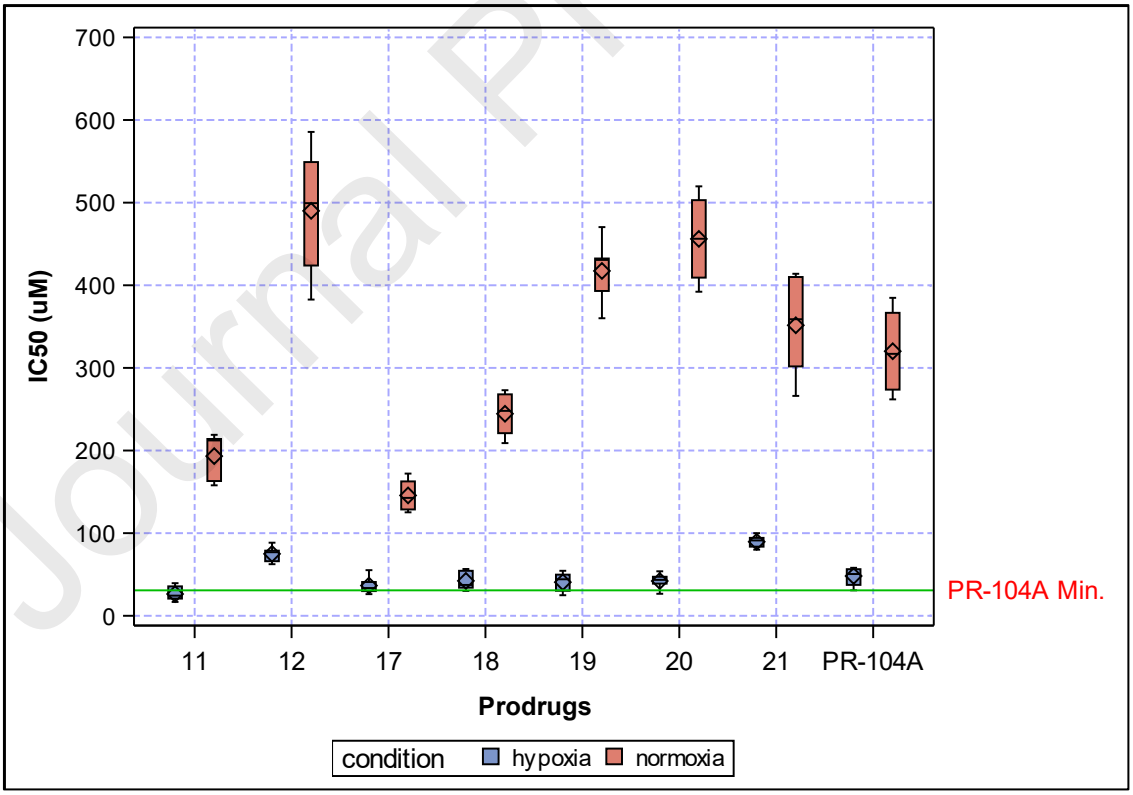


Fig. 2: cytotoxicity activities of prodrugs in PC-3 cells, under hypoxic and normoxic conditions

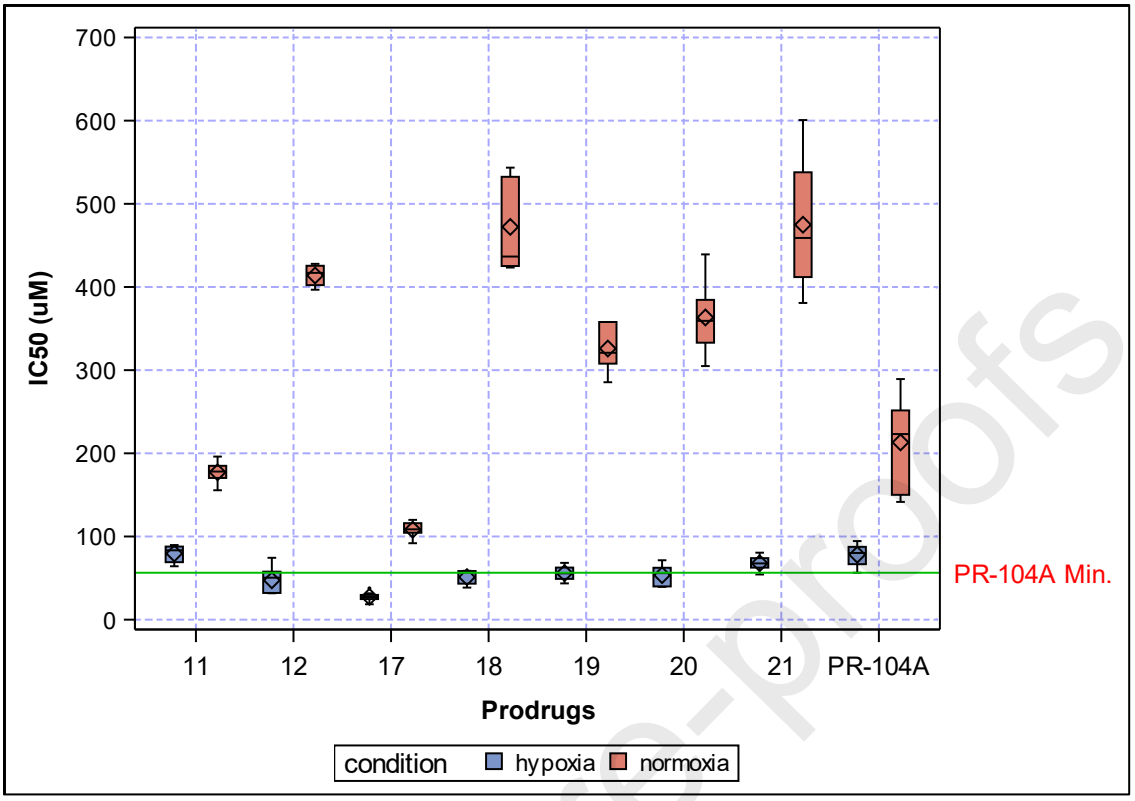


Fig. 3: cytotoxicity activities of prodrugs in DU-145 cells, under hypoxic and normoxic conditions

Analysis of the data did yield some structure-activity trends concerning the explored modifications (i.e., carboxylic acid/alcohol and mustard leaving group). Compounds with carboxylic acid moieties tended to have lower cytotoxicity values under normoxia. At first glance, this may be inferred to a lower cellular uptake profile due to the negatively charged carboxylate under physiological conditions. However, the cytotoxicities of the DNBMs containing carboxylic acids were mostly consistent with the cytotoxicities of the alcohol bearing analogs under hypoxic conditions. If these charged compounds have lower cellular uptake values, this suggests that the cytotoxicity of the carboxylic acid analogs was inherently higher than the alcohol derivatives. At any rate, the decrease in cytotoxicity under normoxic conditions and the maintenance of cytotoxicity under hypoxic conditions led to generally larger hypoxia selectivity ratios for the carboxylic acid analogs relative to the alcohol derivatives. For instance, in the PC-3 cell line, prodrug **20** showed hypoxia selectivity of 11, while its alcoholic analog, **17**, showed a hypoxia selectivity of 3.9. Under our conditions, increasing the leaving group potential did not lead to significant gains in cytotoxicities. However, for DNBM analogs with the triflate leaving group, **18** and **21**, significant declines in cytotoxicities were observed under both normoxic and hypoxic conditions relative to other analogs. This may well be attributable to the greater instability of the triflate constructs in solution superseding any potential cytotoxicity gains due to the greater leaving group potential of this group.

While the cytotoxicities of the constructs in both cell lines were in a relatively narrow range (26 ± 2 to 90 ± 3 μM), there was a significant variance between cell lines for some compounds. For example, compound **11** had the highest cytotoxicity in PC-3 cells but was the worst-performing construct in the DU-145 cell line. Relatively large cell-line dependent variation for a DNBM is not uncommon and has been observed by others.[20] Additionally, a linear regression analysis was performed to examine if a correlation existed between the log $D_{7.4}$ and IC_{50} values, but no statistically significant correlation was found.

Our interest in the DNBM class of hypoxia-activated prodrugs lies in their properties beyond chemotherapeutics, specifically for the potential development of diagnostic agents and other therapeutic approaches. Numerous investigators have explored other bio-reductive prodrugs for this purpose.[25-28] It was with this idea in mind that we explored the impact of modifying the alcohol side chain of PR-104A with a carboxylic acid, to aid in eventual conjugation to other functionalities (i.e., fluorophores, radiolabels and other constructs). None of the modifications explored above substantially influenced the ability of the constructs to activate in hypoxic environments, form intracellular adducts and yield their cytotoxic effects. Due to this tolerance to modification, we intend to further explore the capability of these DNBMs in the development of diagnostic and radiotherapeutic agents.

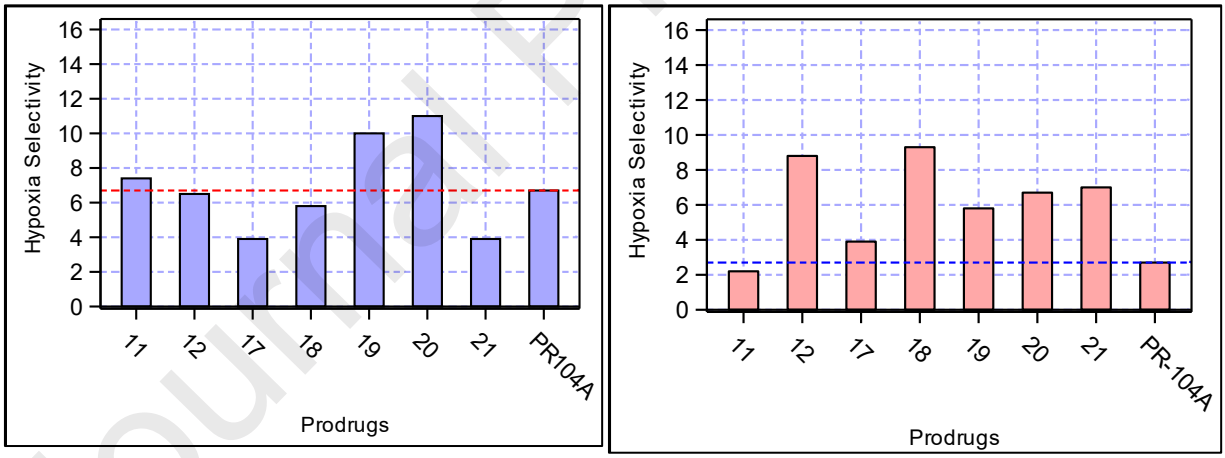


Fig. 4: hypoxia selectivity of prodrugs **11-21** in PC-3 (left) and DU-145 cells (right)

To summarize, we have designed and successfully synthesized a series of DNBMs resembling the hypoxia-selective prodrug PR-104A. With these analogs in hand, we examined to what extent modification of the mustard leaving-group potential and incorporation of a carboxylic acid moiety affects their biological performance. All of the synthesized analogs demonstrated significantly higher cytotoxicities under hypoxic relative to normoxic conditions. This implies that all of the synthesized DNBMs can undergo reduction under hypoxic conditions and convert to the active cytotoxic form of the drug. Increasing the leaving group potential of the DNBM did not lead to notable gains in cytotoxicity.

Incorporation of a carboxylic acid group into the DNBM construct did not negatively impact cytotoxicity and tended to yield better hypoxia-selectivity ratios than analogous alcohol derivatives. In the future, we intend to exploit these DNBMs for their adduct formation capabilities and to develop diagnostic and radiotherapeutic agents.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Refrences:

1. Hockel M, Vaupel P. *J Natl Cancer Inst.* 2001;93(4): 266-276.
2. Vaupel P, Höckel M. *Recombinant Human Erythropoietin (rhEPO) in clinical oncology.* Springer; 2002:127-146.
3. Vaupel P, Mayer A. *Cancer Metastasis Rev.* 2007;26(2): 225-239.
4. Subarsky P, Hill RP. *Clin Exp Metastas.* 2003;20(3): 237-250.
5. Brown JM, Wilson WR. *Nat Rev Cancer.* 2004;4(6): 437.
6. Wilson WR, Hicks KO, Pullen SM, Ferry DM, Helsby NA, Patterson AV. *Radiat Res.* 2007;167(6): 625-636.
7. McKeage MJ, Gu Y, Wilson WR, et al. *BMC cancer.* 2011;11(1): 432.
8. McKeage MJ, Jameson MB, Ramanathan RK, et al. *BMC cancer.* 2012;12(1): 496.
9. Helsby NA, Wheeler SJ, Pruijn FB, et al. *Chem Res Toxicol.* 2003;16(4): 469-478.
10. Palmer BD, Wilson WR, Anderson RF, Boyd M, Denny WA. *J Med Chem.* 1996;39(13): 2518-2528.
11. Hunter FW, Wouters BG, Wilson WR. *Br J Cancer.* 2016;114(10): 1071.
12. Stornetta A, Deng K-CK, Danielli S, et al. *Biochem Pharmacol.* 2018;154: 64-74.
13. Phillips RM. *Cancer Chemoth Pharm.* 2016;77(3): 441-457.
14. Zhou Z, Wagh NK, Ogbomo SM, et al. *J Nucl Med.* 2013;54(9): 1605-1612.
15. Wagh NK, Zhou Z, Ogbomo SM, Shi W, Brusnahan SK, Garrison JC. *Bioconjugate Chem.* 2012;23(3): 527-537.
16. Comer JE. *Drug bioavailability: Estimation of solubility, permeability, absorption and bioavailability.* 2003:21-45.
17. Di L, Kerns EH. *Curr Opin Chem Biol.* 2003;7(3): 402-408.
18. Läppchen T, Rossin R, van Mourik TR, et al. *Nucl Med Biol.* 2017;55: 19-26.
19. Helsby NA, Wheeler SJ, Pruijn FB, et al. *Chem Res Toxicol.* 2003;16(4): 469-478.
20. Patterson AV, Ferry DM, Edmunds SJ, et al. *Clin Cancer Res.* 2007;13(13): 3922-3932.
21. Williams EM, Little RF, Mowday AM, et al. *Biochem J.* 2015;471(2): 131-153.
22. Brookes P, Lawley P. *Biochem J.* 1961;80(3): 496.
23. Ortiz-Prado E, Dunn JF, Vasconez J, Castillo D, Viscor G. *Am J Blood Res.* 2019;9(1): 1-14.
24. McKeown SR. *Br J Radiol.* 2014;87(1035): 20130676.

25. Duszynski CC, Avati V, Lapointe AP, Scholkmann F, Dunn JF, Swain MG. *Hepatology*. 2020;71(4): 1408-1420.
26. Hoigebazar L, Jeong JM, Lee J-Y, et al. *J Med Chem.* 2012;55: 3155-3162.
27. Bejot R, Kersemans V, Kelly C, Carroll L, King RC, Gouverneur V. *Nucl Med Biol*. 2010;37: 565-575.
28. Das T, Chakraborty S, Banerjee S, Sarma HD, Samuel G, Venkatesh M. *Radiochima Acta.* 2006;94: 375-380.