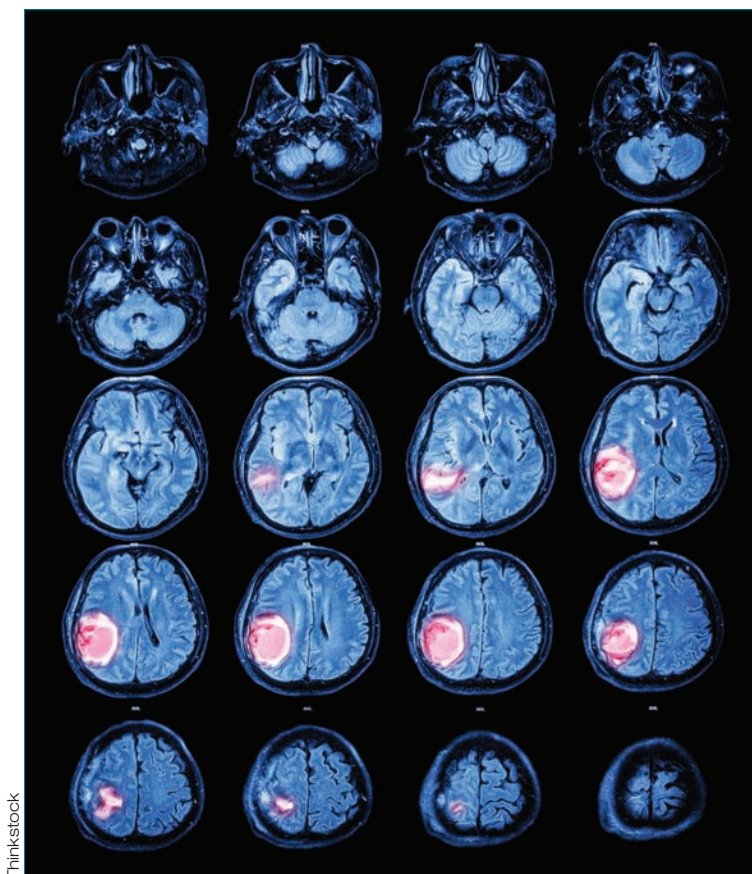


Novel Prodrugs of 6-Diazo-5-Oxo-L-Norleucine With More Brain Delivery

BY RICHARD SIMONEAUX

The World Health Organization considers glioblastoma multiforme (GBM) the most common brain neoplasm, accounting for more than 60-75 percent of astrocyte tumors and 15 percent of all cranial neoplasms. When compared to the strides that have been made in the treatment of other malignancies, such as breast or lung cancer, therapies for GBM have remained largely unchanged over the past 40 years. GBM is also one of the most lethal cancers, with the current standard therapy, surgical tumor resection followed by concomitant co-administration of the radiation-sensitizing DNA-methylating agent temozolamide and radiation therapy only providing mean post-surgical survival rates of 14.6 months.



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Even with this aggressive therapy, the 2-year overall survival rate was 27 percent, with only 5 percent of patients surviving 5 years past their diagnosis. One of the major reasons for this recalcitrance is the impenetrability of the blood-brain barrier. To address this unmet need for effective therapies that effectively cross the blood-brain barrier, Barbara Slusher, PhD, MAS, of Johns Hopkins Drug Discovery, and Jonathan Powell, MD, PhD, Department of Oncology, Johns Hopkins School of Medicine, sought to create prodrugs of the glutamine antagonist 6-diazo-5-oxo-L-norleucine (DON). A report on their initial investigations is included in the *Journal of Medicinal Chemistry* (DOI: 10.1021/acs.jmedchem.6b01069).

Because GBM is a rapidly-proliferating cancer, it often outstrips the vascular system's oxygen and nutrient supply, and consequently becomes reliant upon the amino acid glutamine for metabolism. This versatile amino acid is utilized in GBM for de novo purine synthesis (via PRPP amidotransferase, guanosine monophosphate synthetase,

and FGAR amidotransferase), de novo pyrimidine synthesis (via CTP synthase and carbamoyl phosphate synthase (CAD)), and glutamate production (via glutaminase).

"This so-called 'glutamine addiction' is characteristic of GBM and other proliferative cancers, including pancreatic cancer, acute myelogenous leukemia, and small cell lung cancer," Slusher explained.

Understanding Glutaminase

One enzyme key to this unique metabolism is glutaminase. Slusher further clarified: "Human glutaminase is encoded by two genes—the kidney-type GLS1 isoforms KGA and GAC, and the liver-type GLS2 isoforms GAB and LGA. The kidney-type isoforms are associated with cell proliferation while the liver-type isoforms dominate in quiescent cells." In normal glial cells, glutaminase catalyzes the formation of glutamate, the most commonly-used CNS excitatory neurotransmitter. In GBM, the glutamate produced from glutamine enters the tricarboxylic acid (TCA) cycle, where it serves as an energy source for the tumor in the absence of oxygen and glucose. This ability of glutamate to serve as an alternative energy source in tumors was confirmed mechanistically by utilizing uniformly-labeled ^{13}C -glutamine as a tracer to measure ^{13}C -labeled products (via ^{13}C -nuclear magnetic resonance) obtained from a Burkitt lymphoma P493 cell line model kept under hypoxic and glucose-deficient conditions (*Cell Metab* 2012;15(1):110-21). Moreover, the glutaminase-formed glutamate that is shunted into the TCA cycle or into the synthesis of compounds utilized by the tumor is no longer available for healthy cells to synthesize glutathione, an important antioxidant that could help fight the malignancy and the oxidative damage it causes.

DON, an unnatural amino acid having a structure similar to that of L-glutamine, was first isolated in the 1950s from *Streptomyces* bacteria. "This compound has been shown to alkylate several glutamine-dependent enzymes, including glutaminase, CTP synthetase, FGAR aminotransferase, and NAD synthase," Powell noted. "It is thought that DON may disrupt the de novo purine and pyrimidine synthesis by interaction with PRP amidotransferase and CAD respectively."

Further proof for DON's mechanism of action was obtained when an X-ray crystallography study was done using the kidney-type GLS1 glutaminase isoform KGA. In this study (*Sci Rep* 2014;4:3827), DON was shown to alkylate the active-site serine, SER286, irreversibly forming a covalent bond with the active site nucleophile. During the O-alkylation of the SER286 side-chain hydroxyl group, the diazo group alpha to the ketone moiety is lost as N_2 . Although DON did show some initial success in clinical trials, it was ultimately hampered by its significant dose-limiting toxicities in the glutamine-dependent GI tract.

Although there was considerable evidence that targeting glutaminase could be useful for GBM, as yet, there have been no in vivo data for DON against this malignancy. One other known selective glutaminase inhibitor has been entered into clinical trials, CB-839. A study that tested this compound for efficacy against triple negative breast cancer found the concentration of the drug in the tumor or other bodily tissues was greater than 7 times that found in the brain, showing poor penetrability of the blood-brain barrier (*Mol Cancer Ther* 2014;13(4):890-901). To address the lack of in vivo data for DON against GBM, researchers at Johns Hopkins developed

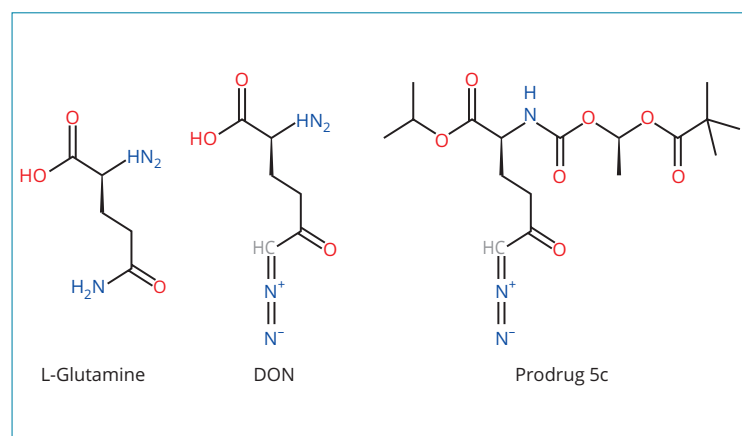
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a U87 flank xenograft mouse model. Mice were dosed at 0.8 mg/kg, i.p. and the desired effect of endogenous glutamine accumulation in the tumor was noted. Additionally, mice dosed at 0.8 mg/kg q.d., i.p. showed a more than 50 percent decrease in tumor size, as compared to the control (vehicle-dosed) mice, which showed constant tumor growth. In spite of the tumor improvement, DON-dosed mice also showed significant toxicity, including weight loss ($12 \pm 4.1\%$), lethargy, ptosis, and hunching. Having shown in vivo efficacy and toxicity, a search was now undertaken to address the shortcomings of DON.

One possible means to circumvent the GI toxicity would be to utilize a prodrug that would increase the concentration of the drug where its activity is desired (the brain in this case) and limit the systemic exposure of the active compound. Initial attempts to mask the reactive diazo moiety afforded highly unstable ketals or cyclic diazo-imines that would not convert back to DON. Attempts to protect the carboxylic acid group as an ester also led to highly unstable compounds, thus suggesting that both the acid and amine moieties of DON required protection. A number of compounds were made with different esters and amine-protecting functionalities, and to test the utility of these compounds, initial plasma stability studies were performed using mouse, monkey and, human plasma. Of the compounds made, the analog shown, 5c, displayed the highest stability in human plasma, with 91 percent of



the prodrug remaining after 1 hour, while for monkeys, 61 percent remained after the same time period. For all prodrugs tested in mouse plasma, none remained after 1 hour. This is a common problem encountered when attempting to perform stability studies on ester-based prodrugs, as they are rapidly metabolized in rodent plasma.

As a result of the stability in human, monkey, dog, and miniature swine plasmas, compound 5c was chosen for further evaluation in pharmacokinetic (PK) studies. Plasma studies in non-human primates were conducted in a pair of pigtail macaques. The studies were done in these higher order animals because their PK metabolism profile more closely mimicked that present in humans. For these studies, the cerebrospinal fluid concentrations of DON were measured as a proxy for brain levels. Slusher commented, "Aside from the practical aspect of not having to euthanize the animal, one can sample the fluid at multiple time points after drug administration." She also noted "direct comparisons can be made by testing the same fluid in humans." These monkeys were dosed with DON or 5c at 1.6 mg/kg i.v. (equimolar DON doses). For DON, the C_{max} plasma level was 12.6 nmol/mL at a T_{max} of 0.25 hr, displaying an AUC of 42.7 hr·nmol/mL. For 5c, these figures were 2.23 nmol/mL at 0.25 hr and 5.71 hr·nmol/mL, respectively. These results showed that the concentration of DON in the plasma was 7-fold lower for the prodrug 5c. This situation

was reversed for the CSF levels, where the concentration of DON in the CSF was higher following 5c dosing, resulting in a 10-fold higher CSF/plasma ratio as compared to the administration of the parent compound, DON.

To corroborate the in vivo results, an in vitro stability study was performed using pig brain homogenate. The results of this study clearly showed the prodrug steadily disappeared in the brain homogenate over time and was efficiently activated to DON with a half-life of 35 minutes. Regarding the distribution of DON in

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vivo, Slusher revealed, "We have completed preliminary tissue distribution studies in miniature swine with DON and our prodrugs." She further noted, "While the prodrug provided more DON delivery to the brain, it delivered significantly less DON to GI tissues (a major toxicity site). The prodrug also caused less adverse clinical effects in the swine including less anorexia, diarrhea, and reduced movement versus DON administration. In addition, on necropsy, DON administration caused more severe GI toxicity versus the prodrug."

It is of interest to note that the amine-protecting moiety (termed "Me-POM" in their paper) introduces a stereogenic center at the masked acetaldehyde carbon, thus giving a pair of chromatographically-separable diastereomers. Slusher noted "the diastereomer shown was the one utilized for initial pharmacokinetic studies, however, when subsequent tests were done on the other diastereomer, its properties were shown to mirror those of the initially tested prodrug 5c."

She further explained that "additional prodrugs have been made that have enhanced PK properties relative to 5c. The data surrounding those compounds will be discussed in a forthcoming *J Med Chem* paper." She did reveal information for one of these analogs "We did synthesize the analogous 'di-Me-POM,' which has a masked acetone equivalent that gives a single enantiomeric product instead of a pair of diastereomers requiring separation. This compound did show better PK properties than the Me-POM analog 5c."

Next Steps for GBM

With regards to the future of this project, Slusher noted that "because there is no effective higher-order animal model for GBM, we plan to show that DON is efficacious against GBM in mouse models and then use large animal species to evaluate our prodrugs' pharmacokinetics and preferential DON delivery in the brain."

"We hope to find a partner to work with us to bring this project to a phase I-Ib stage in the next 1-2 years," Powell emphasized. "We feel that a major advantage of the DON prodrug program is that the active compound targets a number of glutamine-utilizing enzymes relevant to rapidly proliferating tumor cells. In doing so, this compound not only disrupts a potential energy source, but also hinders the de novo synthesis of both purine and pyrimidine nucleotides. These compounds may also have utility in treating other types of tumors. Enhanced results have also been observed with these compounds in murine immunotherapy studies." **OT**

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