

## **SUPERTITLE: CANCER THERAPY**

### **TITLE: The Next-Generation of DNMT Inhibitors**

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#### **STANDFIRST [300-360 characters with spaces]**

DNA methyltransferase inhibitors (DNMTi) are widely used in preclinical and clinical studies, but poor pharmacokinetics and low efficacy in solid tumours limit their therapeutic use. A new study reports the development and characterization of a specific, non-covalent DNMTi with more durable DNA hypomethylation and lower toxicity than nucleoside analogues.

#### **MAIN TEXT [1000-1500 words]**

DNA can be modified by different epigenetic marks such as the addition of methyl groups, and cancer cells display a highly dysregulated DNA methylation landscape. The reliance of cancer cells on DNA methylation and the reversibility of this modification make it a promising target for cancer therapy. DNA methylation is deposited by the canonical DNA methyltransferases DNMT1, DNMT3A, and DNMT3B. The maintenance methyltransferase, DNMT1, binds to hemi-methylated DNA during DNA replication and mediates heritable DNA methylation. DNMT3A and DNMT3B, as *de novo* methyltransferases, preferentially bind to non-methylated DNA and generate new methylation patterns (**ref. 1**). Preclinical and clinical studies indicate that DNA hypomethylation can exert anti-cancer effects through the activation of silenced tumour suppressor genes and repression of oncogenes as well as the induction of “viral mimicry”, a cell state that is characterized by the transcriptional reactivation of normally dormant repeat elements. In this state, accumulated cytosolic nucleic acids derived from these reactivated repeats, such as double-stranded RNAs (dsRNAs), are recognized by pattern recognition receptors. This recognition engages the innate and adaptive immune systems, resulting in a decrease in cancer cell fitness (**ref. 2,3**). In this issue of *Nature Cancer*, Pappalardi and colleagues describe the development of GSK3685032, a novel, reversible, and selective DNMT1 inhibitor that was identified through a high-throughput enzymatic assay performed on 1.8 million small molecules. The authors demonstrate that GSK3685032 is less toxic and has higher, more durable hypomethylating activity

compared to current DNA hypomethylating agents (HMAs), indicating that its use as a monotherapy or potentially in combination with other compounds can translate to better clinical outcomes (**ref. 4**).

The first generation of HMAs consists of the nucleoside analogues 5-azacytidine (azacytidine or AZA) and 5-aza-2'-deoxycytidine (decitabine or DAC). Mechanistically, DAC incorporates solely into DNA while AZA incorporates into RNA and DNA. Once incorporated into the replicating DNA, these cytidine analogues covalently trap the DNMTs to DNA, resulting in degradation of DNMTs and consequent DNA hypomethylation (**ref. 5**). HMA activity is dose-dependent, with higher doses inducing cytotoxic DNA damage. Even though these agents have been shown to be beneficial in the treatment of different hematological malignancies such as myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML), there remain limitations in their administration and efficacy. *In vivo*, they are deaminated and eliminated by cytidine deaminase, resulting in a short half-life of around 30-50 minutes. HMAs also have a degree of selectivity for rapidly dividing transformed cells compared to normal cells as they act through integration into newly synthesized strands of replicating DNA but can still result in a host of side-effects including thrombocytopenia and neutropenia. Aside from AZA and DAC, there are some recently identified non-covalent inhibitors of DNMTs, such as RG-108 and SGI-1027, that have limited use due to their low potency and selectivity (**ref. 6**). In contrast to DAC and AZA, which irreversibly inhibit the activity of all three canonical DNMTs, GSK3685032 acts as a competitive inhibitor of DNMT1 via competition with DNMT1 active-site loop and target recognition domain to incorporate into the hemi-methylated DNA (**Fig. 1**).

To determine the killing activity of a selective DNMT1 inhibitor in cancer cells, Pappalardi and colleagues examined the effect of GSK3685032 on a panel of 51 hematological cancer cell lines including 15 leukemia, 29 lymphoma, and 7 multiple myeloma cell lines. On the majority of tested cell lines, GSK3685032 treatment showed anti-proliferative cytostatic effects starting 3 days post-treatment with increasing dose- and time-dependent potency through 6 days. Comparatively, DAC-induced anti-tumour effects were mediated through cytotoxicity rather than cytostatic effects while the inactive analog GSK3510477 had no effect. Moreover, the authors demonstrate that GSK3685032 is both a more potent and a more selective inhibitor than other non-nucleoside HMAs, including RG-108 and SGI-1027. Another interesting finding was that GSK3685032 led to time- and dose-dependent global DNA hypomethylation and associated transcriptional activation (**ref. 4**). Pathway analysis revealed that, similar to DAC and AZA (**ref. 2,3**), gene signatures upregulated by GSK3685032 treatment include those involved in interferon signaling, viral sensing, and antigen presentation. Activation of these pathways was linked to re-expression of human endogenous retroviruses (hERVs), but further work would be required to delineate the ability of this selective inhibitor to activate other retrotransposon families such as inverted repeat-Alus (IR-Alus), which belong to the SINE family and have recently been shown to be a major source of immunogenic dsRNAs (**ref. 7**). In contrast to DAC, which displayed diminishing catalytic activity at doses above 1  $\mu$ M, GSK3685032 showed sustained dose-dependent increases in hypomethylation activity at higher doses up to 10  $\mu$ M. Furthermore, DAC induced DNA damage and significantly reduced blood cell components such as neutrophils, red blood cells, and platelets in

xenograft mouse models of AML. On the other hand, *in vivo* administration of GSK3685032 (1-45mg/kg, subcutaneously, twice daily) was better tolerated, with decreased impact on blood cell components at higher doses\which recovered following drug withdrawal during four weeks. Despite these decreased side effects, GSK3685032 showed more potent tumour growth inhibition and significantly longer survival benefit compared to DAC. Further studies will be necessary to assess if the drug resistance arises over the prolong administration of this compound and to investigate the persistence of these effects and the potential timings of tumour recurrence during long-term treatments.

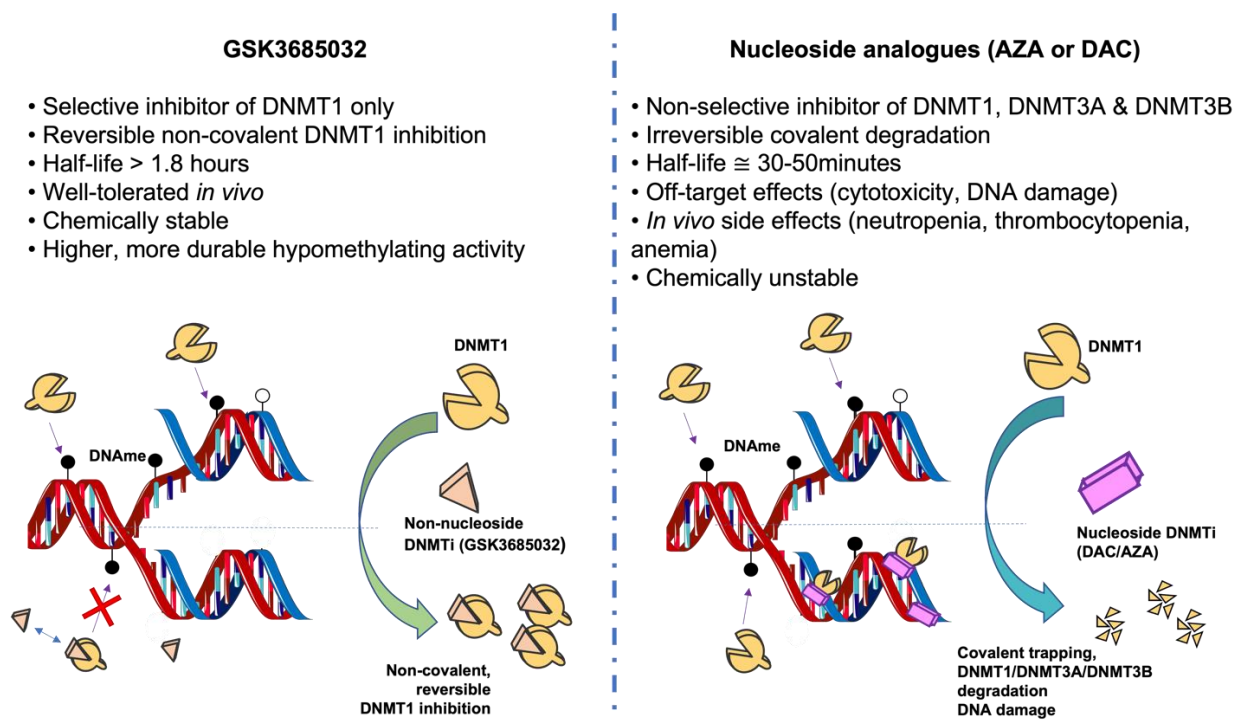
Due to its selectivity for DNMT1 through a unique non-covalent mechanism, GSK3685032 allows study of the effects of disrupting maintenance methylation following DNA replication while avoiding DNA damage-induced off-target effects produced by traditional HMAs. As a result, GSK3685032 overcomes the dose-limiting toxicities seen with AZA and DAC treatment and can produce a greater decrease in global DNA methylation (83% maximal response compared to 70% with DAC.) In particular, the *in vivo* work by Pappalardi and colleagues underscores GSK3685032 as a next-generation epigenetic therapy. GSK3685032 can also benefit from the extensive body of preclinical work using DAC and AZA. For example, it will be critical to expand this initial study to immunocompetent mice, in order to assess the effects of GSK3685032 on the adaptive immune system, since DAC was previously shown to enhance the anti-tumour cytolytic activity of CD8+ T cells (**ref. 8**) and to potentiate the effects of immune checkpoint blockade (**ref. 3**). Additionally, there is compelling preclinical and clinical evidence that DAC can form the basis of potent combination therapies, such as with the BCL2 inhibitor venetoclax (**ref. 9**) or with inhibition of the adenosine-to-inosine editor ADAR1 (**ref. 7**). As both combinations rely mechanistically on the binding of dsRNA species to cytosolic pattern recognition receptors like MDA5, there is a strong rationale for testing venetoclax and ADAR1 inhibition in concert with GSK3685032.

A few outstanding questions about the pharmacological properties of GSK3685032 need to be addressed to support its potential clinical utility. Despite good tolerance in mice, non-specific on-target toxicity in normal cells would need to be thoroughly addressed in follow-up studies. Nevertheless, it would be of great interest to see if GSK3685032 could overcome the clinical deficiencies of traditional HMAs. For instance, it is possible that the higher tolerability and increased target engagement of GSK3685032 could translate into higher efficacy in solid tumours, where AZA and DAC have thus far proven to be ineffective. Observing the effects of prolonged GSK3685032 would also be prudent, as the majority of hematological cancer patients that respond to HMA therapy experience relapse within two years (**ref. 10**).

Overall, Pappalardi and colleagues describe a novel ‘next-generation’ hypomethylating agent specific for DNMT1 that will likely become an important addition to the epigenetic therapy toolkit. Because of its lack of off-target effects and improved demethylation potential, GSK3685032 represents both an exciting therapeutic opportunity as well as a novel tool to study DNA methylation biology.

## References:

1. Jones, P.A., & Liang, G. *Nat. Rev. Genet.* 10(11): 805-11 (2009).
2. Roulois, D. et al. *Cell.* 162(5): 961-73 (2015).
3. Chiappinelli, K.B. et al. *Cell.* 162(5): 974-86 (2015).
4. Pappalardi, M.B. et al. *Nat. Cancer.* (2021).
5. Egger, G., Liang, G., Aparicio, A., & Jones, P.A. *Nature.* 429(6990): 457-63 (2004).
6. Mehdipour, P., Murphy, T., & De Carvalho, D.D. *Pharmacol. Ther.* 205: 107416 (2020).
7. Mehdipour, P. et al. *Nature.* 588(7836): 169-173 (2020).
8. Loo Yau, H. et al. *Mol Cell.* 81(7): 1469-1483.e8 (2021).
9. Fresquet, V. et al. *Cancer Discov.* 11(5): 1268-1285 (2021).
10. Derissen, E.J.B., Beijnen, J.H., & Schellens, J.H.M. *Oncologist.* 18(5): 619-24 (2013).



**Fig 1. Mechanism of DNMT1 inhibition by GSK3685032 compared to nucleoside analogue inhibitors AZA and DAC.** GSK3685032 acts as a competitive inhibitor, competing with the DNMT1 active-site loop for hemimethylated DNA and resulting in the selective and reversible inhibition of DNMT1 activity. As cytidine analogues, DAC and AZA incorporate into the new strand of replicating DNA and covalently trap DNMT1 to DNA, leading to the degradation of all DNMTs and DNA damage.