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Leveraging fluorinated glucosamine action to boost anti-tumor immunity

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Summary

N-acetylglucosaminyl glycans are key regulators of the vitality and effector function of anti-tumor T cells. When galectin-1 (Gal-1) binds N-acetylglucosamines on select membrane glycoproteins on anti-tumor T cells, these cells either undergo apoptosis or become immunoregulatory. Methods designed to antagonize expression or function of these N-acetylglucosamines on N- and O-glycans have thus intensified. Since tumors can produce an abundance of Gal-1, Gal-1 is considered a critical factor for protecting tumor cells from T cell-mediated anti-tumor activity. Recent efforts have capitalized on the anti-N-acetylglucosamine action of fluorinated glucosamines to treat anti-tumor T cells, resulting in diminished Gal-1-binding and higher anti-tumor T cell levels. In this perspective, the prospect of fluorinated glucosamines in eliminating N-acetylglucosamines on anti-tumor T cells to boost anti-tumor immunity is presented.

Keywords

Galectin-1; Glucosamine; Anti-tumor T cell Immunity; Galectin-1 Ligands

A. Historical View of Fluorinated Glucosamine Analogs in Pharmaceutical Development

Nearly 35-years ago, acetylated and fluorinated derivatives of D-glucosamine were synthesized and evaluated as cytotoxic anti-cancer agents [1–3]. The intent was to create analogs of natural-occurring hexosamines that could passively enter cancer cells, compete for nucleotide precursors and inhibit ongoing glycoconjugate and RNA/DNA synthesis pathways [1–3]. This metabolic antagonism could thus potentially alter the expression of cell surface glycoconjugates and/or attenuate cell proliferation. As such, it was proposed that glucosamine analog treatment could change the antigenicity of cancer cell surface and boost immune-mediated mechanisms or simply block cancer growth. Indeed, though evidence suggested that these glucosamine analogs could be metabolized by cancer cells [1–3], precisely how they blocked glycan formation or whether nucleotide-sugar analog conjugates were even formed and utilized by glycosyltransferases to incorporate into growing oligosaccharide chains was still unknown.

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It was later shown that, at non-growth inhibitory concentrations, the fully acetylated and 4-fluorinated glucosamine analog, 2-acetamido-2,4-dideoxy-1,3,6-tri-O-acetyl-4-fluoro-D-glucopyranose (4-F-GlcNAc), could alter the structure and function of N- and O-glycans on ovarian and colon cancer cell glycoproteins [2,4–8]. These alterations inhibited human ovarian cancer (OCa) and colon cancer (CCa) cell binding to galectin-1 (Gal-1) and endothelial (E)-selectin, respectively [2,4–8]. These results provided hope that modulating N-acetyllactosamine (galactose β 1,4 N-acetylglucosamine) synthesis essential for both Gal-1- and E-selectin-binding activity, could actually interfere with lectin-mediated cell adhesion events critically involved in the metastatic process. In fact, because OCa cell adhesion to the extracellular matrix underlying the peritoneal lining and CCa cell trafficking to liver are, in part, mediated by Gal-1 and E-selectin, respectively, there remains much promise for these sugar analogs to be developed as anti-metastatic therapeutics [4–18]. Nevertheless, these results offered the experimental rationale for targeting glyco-metabolically active cells distinct from cancer cells, where glycoconjugate processing and function provide key cell behavioral features without posing a significant threat to cell viability.

Subsequent research on fluorinated glucosamine analogs transitioned in intent to altering key glycan-recognition determinants on skin-homing T cells that initiate their entry into skin [19]. Effector/memory T cells educated to enter dermal tissues require the E-selectin-binding glycan, sialyl Lewis X, for binding E-selectin constitutively expressed on dermal microvessels and for initiating a cascade of adhesive events that facilitates diapedesis. Given evidence on E-selectin-binding glycan downregulation in 4-F-GlcNAc-treated CCa cells, it was hypothesized that 4-F-GlcNAc treatment could inhibit sialyl Lewis X synthesis on skin-homing T cells and block E-selectin-mediated adhesion to dermal microvessels and recruitment into inflamed skin. Indeed, studies showed that antigen-dependent CD4⁺ T cell activation in skin-draining lymph nodes triggered sialyl Lewis X biosynthesis that conferred a susceptibility to 4-F-GlcNAc antagonism [19,20]. Treatment with 4-F-GlcNAc reduced the level of sialyl Lewis X and corresponding E-selectin-binding activity, which blunted antigen-dependent T cell-mediated inflammation in the skin [9–22]. These inhibitory effects were similarly found on human leukemic cells treated with a peracetylated 4-fluorinated N-acetylgalactosamine analog [23].

To understand more globally the anti-carbohydrate effects on T cells by fluorinated glucosamine analogs, further evaluations focused on analysis of Gal-1 binding to T cells following 4-F-GlcNAc treatment. Considering Gal-1's key role in binding and inducing apoptosis or immunoregulatory activity in effector CD4⁺ T cells [24–30], understanding the relative difference between inhibition of Gal-1-binding and E-selectin-binding was an important distinction to ascertain. It can be construed that inhibiting the N-acetyllactosamine lattice required for Gal-1-mediated inflammatory silencing of activated/effector T cells may paradoxically offset a deficit in T cell trafficking due to lowering of E-selectin-binding glycans. It was, in fact, demonstrated that the expression of Gal-1-binding glycans on activated CD4⁺ T cells was more sensitive than E-selectin-binding glycans to 4-F-GlcNAc treatment [31]. In support of this observation, earlier studies analyzing the specificity of glycoconjugate action showed that 4-F-GlcNAc preferentially limits the synthesis of N-acetyllactosamines in N- and O-glycans on glycoproteins and not on neolactoglycosphingolipids, which can also function as E-selectin-binding glycans [19–21,31–35].

Collectively, these findings suggested that Gal-1-binding glycans and related triggering of downstream immune tolerogenic pathways may be a more amenable target for 4-F-GlcNAc treatment. This notion posed new questions on how 4-F-GlcNAc can most effectively be used for N-acetyllactosamine antagonism and whether 4-F-GlcNAc should indeed be

developed as an anti-inflammatory therapeutic. We have thusly postulated that interfering with the synthesis of Gal-1-binding glycans in effector T cells would be perhaps more applicable in the context of cancer immunotherapy, whereby anti-tumor T cells treated with 4-F-GlcNAc would be resistant to Gal-1-mediated tumor immune evasion [31].

B. Mechanism of Fluorinated Glucosamine Anti-Carbohydrate Action

Early evidence suggested that fully acetylated variants of glucosamine could effectively traverse a cancer cell's plasma membrane and sequester endogenous UTP and CTP pools necessary for steady-state RNA/DNA biosynthesis, which elicited a cytotoxic effect in cancer cells [2,3]. Furthermore, it was found that diversion of endogenous UTP pools by exogenous glucosamine analog treatments could elevate UDP-N-acetylglucosamine levels, thereby lowering native pools of other nucleotide-sugars, such as UDP-galactose and UDP-N-acetylgalactosamine needed for N- and O-glycan extension [2,3].

Fluorinated glucosamine analogs were synthesized by addition of a fluorine atom, which cannot form a glycosidic bond, at strategic pyranose ring carbons that could potentially antagonize oligosaccharide elongation [1–3]. As such, by substituting fluorine for a hydroxyl group at the carbon-4 position, it was theorized that, upon entering a cell and incorporation into a growing poly-N-acetylglucosaminyl chain, 4-F-GlcNAc could block glycosidic bonding of a galactose residue at the carbon-4 position. All data on the anti-glycoconjugate effects of 4-F-GlcNAc efficacy subsequently, in fact, indicated that synthesis of (galactose β 1,4 N-acetylglucosamine)_n residues and related sialyl Lewis X moieties were inhibited [19–22]. More recent structural data provided a more refined assessment in that N-acetylglucosamines and sialyl Lewis X on N-glycans and on core 2 O-glycans were reduced and the content and structural diversity of tri- and tetra-antennary N-glycans and of O-glycans were reduced, while biantennary N-glycans were increased. However, MALDI-TOF mass spectrometry analysis did not reveal any m/z ratios relating to the presence of fluorine atoms in N- and O-glycans released from 4-F-GlcNAc-treated human T cells and leukemic cells, indicating that 4-F-GlcNAc did not in fact incorporate into and truncate glycan chains [31]. 4-F-GlcNAc treatment also neither affected the expression nor activity of N-acetylglucosamine-synthesizing enzymes or the level of sialyl Lewis X on glycolipids [31]. As expected though, 4-F-GlcNAc did significantly reduce intracellular levels of UDP-GlcNAc [31], which was validated by another group assessing the mechanism of acetylated and fluorinated glucosamine analog action [36]. What was also noted from this recent work was the first evidence of UDP-4-F-GlcNAc donor sugar in 4-F-GlcNAc-treated cancer cells [36].

Cumulatively, data on fluorinated glucosamine action and 4-F-GlcNAc anti-carbohydrate efficacy indicate that Gal-1- and E-selectin-binding reductions are not caused by direct 4-F-GlcNAc incorporation into oligosaccharides and consequent chain termination, but rather by shunting endogenous UDP-GlcNAc synthesis towards production of UDP-4-F-GlcNAc (Figure 1). In that there is no evidence of glycan incorporation of fluorinated analogs, the fluorine residue at the carbon-4 position in UDP-4-F-GlcNAc is likely hindering the ability of N-acetylglucosaminyltransferases to transfer the 4-F-GlcNAc sugar to an oligosaccharide acceptor [31,36]. Alternatively, in addition to titrating out pools of UTP, another potential mode of action is that 4-F-GlcNAc is irreversibly binding and inactivating UDP-acetylglucosamine pyrophosphorylase (UAP) directly. These possibilities need to be investigated further.

C. Blocking N-acetyllactosamine Synthesis in CD8⁺ T cells to Improve Anti-tumor Immunity

The presence of tumor-infiltrating lymphocytes, in particular, CTLs, and their implication in cancer patient survival and prognosis is not clear [37–40]. Moreover, adoptive transfer of autologous anti-melanoma CTLs has shown only a modest improvement in patients with advanced disease [41–43]. These observations suggest that melanomas can innately avert the immune system. Galectins, in particular Gal-1 and Gal-3, have been shown to be produced at high levels by melanoma cells [44–46] as well as certain lymphomas, such as Hodgkin's lymphoma [47–49] and cutaneous T cell lymphoma [27], and found to elicit potent immunosuppressive features [27,47,48,50]. In fact melanoma-derived Gal-1 has been shown to efficiently subdue the effector function of T helper (Th) 1 and Th17 cell subsets as well as CTLs [28,29,50], all of which express a high level of Gal-1-binding N-acetyllactosamines. Since Gal-1 characteristically binds effector CD4⁺ T cells with anti-tumor activity and CTLs and causes apoptosis/tolerization, we have hypothesized that reducing Gal-1-binding to N-acetyllactosamines on anti-tumor T cells could significantly elevate anti-tumor T cell immune activity, particularly against tumors that express copious amounts of Gal-1.

Accordingly, most recent efforts in 4-F-GlcNAc development have centered on anti-tumor studies with the intent of lowering Gal-1-binding glycans, alleviating Gal-1-dependent immunoregulation and boosting anti-tumor T cell immunity. Our laboratory has accumulated some exciting new *in vivo* data on the use of 4-F-GlcNAc to treat melanomas and lymphomas. Melanoma was not only included as a tumor model based on its high Gal-1 expression, but that diminution of E-selectin-binding glycan would be inconsequential. Since microvessels within melanomas do not express E-selectin [51], 4-F-GlcNAc-treated T cells could theoretically still infiltrate melanomas while evading Gal-1-dependent control to elicit their effector function. Using non-toxic doses of 4-F-GlcNAc in mice bearing melanoma or lymphoma, we found that tumor growth was grossly attenuated [52]. These results were, in part, due to 4-F-GlcNAc-dependent sparing of Gal-1-mediated apoptosis of IFN- γ - and IL-17-producing CD4⁺ T cells and of melanoma specific CTLs by reducing their surface content of Gal-1-binding N-acetyllactosamines. In other words, there was a shift in the effector - regulatory T cell balance towards more Th1 and Th17 cells and more CTLs, and less immune regulating IL-10⁺ T cells generated by tumor-derived Gal-1 [26,27,52]. These findings reinforced the importance of N-acetyllactosamines in controlling the fate and function of effector CD4⁺ T cell and CTL subsets and indicated that treating melanomas and lymphomas, which abundantly express Gal-1 and likely other immunosuppressive galectins, with 4-F-GlcNAc could prove to be therapeutically efficacious [52]. In all, these data highlight the promise of 4-F-GlcNAc to limit tumor growth by boosting T cell-mediated immunity [53].

D. Conclusion and Future Prospects of Fluorinated Glucosamine Analogs

There exists a long standing history on the development of fluorinated glucosamine analogs for the metabolic antagonism of glycoconjugate and DNA/RNA synthesis. While early studies focused on the merits of cytotoxic-induction in cancer cells by 4-F-GlcNAc, subsequent efforts re-considered these intents and began to evaluate the targeting of glyco-metabolically-active T cells. In this case, the main objective was to selectively target these T cells, which could rapidly uptake glucosamine analogs to antagonize UDP-GlcNAc production and block the synthesis of sialyl Lewis X necessary for E-selectin-mediated trafficking to inflamed skin. During attempts to illuminate the mechanism of 4-F-GlcNAc-glycan alterations and lectin-binding activities, it was appreciated that Gal-1-binding was more efficiently inhibited than E-selectin-binding activity. These results encouraged later efforts to evaluate 4-F-GlcNAc as an inhibitor of Gal-1-binding N-acetyllactosamine

synthesis in T cells and blocker of the immunoregulatory control of tumor-derived Gal-1 (Figure 2A).

As such, studies from our laboratory showed that 4-F-GlcNAc elicited potent anti-tumor activity by increasing the quantity of effector T cells and reducing the level of immune regulating IL-10⁺ T cells characteristically generated by tumor-derived Gal-1 [26,27,52,53] (Figure 2B). However, much skepticism remains as this mode of therapy could theoretically alter glycosylation in other glyco-metabolically-active leukocytes, such as activated antigen-presenting cells and hematopoietic progenitor cells, wherein lectin-binding events are known to affect their immunologic behavior [54–57]. Future studies are needed to ascertain the relative specificity of anti-glycosylation effects on non-T cells in 4-F-GlcNAc-treated animals.

As a prospective approach, which provides ultimate targeting efficiency, we are pursuing efforts to study 4-F-GlcNAc in the setting of adoptive T cell cancer therapy. Adoptive transfer of autologous tumor-specific CTLs as an anti-cancer therapy is a promising, though imperfect, approach to boost anti-tumor immunity [58–60]. We believe that 4-F-GlcNAc treatment of *in vitro*-expanded tumor Ag-specific CTLs will generate CTLs lacking not only Gal-1-binding N-acetylglucosamines but potentially Gal-9-binding TIM-3 and other immunoregulatory galectin ligands, enhancing their tumoricidal activity due to their insensitivity to galectin(s)-dependent regulation (Figure 2C). Since CTLs express an abundance of N-acetylglucosamines, tailoring 4-F-GlcNAc treatment with this *ex vivo* approach could improve the quantity, longevity and anti-tumor activity of CTLs, particularly against tumors that overexpress Gal-1, Gal-9 and other immunoregulatory galectins. Whereas a neutralizing antibody against a single galectin could help alleviate respective galectin-dependent immunoregulation, 4-F-GlcNAc's ability to potentially inhibit the synthesis of Gal-1, Gal-3 and Gal-9 binding glycans on CTLs could indeed offer a multifaceted strategy, in which multiple galectin regulators are neutralized [53]. This N-acetylglucosamine reduction strategy could even be advantageous to help boost the potency of cancer vaccines or viral vaccines for other diseases, wherein tumor- and virus-specific CTLs are key regulators of an efficacious immunologic response, though have been shown to be negatively regulated by Gal-9 [61,62]. Moreover, combining 4-F-GlcNAc with a vaccine delivery method, such as skin scarification, can perhaps synergize the efficacy of immunologic response and help maintain protective immunity [63,64]. The mechanism and functional consequences of 4-F-GlcNAc efficacy on CTLs have reinvigorated the prospect of using fluorinated glucosamines as a novel immunotherapeutic adjuvant for treating cancer and for boosting vaccine efficacy against infectious diseases.

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Highlights

- Galectin-1 (Gal-1) is commonly expressed at a high level by tumor cells and causes immune suppression of anti-tumor T cells.
- Gal-1 – Gal-1-binding carbohydrate interactions play a pivotal role in shaping the effector function of anti-tumor T cells.
- Gal-1-binding carbohydrates, known as N-acetyllactosaminyl glycans, are targetable entities on anti-tumor T cells.
- Fluorinated glucosamine analogs are effective metabolic inhibitors of N-acetyllactosamine formation and, thus,
- potentially efficacious methods for thwarting Galectin-1-mediated immune suppression of effector anti-tumor T cells.

A Glycometabolically-active Gal-1 Ligand⁺ T cells

B 4-F-GlcNAc-Treated T cells

Figure 1. Mechanism of Peracetylated 4-Fluorinated Glucosamine Anti-Carbohydrate Action
(A) T cells actively synthesizing cell surface glycoconjugates are sensitive to glycometabolic antagonists, such as fully acetylated 4-F-GlcNAc. **(B)** Following passive cellular entry and deacetylation of peracetylated 4-fluorinated-glucosamine, 4-F-GlcNAc is phosphorylated and conjugated to UTP by UDP-acetylglucosamine pyrophosphorylase (UAP). Depending on the cytosolic concentration of 4-F-GlcNAc, endogenous UDP-GlcNAc levels are compromised due to sequestration of UTP. This shunting effect causes reductions in N-acetylglucosamines necessary for extending and branching of N- and O-glycan antennae characteristically bound by Gal-1.

A**Gal-1-mediated T cell Regulation and Melanoma Immune Evasion Hypothesis** **B****4-F-GlcNAc Action and Anti-tumor Immune Activity** **C****Proposed Use & Evaluation of 4-F-GlcNAc**

Figure 2. Neutralization of the Gal-1 – Gal-1 ligand axis to Boost Anti-tumor T cell Immunity
(A) A schematic depiction of tumor immune evasion model in which melanoma-derived Gal-1 can antagonize the anti-tumor efficacy of T cells. **(B)** A cartoon depiction of the metabolic antagonism of Gal-1-binding N-acetylglucosamine synthesis in effector anti-tumor T cells caused by 4-F-GlcNAc and consequent enhancement of anti-tumor immunity. **(C)** A schematic overview of a novel cancer immunotherapeutic strategy to selectively target donor CTLs with 4-F-GlcNAc for escaping Gal-1 control and improving the efficacy of adoptive T cell therapy.