Effects of Alkylation and Immunopotentiation against Ehrlich Ascites Murine Carcinoma In Vivo Using Novel Tetra-O-Acetate Haloacetamido Carbohydrate Analogs

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · MAY 2015

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2015.05.022

4 AUTHORS, INCLUDING:

Matthew Trendowski
Syracuse University
22 PUBLICATIONS **85** CITATIONS
SEE PROFILE

Timothy D Christen
Syracuse University
6 PUBLICATIONS **16** CITATIONS
SEE PROFILE

Thomas P. Fondy
Syracuse University
67 PUBLICATIONS **932** CITATIONS
SEE PROFILE

European Journal of Medicinal Chemistry 98 (2015) 149–159

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Research paper

Effects of alkylation and immunopotentiation against Ehrlich ascites murine carcinoma in vivo using novel tetra-O-acetate haloacetamido carbohydrate analogs

Matthew Trendowski*, Timothy D. Christen, Joseph N. Zoino, Thomas P. Fondy

Department of Biology, Syracuse University, 107 College Place, Syracuse, NY 13244, USA

ARTICLE INFO

Article history:
Received 1 April 2015
Received in revised form
9 May 2015
Accepted 14 May 2015
Available online 18 May 2015

Keywords:
Alkylating agents
Leukocyte influx
Carbohydrate analogs
Chemotherapy
Immunopotentiation

ABSTRACT

Tetra-O-acetate haloacetamido carbohydrate analogs (Tet-OAHCs) are novel alkylating agents that appear to have alkylating activity at the plasma membrane, specificity against neoplastic cells, and may potentiate host leukocyte influx. This study sought to characterize the chemical attributes and *in vivo* activity of Tet-OAHCs. Four Tet-OAHCs were assessed for their partition coefficient and alkylating activity to determine cellular environments where adduct formation would be favorable. *In vitro*, IC_{50} values of all four Tet-OAHCs were determined against Ehrlich ascites murine carcinoma, as well as two leukemias (U937 human monocytic leukemia and L1210 murine lymphoid leukemia) to assess their cytotoxicity in multiple neoplastic cell lines. *In vivo*, B6D2F1 and CD2F1 mice were challenged i.p. with Ehrlich ascites carcinoma prior to, or after being treated with a single dose of one of the analogs. Finally, a quantitative comparison of host leukocyte influx between Tet-OAHCs and other alkylating agents was performed to confirm previous *in vivo* observations that the tetra-O-acetate carbohydrate moiety is important for inducing a host leukocyte response in murine models.

The results can be summarized as follows: 1) Tet-OAHCs appear to demonstrate high alkylating activity in amphiphilic environments. 2) All four congeners have comparable *in vitro* cytotoxicities against the neoplastic cell lines examined. 3) The analogs demonstrate marked *in vivo* activity in both B6D2F1 and CD2F1 mice challenged with a lethal dose of Ehrlich ascites carcinoma, and frequently produce long term survival at 60 days, which is not observed in simple halo derivatives or two currently approved antineoplastic agents (daunorubicin and mechlorethamine). These effects are observed when the agents are administered either before or after the tumor challenge. 4) The carbohydrate moiety appears to be important for potentiating host leukocyte influx, as Tet-OAHCs, but not other alkylating agents demonstrated such activity *in vivo*.

1. Introduction

Alkylating agents have been a mainstay in cancer chemotherapy due to their ability to readily interfere with the rapid mitotic progression of malignant cells. These agents are known for alkylating nucleophilic DNA (often at N-7 in guanine bases), prompting the formation of DNA adducts, crosslinking in the presence of difunctional agents, and ultimately apoptosis [1,2]. Although alkylating agents and related platinating agents have been vital for the success of current chemotherapeutic protocols, there are notable limitations. Their crosslinking capability is considerably reduced in the presence of the DNA-repair enzyme O-6-methylguanine-DNA methyltransferase (MGMT) [1,3,4]. Alkylating-like agents also face a similar problem of rapid DNA repair as the propensity of nucleotide excision repair (NER) is ever-present in a substantial variety of cancers [2]. Therefore, the ability of malignant cells to repair damaged DNA is an apparent constraint on the efficacy of currently approved alkylating agents.

Although DNA has been the nucleophilic target of all clinically approved alkylating agents, it may also be possible to alkylate the plasma membrane of malignant cells to exert antineoplastic activity. It has been known for quite some time that plasma membrane proteins exposed on the cell surface have important biological functions, such as cell signaling, ion transport, and cell–cell and cell-matrix adhesion interactions [5–8]. Due to recent advances in genomic, transcriptomic, and proteomic analysis, it has

* Corresponding author.
E-mail address: mrtrendo@syr.edu (M. Trendowski).

http://dx.doi.org/10.1016/j.ejmech.2015.05.022

been elucidated that the expression level of many plasma membrane proteins is altered in malignant cells [5,6,9]. Such protein alterations often confer metastatic properties, creating a target for monoclonal antibodies and other forms of immunotherapy. While these approaches target specific aberrancies on the cell surface, alkylating agents would react nonspecifically with functional groups on the exterior of the plasma membrane, circumventing the need for a designated target to be present.

Although cell-surface biochemistry is a suitable target for chemotherapeutic investigation, the characteristics of agents that affect exterior plasma membrane signaling to produce therapeutic benefit are less inherent. A logical target may be cell surface glycoconjugates, as they are pivotal in surface membrane biochemistry, and altering such signaling through alkylation could have considerable antineoplastic potential. Carbohydrates are involved in the adhesion of cells to substrates, as well as their adherence to each other. They have been shown to change in accessibility as a function of the cell cycle, and have been indicated to play a pivotal role in cell differentiation [9,10]. Further, cell surface carbohydrates have a profound influence on host immune response. Lectin-like carbohydrate binding sites are integral for the interaction of cytokines with their targets [11–13]. Carbohydrates are also involved in chemotaxis of granulocytes. This is exemplified by N-acetylated-hexosamines, as they potentiate neutrophil recognition and phagocytosis of polyglutaraldehyde microspheres when covalently bound [14]. Mononuclear agranulocytes are also considerably influenced by carbohydrate moieties, as macrophages possess carbohydrate-binding sites related to recognition, binding, and processing of target molecules and cells. Even the humoral immune response is markedly characterized by carbohydrate dependence, as T-lymphocytes have lectin-like carbohydrate receptors that have been shown to affect antigen-specific *in vitro* assays [14,15].

We have previously shown that halo carbonyls with antineoplastic activity *in vivo* (including haloacetamides) act as non-charged lipophilic or amphiphilic electrophiles [14–16]. Further, the compounds with the most antitumor activity in these series are strong alkylating agents, and react by S_N2 mechanisms, enabling a concerted reaction to occur in lipophilic environments (such as the plasma membrane). Since antitumor activity is dependent on the polarity and alkylating activity of halo carbonyls, it has been postulated that these analogs exert their effects by alkylating cell surface nucleophiles in addition to intracellular nucleophiles (such as DNA or proteins not found at the plasma membrane). Due to the potential importance a carbohydrate moiety could have in developing novel alkylating agents with plasma membrane activity, and to improve the specificity of haloacetamides, we have synthesized novel tetra-O-acetate haloacetamido carbohydrate analogs (Tet-OAHCs). These compounds have the R group substituted with carbohydrate derivatives found on the plasma membrane (Fig. 1), and appear to have high alkylating activity, marked antitumor activity *in vivo* against solid tumors and disseminated cancers [14,15,17], and may potentiate host leukocyte influx at the site of administration and tumor challenge [17]. Acetylation of carbohydrate hydroxyl groups has proven to be necessary, as attaching a hydrophilic moiety to a haloacetamide significantly reduces antitumor activity, as observed by haloacetamides with free sugars [14,15].

In regards to potentiating leukocyte influx, we have shown that when N-bromoacetyl-β-D-glucosamine tetra-O-acetate produced long term survival in mice challenged with Ehrlich ascites murine carcinoma after a single injection (0.11 mmol/kg), it was observed that the sites of tumor challenge and drug administration had a rapid granulocyte influx [17]. Further, N-bromoacetyl-β-D-galactosamine tetra-O-acetate injected i.p. at the same dose one day

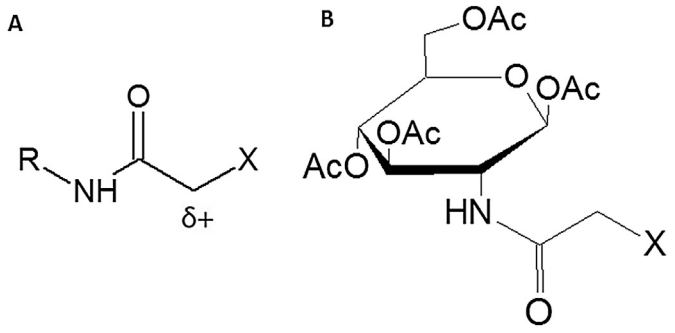

Fig. 1. Characterization of tetra-O-acetate haloacetamido carbohydrate analogs. A) General structure of haloacetamides. Reactivity at the CH_2X group is controlled by the electron withdrawing or donating power of the R group, and the corresponding pK_a of the parent amine. The hydrogen bond donating and accepting properties of the amide nitrogen allow the reactivity to be modulated by the dielectric constant of the *in vivo* environment. B) Basic structure of tetra-O-acetate haloacetamido carbohydrates (Tet-OAHCs).

prior to a challenge of 1×10^6 Ehrlich ascites carcinoma cells conferred substantial resistance to lethal tumor outgrowth when compared to vehicle-treated control animals. Although potential plasma membrane alkylating chlorohydroxyacetone-benzoate derivatives were shown to produce some long term survival in the same tumor system [18], they were not associated with an increased granulocyte influx, nor did pretreatment of such compounds one day prior to tumor challenge confer any noticeable resistance.

Although our previous experimental data suggest that Tet-OAHCs are potent alkylating agents capable of potentiating leukocyte influx, most of the data have been circumstantial. We have yet to definitively confirm the partition coefficient and alkylating activity of these congeners as we previously have with other halo carbonyls, as well as demonstrate that the increased immune response *in vivo* can be quantitatively attributed to the acetylated carbohydrate moiety. Therefore, the present study intends to further characterize the chemical attributes of the analogs by evaluating their partition coefficients and alkylating activities. In addition, we will confirm the *in vitro* antineoplastic activity of Tet-OAHCs by assessing their cytotoxicity against Ehrlich ascites carcinoma, as well as U937 human monocytic leukemia and L1210 murine lymphoid leukemia. *In vivo* antineoplastic activity will be assessed by challenging large cohorts of B6D2F1 and CD2F1 mice with a normally lethal i.p. injection of Ehrlich ascites carcinoma cells, prior to being treated with a single dose of one of the analogs, while also acquiring the ED_{50}, LD_{50}, and therapeutic indices of these congeners. We also compare the ability of Tet-OAHCs to potentiate long term survival in mice challenged with Ehrlich ascites carcinoma with clinically approved agents (daunorubicin and mechlorethamine). Finally, a quantitative comparison of host leukocyte influx between Tet-OAHCs and another lipophilic monofunctional non-carbohydrate alkylating agent that appears to have activity toward the plasma membrane, bromohydroxyacetone benzoate (BrHAB), will be performed to confirm previous *in vivo* observations that the tetra-O-acetate carbohydrate moiety is important for inducing a host leukocyte response in murine models.

2. Materials and methods

2.1. Preparation of neoplastic cell lines

Ehrlich ascites murine carcinoma (ATCC® CCL-77), L1210 murine lymphoid leukemia (ATCC® CCL-219), and U937 human monocytic leukemia (ATCC® CRL-1593.2) were acquired commercially. Ehrlich

ascites carcinoma was seeded at 4×10^4 cells/cm^2 in 75 cm^2 culture flasks and suspended in NCTC 135 medium supplemented with 10% fetal bovine serum. Suspension leukemia cell lines were seeded at similar concentrations (5.2×10^4 cells/ml) in 75 cm^2 flasks, but were suspended in 20% fetal bovine serum (FBS) in Iscove's medium without glutamine, with the following added: 200 units/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamicin sulfate, 40 μM glutamine (50 μl of 2 mM glutamine per 5 ml medium), and 50 μl of amphotericin B (2.5 μg/ml concentration) per 5 ml of medium. Flasks were incubated in 5% CO_2 at 37 °C. Once confluent, Ehrlich ascites carcinoma cells were trypsinized with 0.05% trypsin-EDTA solution 1X (Sigma–Aldrich Corp., St. Louis, MO, USA) for 5 min at 37 °C, dislodged by a sharp knocking of the flasks during that period, washed, diluted to 10 ml with fresh complete medium, and cells were seeded into 75 cm^2 culture flasks at a 1:3 subcultivation ratio.

A Z2 Beckman–Coulter® Particle Count and Size Analyzer (Beckman Coulter Inc., Brea, CA, USA) along with a Bio-Rad® TC20 Automated Cell Counter (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to assess the inhibitory effects of the four Tet-OAHCs. Cell viability was determined with trypan blue staining and analysis with the TC20 cell counter. In addition, cell viability was assessed with a XTT Cell Proliferation Assay Kit (ATCC® 30–1011K). 100 μl of cells were seeded per well into a flat-bottom 96-well microtiter plate in triplicate for each cell dilution. The plate was incubated for 24 h prior to addition of XTT solution. Cells were then incubated for an additional 2 h before the wavelength was read.

2.2. Syntheses of tetra-O-acetate haloacetamido carbohydrate analogs

The molecular structures of all four 2-deoxy-2-bromoacetamido-D-hexose 1,3,4,6 tetra-O-acetates examined in the present study, N-bromoacetyl-α-D-glucosamine tetra-O-acetate (Br-Tet-O-α-glucosamine), N-bromoacetyl-β-D-glucosamine tetra-O-acetate (Br-Tet-O-β-glucosamine), N-bromoacetyl-β-D-galactosamine tetra-O-acetate, (Br-Tet-O-β-galactosamine), and N-bromoacetyl-α-D-mannosamine tetra-O-acetate (Br-Tet-O-α-mannosamine), are presented in Fig. 2A, and were synthesized as previously described [14,15,17]. Bromoacetamido analogs were synthesized as these congeners have demonstrated more antineoplastic activity than other halogen acetamido analogs we have investigated [15]. Simple haloacetamides, haloacetates, and non-hexose haloacetamido derivatives examined in the present study were acquired commercially (Sigma–Aldrich Corp.), or were synthesized from derivatives similar in structure.

2.3. Assessing the partition coefficients and alkylating activities of tetra-O-acetate haloacetamido carbohydrate analogs

The partition coefficient between n-octanol and water was determined by A_{240} measurement of solutions of the various compounds in either n-octanol or water prior to extraction, and in both phases after extraction. This method has been checked using compounds of known log P, by high performance liquid chromatography, thin layer chromatography, and by organic halide analysis. Analogs with log P values ranging from −2 to +2 were denoted as having an intermediate partition coefficient. Alkylating activity at 37 °C in aqueous conditions, pH 7.1, was determined by an assay that used the initial pseudo-first-order rate constant of halide loss in the presence of 5% pyridine-acetate (Fig. 2B). This was done to determine alkylating activity as a function of the dielectric constant of the solvent medium. In addition, this assay can be used to monitor the rate of alkylation in different solvent media, which will be important in future attempts to design membrane-directed agents that affect electron donors in a medium of low polarity, as is the case with the plasma membrane. Alkylating activity was assessed by determining the mM X^- loss/min/mM RX for each compound. Values were in accordance to those acquired by the Friedman and Boger S_N2 nucleophilic substitution method using p-nitrobenzylpyridine in a solvent of 83% acetone with 0.1 M inorganic phosphate (Pi), pH 7.2 and temperature at 37 °C [19].

2.4. Ehrlich ascites murine carcinoma in vivo

B6D2F1 and CD2F1 mice were challenged with a normally lethal i.p. injection of 2.5×10^7 Ehrlich ascites carcinoma cells, prior to being treated with one of the analogs. The 2.5×10^7 cell injection of Ehrlich ascites carcinoma is ten times the minimum dose lethal to 95% of B6D2F1 mice [17], and produced a median survival of 16 days when mice were treated only with the vehicle or left untreated.

2.5. Formulation of tetra-O-acetate haloacetamido carbohydrate analogs for assessing In vivo antineoplastic activity

Br-Tet-O-α-glucosamine, Br-Tet-O-β-glucosamine, Br-Tet-O-β-galactosamine, and Br-Tet-O-α-mannosamine were suspended in 10% tween 80 made up to 0.85% NaCl solution. The agents were then administered as single i.p. injections.

2.6. Assessing the leukocyte influx in B6D2F1 mice following administration of tetra-O-acetate haloacetamido carbohydrate analogs

B6D2F1 mice were administered a single ED_{50} dose i.p. of Br-Tet-O-β-glucosamine or Br-Tet-O-β-galactosamine (ED_{50} against 2.5×10^7 Ehrlich ascites carcinoma cells injected i.p. 24 h prior to administration of the analogs). 8 mice served as background controls for peritoneal cells in B6D2F1 mice. Untreated controls were sacrificed one day prior to drug administration in the other treatment groups. All mice had their total peritoneal contents recovered, counted, and analyzed with Wright-Giemsa stain.

3. Results

3.1. In vitro efficacy of tetra-O-acetate haloacetamido carbohydrate analogs

Tet-OAHCs appeared to have similar activity against suspension cell lines (U937 and L1210) and the adherent Ehrlich ascites carcinoma cells, with comparable IC_{50} values being attained after treatment for 48 h (Fig. 3). Two exceptions were the increased cytotoxicity Br-Tet-O-α-glucosamine had towards U937 (2.10 μM), and the slight, but noticeably decreased cytotoxicity of Br-Tet-O-α-mannosamine against all cell lines.

3.2. Efficacy of tetra-O-acetate haloacetamido carbohydrate analogs on potentiating long term survival in Ehrlich ascites murine carcinoma

All four Tet-OAHCs appeared to potentiate long term survival to at least 60 days when administered as a single i.p. injection in both B6D2F1 and CD2F1 mice challenged i.p. with a normally lethal dose of 2.5×10^7 Ehrlich ascites carcinoma cells (Table 1). While mice treated only with the vehicle lived for a median of 16 days, CD2F1 mice treated with Br-Tet-O-β-glucosamine reached 60 day survival at a rate of 96.4% (27/28 mice), with successful treatment requiring as little as 0.07 mmol/kg. Similar results were attained in the

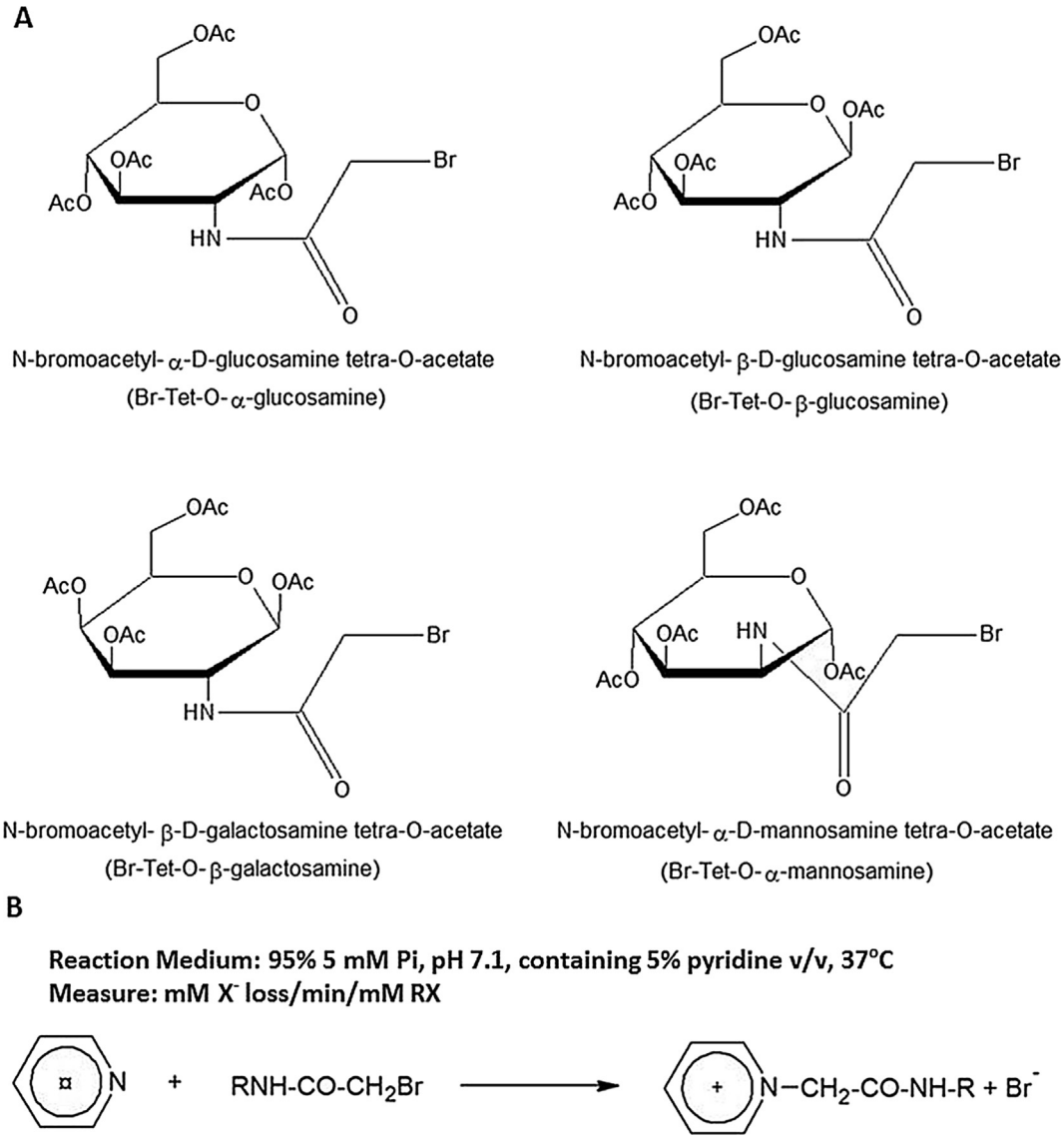

Fig. 2. Molecular structures of 2-deoxy-2-bromoacetamido-D-hexose 1,3,4,6 tetra-O-acetates, and the assay used to determine their alkylating activity. A) Four Tet-OAHCs were examined in the present study. Abbreviations of the compounds used are given underneath the formal name. B) Assay used to determine alkylating activity of Tet-OAHCs.

B6D2F1 mice cohort treated with a single dose of Br-Tet-O-β-glucosamine (ranging from 0.09 to 0.15 mmol/kg), as these mice were alive 60 days after the initial tumor challenge at a rate of 91.9% (34/37 mice). Br-Tet-O-β-galactosamine also exhibited marked antitumor activity, with 62.5% (5/8 mice) CD2F1 mice surviving to at least 60 days when treated with a single dose ranging from 0.05 to 0.08 mmol/kg, and 81.8% (166/203 mice) B6D2F1 mice surviving the same length of time when treated with 0.06–0.15 mmol/kg. Br-Tet-O-α-mannosamine was only examined in CD2F1 mice, but elicited a 90% (18/20 mice) survival rate 60 days after the initial tumor challenge when administered at 0.13–0.29 mmol/kg 0.045–0.11 mmol/kg. Br-Tet-O-α-glucosamine potentiated long term survival at 58.3% (7/12 mice), with three of those deaths possibly attributed to drug toxicity, as the LD_{50} of the tetra-O-acetate derivative of Br-Tet-O-α-glucosamine is 0.11 mmol/kg (three mice received the LD_{50} in this cohort). Nevertheless, all Tet-OAHCs examined potentiated long term survival at a much higher rate than daunorubicin, including when the anthracycline was administered on two consecutive days (4/20 mice), rather than a single day (0/10 mice; Table 1).

Interestingly, it appears that the functional group used to make the carbohydrate more lipophilic has a profound influence on antineoplastic activity as a tetra-O-propionate derivative of β-glucosamine did not produce any long term survival in CD2F1 mice at 60 days (0/8 mice), even when administered at a higher concentration (0.75 mmol/kg) in comparison to the other bromoacetamido-D-hexose analogs (Table 1). In addition, it appears that the Tet-OAHCs have similar ED_{50} values (0.04 mmol/kg to 0.12 mmol/kg), but variable LD_{50}values (0.11 mmol/kg to 0.41 mmol/kg), producing therapeutic indices ranging from 2.8 (Br-Tet-O-α-glucosamine and Br-Tet-O-α-mannosamine in CD2F1 mice) to 5.2 (Br-Tet-O-β-glucosamine in CD2F1 mice).

3.3. Efficacy of simple haloacetamides, haloacetates, and non-hexose haloacetamido derivatives in CD2F1 Ehrlich ascites tumor-bearing mice

Tet-OAHCs appear to have potent antineoplastic activity *in vivo* against Ehrlich ascites carcinoma not observed in other haloacetamides and haloacetates (Table 2). While Tet-OAHCs could

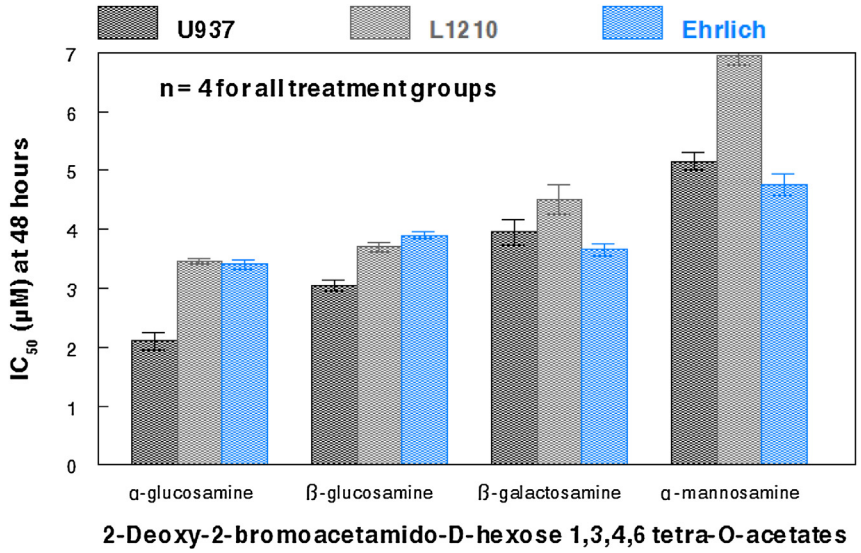

Fig. 3. 50% inhibitory concentrations of 2-deoxy-2-bromoacetamido-D-hexose 1,3,4,6 tetra-O-acetates at 48 h against U937 human monocytic leukemia, L1210 murine lymphoid leukemia, and Ehrlich ascites murine carcinoma. IC_{50} values were assessed with two separate cell counters and trypan blue exclusion, as well as with an XTT kit. Bars represent standard error of the mean (SEM) of 4 individual Tet-OAHC treatments for all cell lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Efficacy of 2-deoxy-2-bromoacetamido-D-hexose 1,3,4,6 tetra-O-acetates and daunorubicin in ehrlich ascites tumor-bearing mice.

Agent	Mice	LD_{50}/ED_{50} (mmol/kg)	Survivors at 60 days (number/treated)	Dose range (mmol/kg)	Therapeutic Index
β-Glucosamine					
Tetra-O-Acetate	CDF	0.31/0.06	27/28	0.07–0.37	5.2
	BDF	[a]0.36/0.07	34/37	0.09–0.15	5.1
Tetra-O-Propionate	CDF	0.89/NE	0/8	0.75	–
β-Galactosamine	CDF	0.22/0.05	5/8	0.05–0.08	4.4
	BDF	0.24/0.07	166/203	0.06–0.15	3.4
α-Mannosamine	CDF	[a]0.33/0.12	18/20	0.13–0.29	2.8
α-Glucosamine	CDF	[a]0.11/0.04	7/12	0.045–0.11	2.8
Daunorubicin	CDF	5.78×10^{-3}/NE	0/10	5.68×10^{-4} (1x)[b]	–
			4/20	5.68×10^{-4} (2x)[b]	–

[a] LD_{50} values were found in tumor challenged mice. Other LD50 values were found in nontumor-bearing mice.
[b] Daunorubicin was administered on either day 1 only, or days 1 and 2, while the other agents were administered only on day 1. ED_{50} refers to a dose that elicited 50% survivors at day 40. Therapeutic index = LD_{50}/ED_{50}. Mice were given a single i.p. injection (other than daunorubicin 2x) of all agents one day after an i.p. tumor challenge of 2.5×10^7 Ehrlich ascites carcinoma cells. BDF refers to B6D2F1 mice, while CDF refers to CD2F1 mice. NE: not evaluated.

attain long term survival 60 days after the initial tumor challenge at rates as high as 96.4%, haloacetamides and haloacetates not conjugated to an acetylated carbohydrate hardly ever elicited such a response. None of the 6 non-hexose haloacetamides examined potentiated long term survival to 60 days, even when $ClCH_2CONH_2$ was administered at 0.81 mmol/kg (LD_{50} = 0.86 in CD2F1 mice). While one of the halo acetates ($BrCH_2COO^-$) produced a survival rate of 25% (4/16 mice) 60 days after the initial tumor challenge, no other compound in Table 2 potentiated long term survival at a rate higher than 8% ($CH_3C(CH_2OH)_2NHCOCH_2Br$; 2/25 mice), and most did not have any survivors at the 60 day mark. Further, $BrCH_2COO^-$ had a therapeutic index of 1.06, and requires too high of a concentration to elicit any sustainable therapeutic benefit.

3.4. Effects of partition coefficient and alkylating activity on antineoplastic activity of haloacetamides in CD2F1 mice challenged with Ehrlich ascites murine carcinoma

It appears that both partition coefficient and alkylating activity have a substantial influence on the antineoplastic activity of haloacetamides in Ehrlich ascites tumor-bearing CD2F1 mice (Table 3). Br-Tet-O-β-glucosamine, which potentiated a 96.4% survival rate at 60 days had alkylating activity = 8.6×10^{-2} mM X^- loss/min/mM RX, with an intermediate log P value of 0.23. In addition to Br-Tet-O-β-glucosamine, the other active Tet-OAHCs had similar alkylating activities and intermediate partition coefficients. Interestingly, while the tetra-O-propionate β-glucosamine bromoacetamide had the highest alkylating activity (9.6×10^{-2} mM X^- loss/min/mM RX), it did not produce any long term survival in CD2F1 mice challenged with Ehrlich ascites carcinoma (Table 1). Interestingly, this tetra-O-propionate derivative had a lipophilic log P value of 2.25, suggesting that increasing the lipophilicity of Tet-OAHCs beyond an intermediate range might be detrimental to their efficacy. This is further supported by the non-carbohydrate bromoacetamide 2-bromoacetamido-4-nitrophenol, which has an alkylating activity higher than two of the active Tet-OAHCs (7.7×10^{-2} mM X^- loss/min/mM RX), but is more lipophilic (log P = 1.78), and is less active against Ehrlich ascites carcinoma; this compound was assessed for antineoplastic activity in Ehrlich ascites tumor-bearing mice in Ref. [17]. We have previously demonstrated that the free sugar β-glucosamine bromoacetamide has considerably reduced antineoplastic activity [17], and this may now also be attributed to its relatively low alkylating activity

Table 2
Activity levels of simple haloacetamides, haloacetates, and non-hexose haloacetamide derivatives in CD2F1 ehrlich ascites tumor-bearing mice.

Compound	LD_{50}/ED_{50} (mmol/kg)	Survivors at 60 days (number/treated)	Dose range (mmol/kg)	Medium Life vs control (%)	Therapeutic Index
Haloacetamides					
$ClCH_2CONH_2$	0.86/NE	0/5	0.81	100	<1
$BrCH_2CONH_2$	0.24/NE	0/15	0.11–0.25	100	<1
ICH_2CONH_2	0.19/NE	0/5	0.15	100	<1
$BrCH_2CONHEt$	0.52/NE	0/8	0.30–0.45	100	<1
$BrCH_2CONHnPr$	0.62/NE	0/25	0.12–0.40	100	<1
$BrCH_2CONHnHex$	0.36/NE	0/7	0.26	100	<1
Haloacetates					
$BrCH_2COO^-$	0.49/NE	4/16	0.30–0.65	100	<1
$BrCH_2COOMe$	0.20/NE	0/5	0.15	100	<1
$BrCH_2COOEt$	0.32/NE	0/28	0.17–0.37	100	<1
Derivatives of 2-Methyl-Amino 1,3 Propane Diol					
$CH_3C(CH_2OH)_2NHCOCH_2Br$	0.18/0.17	2/25	0.04–0.15	107	1.06
Mono-O-Acetate	0.46/NE	1/15	0.17–0.37	121	<1
Di-O-Acetate	0.53/NE	0/15	0.11–0.26	117	<1
Derivative of Tris-Hydroxymethyl Amino-Methane					
$C(CH_2OH)_3NHCOCH_2Br$	0.22/NE	0/10	0.10–0.15	119	<1

Derivatives had alkylating activity and log P values spanning the range of active haloacetamido compounds. Derivatives of 2-methyl-amino 1,3 propane diol: $CH_3C(CH_2OH)_2NHCOCH_2Br$ (alkylating activity: 3.6, log P: −0.43); mono-O-acetate (alkylating activity: 4.5, log P:-0.07); di-O-acetate (alkylating activity: 5.7, log P: 0.90). Derivative of tris-hydroxymethyl amino methane: $C(CH_2OH)_3NHCOCH_2Br$ (alkylating activity: 2.9, log P: −1.14). NE refers to ED_{50} values that were not evaluated.

Table 3
Effects of partition coefficient and alkylating activity on antitumor activities of haloacetamides in ehrlich ascites tumor-bearing CD2F1 mice.

Compounds	Active	Log P	Alkylating Activity
N-Bromo-D-Hexosamines			
β-Glucosamines: Tetra-O-Acetate	Yes	0.23	8.6
Tetra-O-Propionate	No	2.25	9.6
Free Sugar	No	−3.91	2.1
β-Galactosamine: Tetra-O-Acetate	Yes	0.21	8.8
α-Mannosamine: Tetra-O-Acetate	Yes	0.24	4.6
α-Glucosamine: Tetra-O-Acetate	Yes	0.22	6.3
Other Bromine-Containing Compounds			
2-Bromoacetamido-4-Nitrophenol	Weak	1.78	7.7
$BrCH_2CONHEt$	No	0.37	3.6
$BrCH_2CONHnPro$	No	0.91	3.6
$BrCH_2CONHnHex$	No	2.18	3.6
$BrCH_2CONH_2$	No	−0.52	2.6
$BrCH_2COO^-$	No	−1.95	3.4
$BrCH_2COOMe$	No	0.77	3.9
$BrCH_2COOEt$	No1.30	3.7	

(2.1×10^{-2} mM X^- loss/min/mM RX), in addition to its high hydrophilicity (log P = −3.91).

Based on the potential relationship of alkylating activity and partition coefficient in regards to antineoplastic activity, structure-activity plots were developed for Tet-OAHCs and other halo carbonyls (Fig. 4). From these analyses, it appears that alkylating activity does not have a marked effect on host toxicity ($p = 0.22$), but that increasing log P values appear to reduce toxicity ($p = 0.0004$). The potential importance of these findings will be elaborated upon in the discussion.

3.5. Extent of leukocyte influx in B6D2F1 mice following administration of tetra-O-acetate haloacetamido carbohydrate analogs

A quantitative comparison of host leukocyte influx between Tet-OAHCs and another monofunctional non-carbohydrate alkylating agent (BrHAB) is shown in Fig. 5. Panel A indicates that only the carbohydrate analogs, and to a lesser extent BrHAB, produced a further increase in non-basophilic granulocytes 24 h post-administration, whereas the early response induced by other agents subsided by this time. The elevated granulocyte levels were still observed in the carbohydrate analog groups 48 h post-drug administration. Panels B and C demonstrate that neutrophils were responsible for the granulocyte early response, while eosinophils induced the persisting response. While the carbohydrate analogs had both neutrophil and eosinophil influx, BrHAB only induced neutrophil influx, corresponding to reduced granulocyte influx levels at the 24 h period. Panel D indicates that monocyte influx was preferentially stimulated in groups A and B (Br-Tet-O-β-glucosamine and Br-Tet-O-β-galactosamine, respectively), and that this effect was still increasing at 72 h post-drug administration. Panel E demonstrates that while the Tet-OAHCs reduced lymphocyte counts immediately after administration, the counts recovered by 72 h post-drug administration. Panel F presents the total cell counts which correspond to the early reduction in lymphocyte counts, and following lymphocyte recovery, are influenced by granulocyte and monocyte-macrophage influx.

3.6. Long term survival of Ehrlich ascites tumor-bearing B6D2F1 and CD2F1 mice by pretreatment with tetra-O-acetate haloacetamido carbohydrate analogs

We have previously observed that apparent cures of preexisting Ehrlich tumors in mice with Tet-OAHCs are associated with the elevated influx of host granulocytes that is not observed with ineffective agents [17]. Based on the leukocyte influx potentiated by Tet-OAHCs Br-Tet-O-β-glucosamine and Br-Tet-O-β-galactosamine, it is possible that these congeners are exerting antitumor effects *in vivo* in part by altering host response at the tumor site. In order to further examine the importance of carbohydrate specificity in eliciting therapeutic benefits, Br-Tet-O-β-glucosamine, Br-Tet-O-β-galactosamine, a monfunctional alkylating agent (BrHAB), and a difunctional alkylating agent (mechlorethamine) were administered as a single i.p. dose 24 h prior to B6D2F1 and CD2F1 mice being challenged with 1, 2, or 5×10^6 Ehrlich ascites carcinoma cells (Table 4). While pretreatment of both B6D2F1 and CD2F1 mice with either Br-Tet-O-β-glucosamine or Br-Tet-O-β-galactosamine consistently potentiated long term survival at day 60 for each concentration of tumor challenge, these potent antineoplastic effects were not observed with BrHAB or mechlorethamine. Such findings indicate that alkylating activity alone is not responsible for the consistent long term survival elicited by pretreating mice with Tet-OAHCs, and that immunopotentiation may be an important component of their antitumor effects in mice challenged with

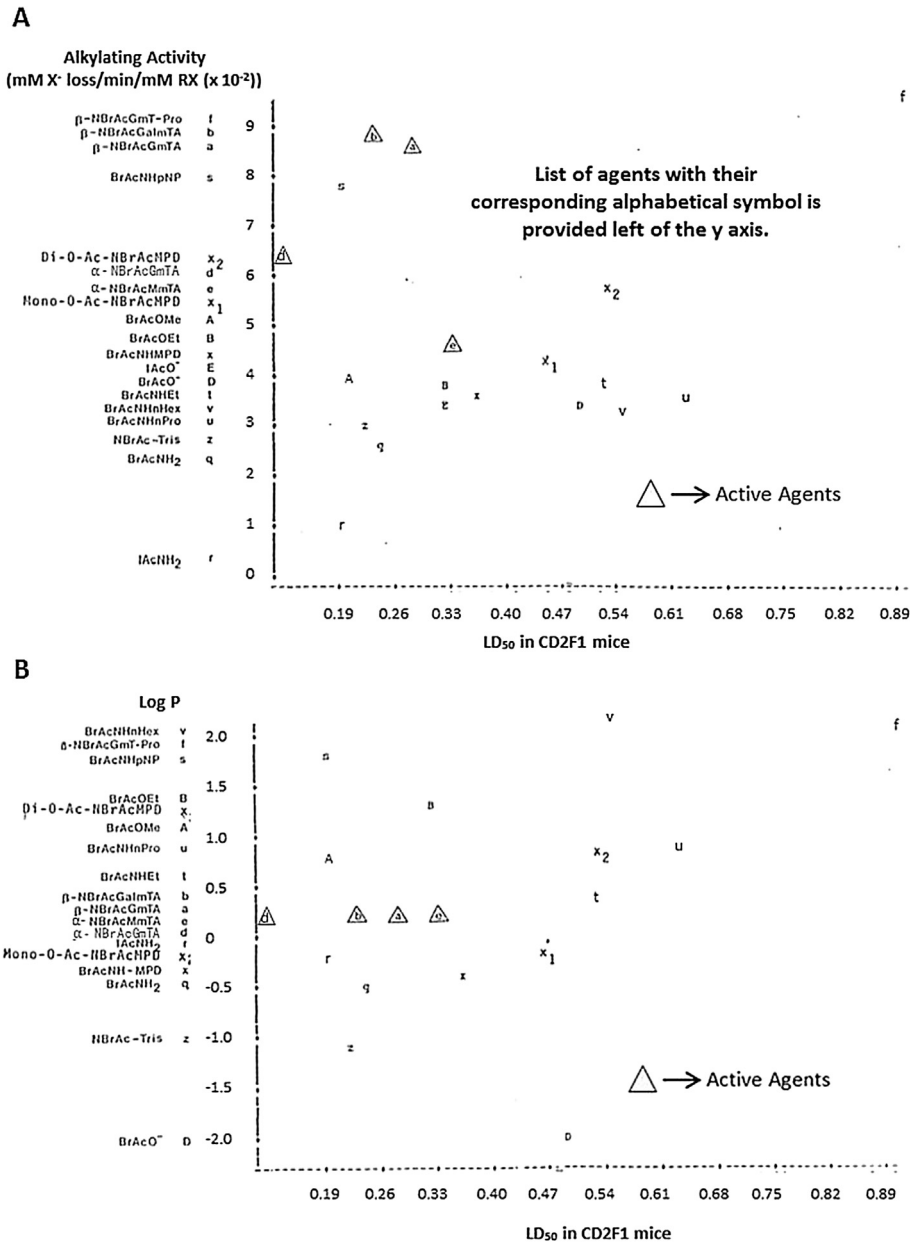

Fig. 4. Structure-activity plots of alkylating activity and partition coefficient vs. host toxicity for tetra-O-acetate haloacetamido carbohydrate analogs and other halo carbonyls. A) Alkylating activity vs. LD_{50} in CD2F1 mice. Correlation coefficients: + 0.24 for all 19 compounds; + 0.35 for the 15 non-aromatic haloacetamido derivatives ($p = 0.22$ for compounds a through z, excluding s). B) Log P vs. LD_{50} in CD2F1 mice. Correlation coefficients: + 0.38 for all 18 compounds ($p = 0.12$); +0.82 for the 14 non-aromatic haloacetamido derivatives ($p = 0.0004$ for compounds a through z, excluding s). Important abbreviations used are as follows: Galm (galactosamine), Gm (glucosamine), Mm (mannosamine), Pro (propionate) and TA (tetra-O-acetate).

Ehrlich ascites carcinoma.

4. Discussion

Tet-OAHCs are novel alkylating agents with intermediate partition coefficients that appear to have *in vitro* activity against suspension (U937 and L1210), and adherent (Ehrlich ascites carcinoma) neoplastic cell lines, as well as potent *in vivo* antineoplastic activity against Ehrlich ascites carcinoma. *In vitro*, Tet-OAHCs had comparable cytotoxicities against the U937 and L1210 leukemias, as well as Ehrlich ascites carcinoma (Fig. 3). *In vivo*, all four analogs consistently produced long term survival to at least 60 days in B6D2F1 and CD2F1 mice when administered as single i.p. injections 24 h after a tumor challenge that typically kills mice in 16 days (Table 1). The high antineoplastic activity these agents demonstrated against Ehrlich ascites carcinoma *in vivo* was highlighted by Br-Tet-O-β-glucosamine, which produced a 96.4% survival rate in CD2F1 mice, and a 91.9% survival rate in B6D2F1 mice. This dramatic antitumor activity is observed in concentrations well below the LD_{50}, suggesting that therapeutic levels of the congeners can be safely administered in mice as a single i.p. dose.

The therapeutic indices of Tet-OAHCs in the present study ranged from 2.8 to 5.2, indicative of a narrow, but feasible therapeutic window for Ehrlich ascites tumor-bearing murine models. By contrast, simple haloacetamides, haloacetates, and non-hexose haloacetamides did not potentiate any notable long term survival

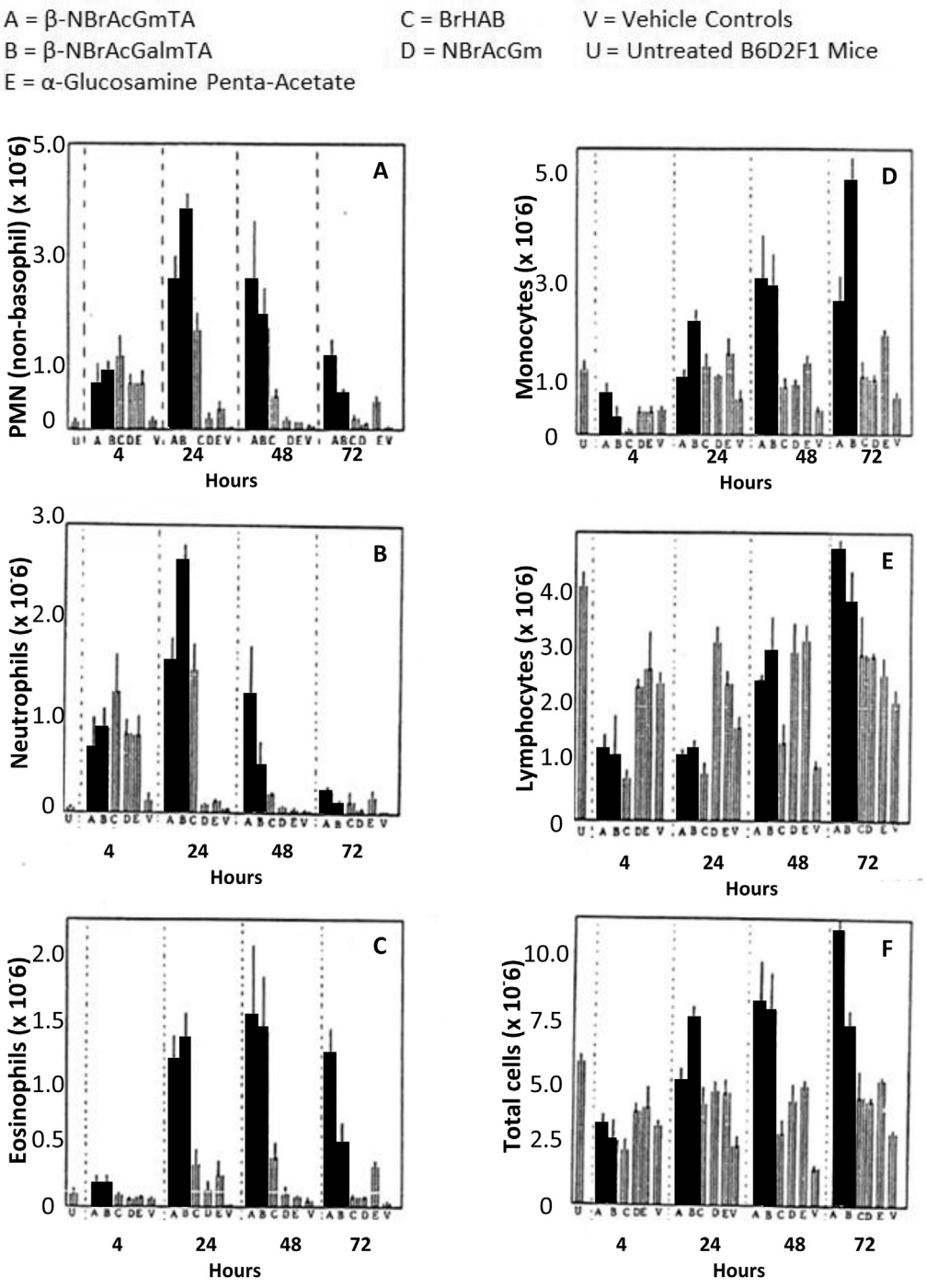

Fig. 5. Effects of tetra-O-acetate haloacetamido carbohydrate analogs on host leukocyte influx. Groups A, B, and C received i.p. injections of 0.065 mmol/kg of the respective compound (see legend above graphs), corresponding to their approximate single ED_{50} doses against 2.5×10^7 Ehrlich tumor cells given 24 h prior to drug administration. Group D received 0.065 mmol/kg i.p. of NBrAcGm (the free sugar derivative of the corresponding carbohydrate analog, group A of the treatment schedules), while group E received the same dosage of glucosamine penta-acetate. Group V was the vehicle controls, and group U served as background controls for peritoneal cells in BD2F1 mice. Total peritoneal contents were recovered, counted and Wright-Giemsa stained. Animals in groups A-V were sacrificed in groups of four at 4, 24, 48, and 72 h post-drug administration. Each treatment group had 16 mice included, except for group U (background controls) in which only 8 were used. Abbreviations used are as follows: β-NBrAcGm-TA (Br-Tet-O-β-glucosamine), β-NBrAcGalm-TA (Br-Tet-O-β-galactosamine), BrHAB (bromohydroxyacetone benzoate), and NBrAcGm (free sugar β-glucosamine bromoacetamide). Bars represent SEM of each population.

as single i.p. injections, even though some of the compounds also demonstrated alkylating activity (Table 3). Further, in the present, as well as in a previous study [17], we have shown that propionate haloacetamido carbohydrate derivatives do not elicit marked antitumor activity (Table 1). Nevertheless, the alkylation assay revealed that the propionate bromoacetamido β-glucosamine derivative had the highest alkylating activity of all the tested haloacetamides (9.6×10^{-2} mM X^- loss/min/mM RX), suggesting that the potent antineoplastic activity of Tet-OAHCs in Ehrlich ascites tumor-bearing mice resides in more than alkylating activity alone. This notion is further validated by Br-Tet-O-α-mannosamine, which had a lower alkylating activity than the other active carbohydrate

Table 4
Efficacy of pretreating B6D2F1 and CD2F1 mice challenged i.p. with ehrlich ascites murine carcinoma with 2-deoxy-2-bromoacetamido-D-hexose 1,3,4,6 tetra-O-acetates, or with non-carbohydrate monofunctional and difunctional alkylating agents 24 h prior to challenge.

Agent	I.P. Dose (mmol/kg)	Relationship to LD_{50} (mmol/kg)	B6D2F1 60 Day survivors I.P. Cell challenge ($\times 10^6$)			CD2F1 60 Day survivors 1×10^6 cell I.P. Challenge
			1.0	2.0	5.0	
β-Glucosamine	0.11	0.27–0.31	16/16	8/8	5/7	14/15
β-Galactosamine	0.11	0.42–0.46	16/16	8/8	8/8	16/16
BrHAB	0.12	0.40–0.50	5/15	1/8	0/7	2/15
Mechlorethamine	0.10	0.45	3/16	–	–	0/16
Tween-Saline[a]	–	–	0/16	–	–	0/8

[a] Vehicle used to deliver agents.

analogs, the propionate bromoacetamido β-glucosamine derivative, and 2-bromoacetamido-4-nitrophenol, but still potentiated long term survival in nearly all CD2F1 mice challenged with Ehrlich ascites carcinoma (18/20 mice; Table 1). The lower alkylating activity (Table 3), and potentially the lower *in vitro*cytotoxicity of Br-Tet-O-α-mannosamine against the three neoplastic cell lines examined (Fig. 3) in comparison to its congeners may be attributed to its unique stereochemistry and subsequent steric effects. The mannose carbohydrate moiety likely positions the bromoacetamido group in an axial position, as opposed to the other three Tet-OAHCs that have the bromoacetamido group in an equatorial position (Fig. 2A).

In addition to being potent electrophiles, it appears that Tet-OAHCs may also act as immunopotentiating agents in both B6D2F1 and CD2F1 mice. While the monofunctional alkylating agent BrHAB had a small, but notable increase in non-basophilic granulocytes 24 h post-administration, BrHAB only induced neutrophil influx, corresponding to reduced granulocyte influx levels at the 24 h period (Fig. 5). By contrast, Br-Tet-O-β-glucosamine and Br-Tet-O-β-galactosamine had both neutrophil and eosinophil influx increase during this period, with the effects lasting in excess of 48 h after the i.p. injection. In addition, monocyte influx was preferentially stimulated by Br-Tet-O-β-glucosamine and Br-Tet-O-β-galactosamine, with increases still being observed at 72 h post-drug administration (Fig. 5D). While it is true that the two congeners temporarily inhibited lymphocyte influx (Fig. 5E), this effect completely dissipated by 72 h, and Tet-OAHCs had the highest overall leukocyte influx by a relatively large margin.

The potential importance of immunopotentiation in the antitumor effects elicited by Tet-OAHCs was further observed when two of the congeners (Br-Tet-O-β-glucosamine and Br-Tet-O-β-galactosamine) were administered i.p. 24 h prior to Ehrlich ascites tumor challenge (Table 4). While neither the monofunctional (BrHAB) or difunctional (mechlorethamine) non-carbohydrate alkylating agent potentiated significant antitumor activity when administered 24 h prior to the challenge, both of the Tet-OAHCs produced extremely high survival rates at 60 days, with all of the B6D2F1 and CD2F1 mice surviving this observation period after being treated with a single i.p. injection of Br-Tet-O-β-galactosamine. This inherent survival difference potentiated by the two Tet-OAHCs and the other alkylating agents again suggest that the antineoplastic activity Tet-OAHCs demonstrate against Ehrlich ascites carcinoma is due to more than potent alkylating activity at the cell surface, or with important intracellular nucleophiles. Due to the observation that Tet-OAHCs potentiate leukocyte influx at the site of administration, as well as the tumor site, it appears that immunopotentiation is at least partially responsible for the marked antitumor activity elicited by the congeners. However, these *in vivo* effects need to be confirmed in other neoplastic cell lines to definitively assess whether Tet-OAHCs potentiate immunotherapeutic benefits in addition to S_N2 alkylating activity.

Analyses of the alkylating activities and partition coefficients of Tet-OAHCs as well as other halo carbonyls revealed important characteristics regarding the optimization of these agents. Although the propionate derivate of bromoacetamido β-glucosamine exhibited the highest alkylating activity and log P value (Table 3), it did not produce any long term survivors in CD2F1 mice challenged with Ehrlich ascites carcinoma (0/8 mice; Table 1). This is particularly striking, as the propionate derivate of bromoacetamido β-glucosamine had the highest LD_{50} value of any of the analogs (Table 1), and was administered at higher concentrations than other bromoacetamido carbohydrates. These effects were further characterized in structure-activity plots (Fig. 4) that compared alkylating activity and log P values to LD_{50} values in CD2F1 mice. Since the active carbohydrate analogs in this series appear to have relatively high alkylating activities and intermediate partition coefficients, these correlations suggest that optimal haloacetamido carbohydrates should have log P values in the range of 0.5–1, and alkylating activity near or perhaps greater than 9×10^{-2} mM X^- loss/min/mM RX. Haloacetamido carbohydrate analogs that should adhere to both principles are proposed in Fig. 6. In addition to monofunctional haloacetamido carbohydrates, difunctional haloacetamido carbohydrates compounds are also posited as a means to potentially increase alkylation rates. It is also well known that difunctional alkylating agents have the propensity to crosslink adjacent nucleophiles [2], and in theory should give haloacetamido carrbohydrates another mechanism by which to damage neoplastic cells. In addition, difunctional haloacetamido carbohydrates are proposed because the present study does not preclude the potential affinity these agents may have for DNA, as is observed with traditional alkylating agents.

As with other novel antineoplastic agents, Tet-OAHCs will need to be used in combination with clinically approved agents to reduce the likelihood of drug resistance, and to improve their overall efficacy. Interestingly, anthracyclines have a marked influence at the cell surface. Anthracyclines are most commonly known as nucleic acid-directed agents, as the compounds intercalate base pairs, produce free radicals, and potently inhibit DNA topoisomerase II function [2]. However, it has also been well established that anthracyclines (particularly doxorubicin) alter the fluidity of neoplastic cell plasma membranes [20,21], and bind phospholipids with considerably affinity [22,23]. Some studies have also indicated that extracellular doxorubicin is important for anticancer activity and that the compound demonstrates marked cytotoxicity without entering the cell [2,24]. Therefore, it may be possible to combine anthracyclines with Tet-OAHCs to elicit potent drug synergy at the cell surface or inside the nucleus. This possibility is made even more intriguing by the fact that daunorubicin was much less effective against Ehrlich ascites carcinoma than the Tet-OAHCs examined in the present study (Table 1), and increasing the *in vitro* and *in vivo* efficacy of anthracyclines in multiple cancer types using haloacetamido carbohydrates and anthracyclines in combination could eventually warrant clinical investigation.

Acetylated haloacetamido carbohydrates are a particularly

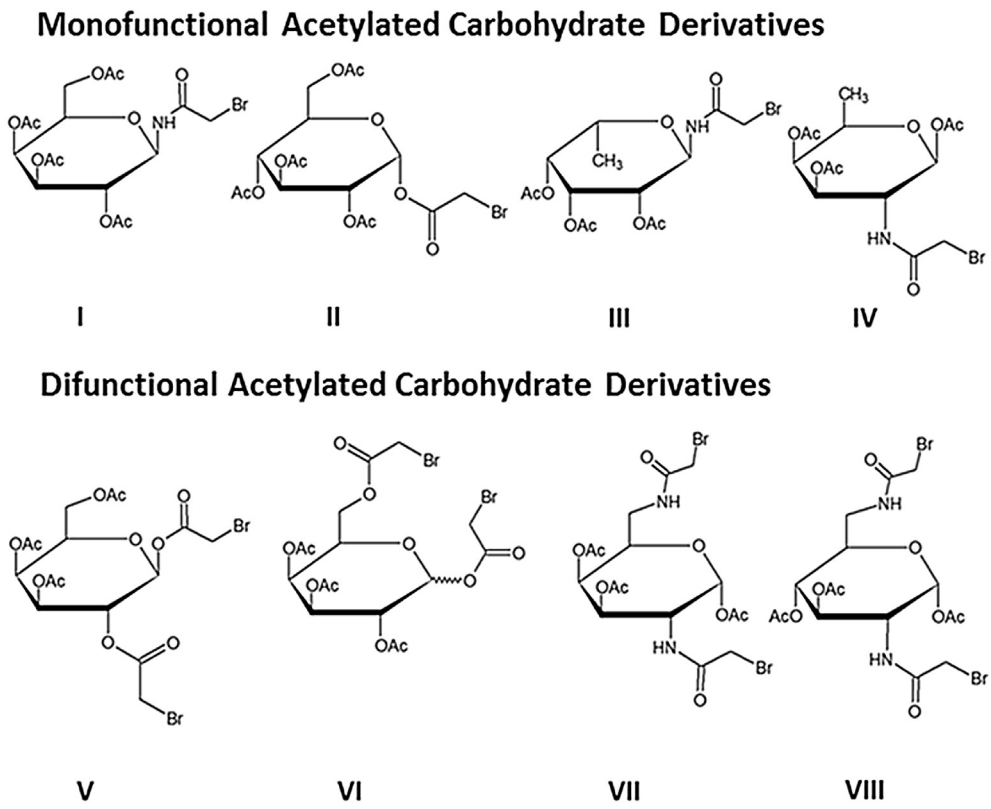

Fig. 6. Proposed acetylated haloacetamido carbohydrate analogs with high alkylating activity and intermediate partition coefficients for preclinical evaluation. Monofunctional derivatives are as follows: I) N-bromoacetyl-galactosylamine-2,3,4,6-tetra-O-acetate, II) 1-O-bromoacetyl-glucose-2,3,4,6-tetra-O-acetate, III) N-bromoacetyl-fucosylamine-2,3,4-tri-O-acetate, and IV) 6-deoxy-N-bromoacetyl-galactosamine-1,3,4-tri-O-acetate. Difunctional derivatives are as follows: V) 1,2-di-O-bromoacetyl-β-D-galactose-3,4,6-tri-O-acetate, VI) 1,6-di-O-bromoacetyl-α or β-D-galactose-2,3,4-tri-O-acetate, VII) 2,6-di-N-bromoacetyl-2,6-dideoxy-2,6-diamino-galactose-1,3,4-tri-O-acetate, and VIII) 2,6-di-N-bromoacetyl-2,6-dideoxy-2,6-diamino-β-D-glucose-1,3,4-tri-O-acetate.

interesting group of alkylating agents, as they appear to potentiate a substantial leukocyte influx at the administration and primary tumor site, indicative of possible immunopotentiation. This is a particularly important observation, as traditional DNA-directed alkylating agents are noted for inducing marked neutropenia in the clinical setting [2,25,26]. An antineoplastic agent that promotes leukocyte influx could be a particularly beneficial supplement to many current chemotherapeutic protocols, and warrants further *in vivo* examination of these novel congeners.

Acknowledgments

Animal studies were approved by an IACUC protocol. The authors would like to extend their sincerest gratitude to two anonymous reviewers who improved the quality of the article. The authors declare no conflicts of interest.

References

[1] D. Fu, J.A. Calvo, L.D. Samson, Balancing repair and tolerance of DNA damage caused by alkylating agents, Nat. Rev. Cancer 12 (2) (2012) 104–120.
[2] B.A. Chabner, D.L. Longo, Cancer Chemotherapy and Biotherapy: Principles and Practice, fifth ed., Lipincott Williams & Wilkins, 2011.
[3] M. Esteller, J. Garcia-Foncillas, E. Andion, S.N. Goodman, O.F. Hidalgo, V. Vanaclocha, S.B. Baylin, J.G. Herman, Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents, N. Engl. J. Med. 343 (19) (2000) 1350–1354.
[4] A. Shiraishi, K. Sakumi, M. Sekiguchi, Increased susceptibility to chemotherapeutic alkylating agents of mice deficient in DNA repair methyltransferase, Carcinogenesis 21 (10) (2000) 1879–1883.
[5] R. Leth-Larsen, R.R. Lund, H.J. Ditzel, Plasma membrane proteomics and its application in clinical cancer biomarker discovery, Mol. Cell. Proteomics 9 (7) (2010) 1369–1382.
[6] Kohnke PL, Mulligan SP, Christopherson RI. Membrane proteomics for leukemia classification and drug target identification. Curr. Opin. Mol. Ther.; 11(6): 603–610.
[7] J.M. Besterman, R.B. Low, Endocytosis: a review of mechanisms and plasma membrane dynamics, Biochem. J. 210 (1) (1983) 1–13.
[8] A.G. Manford, C.J. Stefan, H.L. Yuan, J.A. Macgurn, S.D. Emr, ER-to-plasma membrane tethering proteins regulate cell signaling and ER morphology, Dev. Cell. 23 (6) (2012) 1129–1140.
[9] H.D. Shukla, P. Vaitiekunas, R.J. Cotter, Advances in membrane proteomics and cancer biomarker discovery: current status and future perspective, Proteomics 12 (19–20) (2012) 3085–3104.
[10] A.G. Manford, C.J. Stefan, H.L. Yuan, J.A. Macgurn, S.D. Emr, ER-to-plasma membrane tethering proteins regulate cell signaling and ER morphology, Dev. Cell. 23 (6) (2012) 1129–1140.
[11] J. Middleton, A.M. Patterson, L. Gardner, C. Schmutz, B.A. Ashton, Leukocyte extravasation: chemokine transport and presentation by the endothelium, Blood 100 (12) (2002) 3853–3860.
[12] K. Ebnet, D. Vestweber, Molecular mechanisms that control leukocyte extravasation: the selectins and the chemokines, Histochem Cell. Biol. 112 (1) (1999) 1–23.
[13] K.D. Patel, S.L. Cuvelier, S. Wiehler, Selectins: critical mediators of leukocyte recruitment, Semin. Immunol. 14 (2) (2002) 73–81.
[14] T.P. Fondy, C.A. Emlich, Haloacetamido analogues of 2-amino-2-deoxy-D-mannose. Syntheses and effects on tumor-bearing mice, J. Med. Chem. 24 (7) (1981) 848–852.
[15] T.P. Fondy, S.B. Roberts, Haloacetamido analogues of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose. Syntheses and effects on the Friend murine erythroleukemia, J. Med. Chem. 21 (12) (1978) 1222–1225.
[16] R.W. Pero, P. Babiarz-Tracy, T.P. Fondy, 3-Fluoro-1-hydroxypropan-2-one (fluorohydroxyacetone) and some esters. Syntheses and effects in BDF mice, J. Med. Chem. 20 (5) (1977 May) 644–647.
[17] P. Simon, W.J. Burlingham, R. Conklin, T.P. Fondy, N-bromoacetyl-beta-D-glucosamine tetra-O-acetate and N-bromoacetyl-beta-D-galactosamine tetra-O-acetate as chemotherapeutic agents with immunopotentiating effects in Ehrlich ascites tumor-bearing mice, Cancer Res. 39 (10) (1979) 3897–3902.
[18] Babiarz-Tracy P, McCarthy D, Simon P, Burlingham WJ, Fondy TP. Esters of chlorohydroxyacetone in chemotherapy of murine tumors. Cancer Res.; 40(9): 3274–3280.
[19] O.M. Friedman, E. Boger, Colorimetric estimation of nitrogen mustards in

aqueous media, Anal. Chem. 3 (1961) 906–910.
[20] J.A. Siegfried, K.A. Kennedy, A.C. Sartorelli, T.R. Tritton, The role of membranes in the mechanism of action of the antineoplastic agent adriamycin. Spin-labeling studies with chronically hypoxic and drug-resistant tumor cells, J. Biol. Chem. 258 (1) (1983) 339–343.
[21] M. Sugiyama, T. Sakanashi, K. Okamoto, M. Chinami, T. Hidaka, R. Ogura, Membrane fluidity in Ehrlich ascites tumor cells treated with adriamycin, Biotechnol. Appl. Biochem. 8 (2–3) (1986) 217–221.
[22] E. Goormaghtigh, R. Brasseur, P. Huart, J.M. Ruysschaert, Study of the adriamycin-cardiolipin complex structure using attenuated total reflection infrared spectroscopy, Biochemistry 26 (6) (1987) 1789–1794.
[23] C. Heywang, M. Saint-Pierre Chazalet, C.M. Masson, J. Bolard, Orientation of anthracyclines in lipid monolayers and planar asymmetrical bilayers: a surface-enhanced resonance Raman scattering study, Biophys. J. 75 (5) (1998) 2368–2381.
[24] T.R. Triton, G. Yee, The anticancer agent adriamycin can be actively cytotoxic without entering cells, Science 217 (4556) (1982) 248–250.
[25] A. Nicolini, P. Mancini, P. Ferrari, L. Anselmi, G. Tartarelli, V. Bonazzi, A. Carpi, R. Giardino, Oral low-dose cyclophosphamide in metastatic hormone refractory prostate cancer (MHRPC), Biomed. Pharmacother. 58 (8) (2004) 447–450.
[26] R.A. Weinberg, The Biology of Cancer, second ed., Garland Science, 2013.