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The novel nucleoside transport system exhibited by NB4 cells, *csg*, transports deoxyguanosine analogues, including ara-G

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Abstract

We studied acceptance of various deoxyguanosine analogues by the unique guanosine preferring nucleoside transport system exhibited by NB4 cells, *csg*. Indirect assessment of acceptance using transport inhibition assays revealed that both 1-β-D-arabinofuranosylguanine (ara-G) and 4′-thio-β-D-xylofuranosylguanine (thio-xyl-G) compete with guanosine for the *csg* system, inhibiting guanosine flux by approximately 50%. Direct examination of [^{3}H]-ara-G transport revealed total transport was equally allocated to *csg*, and *es* systems and a total transport rate similar to that determined for guanosine [Flanagan and Meckling-Gill, J Biol Chem 1997;272:18026–32]. Cytotoxicity assays revealed that although both ara-G and thio-xyl-G were capable of competing with guanosine for the *csg* system, neither analogue elicited cytotoxic effects at physiologically relevant concentrations. The analog, 4′-thio-β-D-arabinofuranosylguanine does not gain entry to NB4 cells via the *csg* transport system. Competition assays revealed that this analogue potentiated the inward flux of guanosine and was capable of killing NB4 cells with potency similar to the conventional leukemia drug, ara-C.

Keywords: Nucleoside; Guanosine; *csg*; Acute promyelocytic leukemia; Nucleoside analogues; Ara-G

1. Introduction

Nucleoside analogues must be metabolically activated making their transportability across plasma membranes critical to their pharmacological action. Multiple distinct transport proteins in mammalian cells mediate the uptake or release of nucleosides and nucleoside analogs [1–3]. Classification of these nucleoside-specific membrane transport (NT) processes is based on functional and pharmacological characteristics. For detailed descriptions of the various nucleoside transport processes, see the following reviews [1,4]. Briefly, two equilibrative, bi-directional facilitated diffusion processes, *es*, and *ei*, are distinguished by their sensitivity to nitrobenzylthioinosine (NBMPR), a transport inhibitor, and exhibit broad substrate selectivity. The concentrative Na^+-dependent processes, driven by the sodium electrochemical gradient, (*cif*, *cit*, *cib*, *cs*, *csg*) are distinguishable on the bases of substrate specificity, and inhibitor sensitivity.

Ara-C has remained the mainstay of a variety of combination treatments for AML [5–7] however, resistance to ara-C-based chemotherapy and the non-selective nature of its action is still a major problem in therapy [8,9]. Discovery of an analog with a greater therapeutic index would help to alleviate therapy-associated side effects. In this regard, the possible exploitation of differences in nucleoside transporters available to cancer cells and normal cells represents a promising target. Acute promyelocytic leukemia (APL) represents a subset of AML and is characterized by a *t*(15;17) translocation [10] also demonstrated in the *in vitro* cell line, NB4 [11]. Recently, we characterized the transporter phenotype of NB4 cells and showed that they express multiple NT processes simultaneously; namely, *es*, *ei*, *csg* [12,13]. The *csg* transporter displayed a strong preference for guanosine [12], over other competing natural nucleosides, nucleobases, and nucleoside analogues.

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Abbreviations: APL, acute promyelocytic leukemia; ara-C, cytarabine; ara-G, 1-beta-D-arabinofuranosylguanine; IMDM, Iscove's modified Dulbecco's medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBMPR, nitrobenzylthioinosine; thio-xyl-G, 4′-thio-beta-D-xylofuranosylguanine; NT, nucleoside transport.

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The substrate specificity of the *csg* transporter is dependent on both the presence of an intact ribose ring and the existence of particular functional groups within the base group with 2′-deoxyguanosine, the most successful competitor of guanosine transport [12]. In human cell lines, thus far, the existence of the *csg* transporter is a distinguishing feature of NB4 cells. The inherent preference for guanosine, and the possible cell type specific expression exhibited by the *csg* transporter, may provide a new target for cytotoxic therapy.

In the present study we examined the substrate specificity of the *csg* transporter using various deoxyguanosine analogues including, the novel analogues 4′-thio-β-L-xylofuranosylguanine (thio-xyl-G) and 4′-thio-β-D-arabinofuranosylguanine (thio-ara-G), as well as 1-β-D-arabinofuranosylguanine (ara-G), an analogue currently used clinically to treat T-cell lymphomas [22,23]. We indirectly studied the acceptance of these analogues by *csg* using inhibition assays, and in the case of ara-G, by directly measuring [^{3}H]-ara-G uptake. We also examined the cytotoxic activities of the above guanosine analogues in NB4 cells.

2. Materials and methods

2.1. Cell culture

NB4 cells were maintained in Iscove's modified Dulbecco's medium (IMDM) (Gibco/BRL) with 10% fetal bovine serum (FBS; ICN Biochemicals, Inc.), supplemented with penicillin and streptomycin (50 U/mL) at 37° and 5% CO_2. An initial mycoplasma infection with *M. arginini* was treated with BM cyclin (Boehringer Mannheim) and cells were subsequently screened on a routine basis. Ara-G was purchased from ICN Biochemicals, Inc.; deoxyguanosine analogues, thio-xyl-G and thio-ara-G, were provided by one of the authors, Dr. John Secrist III, Southern Research Institute, AL.

2.2. Transport studies

Cell suspensions were harvested during exponential growth (at a density of $(8.0-9.0) \times 10^5$ cells/mL) by centrifuging at 800 *g* for 8 min at RT. The resulting pellets were washed twice in 25 mL of Na^+ buffer (3 mM K_2HPO_4, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 144 mM NaCl, 20 mM Tris; pH 7.4, osmolarity 300 ± 10 mosm) or Na^+ replacement choline buffer (3 mM K_2HPO_4, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 140 mM choline Cl, 20 mM Tris; pH 7.4, osmolarity 300 ± 10 mosm) and centrifuged as above. Final pellets were resuspended in the appropriate buffer to a final density of $(7.0-8.0) \times 10^6$ cells/mL. Cells were used immediately in the transport assay or after 15–20 min incubation with the nucleoside transport inhibitor, 1 μM NBMPR. Employing rapid assay technology, the uptake of 50 μM [^{3}H]-ara-G was determined using an inhibitor and oil stop procedure at 22° [14] as previously adapted in our laboratory [12]. Uptake curves were generated over a 5 min time course (0, 5, 10, 15, 30, 60, 120, 300 s) and each time point was performed in triplicate. Initial rates were estimated from computer generated (TableCurve®, Jandel Scientific) best-fit equations applied to uptake curves generated in the presence of Na^+, Na^+NBMPR, choline, or choline NBMPR buffer. The linear tangent drawn between 0 and 1 s was used as the initial rate estimate for all time courses. Transport processes, *ci*, *cs*, *es*, and *ei* were determined by subtraction of the curve defined initial rates, as we previously described [12].

2.3. Quantification of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cell suspensions (5.0×10^4 cells/mL) were transferred into each well of a 96-well plate following serial dilution of analogues. Ara-G, and thio-ara-G were dissolved into IMDM medium with 10% FBS and 5% penicillin and streptomycin (50 U/mL). Solubilization of thio-xyl-G required the addition of DMSO. Final concentration of DMSO in 1 mM solution was 6% (v/v) and cells were also prepared in 6% DMSO in absence of thio-xylo-G. Following incubation at 37° for 48 hr, each plate was centrifuged for 8 min at 1000 *g* at RT and the supernatant removed. Fifty microliters of MTT solution (0.5 mg/mL IMDM) was added to each well and plates were incubated for an additional 4 hr at 37°. MTT formazan product was visualized by the addition of 100 μL 2-propanol, with added HCl (0.03%, v/v) and absorbance of measured at 570 nm and IC_{50} values were estimated from computer generated (TableCurve®, Jandel Scientific) best-fit equations for each drug condition.

2.4. Statistical analysis

ANOVA followed by the Student–Newman–Keuls multiple comparisons test was used to make comparisons between [^{3}H]-guanosine fluxes measured in the presence of excess concentrations of analogues/guanosine using Instat™ (GraphPad) for personal computers (Fig. 1). Differences were considered significant when $P < 0.05$.

3. Results

3.1. IC_{50} values of deoxyguanosine analogues

With an IC_{50} value of 2.3 ± 1.2 μM, thio-ara-G exhibited potency similar to ara-C, a nucleoside analog used in virtually all standard induction regimens for AML [15], and has an IC_{50} = 1.2 ± 0.12 μM in NB4 cells [27]. Thio-xyl-G was 100-fold less potent than thio-ara-G with an IC_{50} value of 232.0 ± 3.0 μM. Ara-G displayed an IC_{50} value of 50.7 ± 10.4 μM, exhibiting a potency similar to that

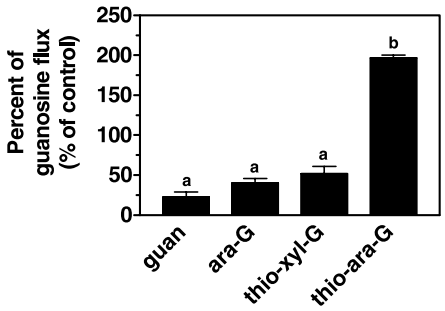

Fig. 1. Substrate specificity of the *csg* transporter in NB4 cells. Uptake of 100 μM [^{3}H]-guanosine was measured in the presence of competing nucleoside analogues, ara-G, thio-ara-G. Uptake was measured after a 5 s incubation of the cells with transport buffer (Na^+ buffer, 100 μM guanosine, and the competitor). The flux of [^{3}H]-guanosine observed in the presence of 1 mM guanosine or 1 mM of the analogues, ara-G, thio-ara-G, and thio-xyl-G is expressed as a percentage of [^{3}H]-guanosine flux measured in the absence of competing compounds (100% flux). Bars represent the mean ± SEM of data obtained in at least three separate experiments. Statistical significance was determined by the application of a Student–Newman–Keuls multiple comparisons test ($P < 0.5$). Bars not sharing a letter are significantly different. Guan: guanosine.

observed in HL-60 cells and much lower than demonstrated in T-cell leukemic lymphoblasts [16]. Guanosine was even less toxic exhibiting an IC_{50} value of approximately 1 mM[2].

3.2. Measurement of [^{3}H]-guanosine flux in the presence of deoxyguanosine analogues

The transport of 100 μM [^{3}H]-guanosine was measured in the presence of 1 mM of the deoxyguanosine analogues, ara-G, thio-ara-G, thio-xyl-G, and guanosine. Ara-G, thio-xylo-G, and guanosine significantly inhibited Na^+-dependent *cs* uptake (*csg*) of 100 μM guanosine at 5 s ($P < 0.05$) by 48, 58 and 74%, respectively (Fig. 1). DMSO-treated cells decreased guanosine flux by approximately 7%. The deoxyguanosine analogue thio-ara-G appeared to assist the transport of guanosine in the presence of Na^+ as demonstrated by a 2-fold increase in [^{3}H]-guanosine flux.

3.3. Characterization of ara-G uptake in NB4 cells

Direct examination of [^{3}H]-ara-G transport indicated that approximately 45% of the total uptake of ara-G occurred via a *cs* route (Fig. 2A and B). Transport in the presence of a Na^+ gradient was almost entirely abrogated by the addition of 1 μM NBMPR and the replacement of Na^+ with choline led to a 50% decrease in total uptake. At 50 μM, [^{3}H]-ara-G uptake reached equilibrium in all experiments within approximately 60 s. Despite transport via the *csg* system, a concentrative system, intracellular [^{3}H]-ara-G increased only 20% above equilibrium and total [^{3}H]-ara-G uptake was maximized by 2 min. A small amount of ara-G transport, approximately 6% of the total transport, occurred in the choline + NBMPR (*ei*) treatment group, however, this process was not saturable and therefore likely represents the rate of diffusion.

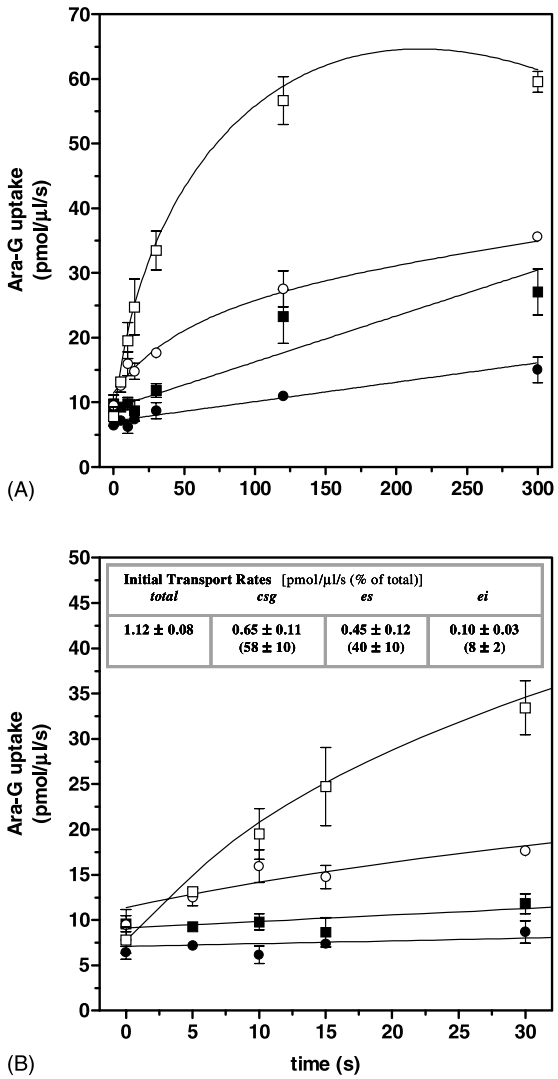

Fig. 2. Uptake of ara-G occurs mainly by *csg* and *es* transporter processes in NB4 cells. (A) Time course of 50 μM ara-G uptake. Uptake was measured in the absence (circles) or presence (squares) of Na^+ with (solid symbols) or without (open symbols) 1 μM NBMPR. (B) Early time points of the uptake curve from A are magnified. The inserted table displays initial transport rates as estimated from computer-generated best-fit equations over the early linear portion of uptake curves as described in Section 2 and in [13]. Transport rates measured in Na^+ buffer alone (open squares) are representative of total transport (*es*, *ei*, *ci*, *csg*), and measurements made in choline buffer (open circles) represent total equilibrative transport (*es* + *ei*). Transport measured in Na^+NBMPR (closed squares) reveals total insensitive transport (*ei* + *ci*) while measurements made in choline NBMPR (closed circles) measure *ei* only. Individual transport processes were resolved by subtraction as follows: choline NBMPR = *ei*; choline − choline NBMPR = *es*; Na + NBMPR − choline NBMPR = *ci*; $Na^+ - Na^+$NBMPR − (choline − choline NBMPR) = *cs*. The data shown are the means ± SEM of triplicate assays and are representative of at least three separate experiments.

4. Discussion

Ara-G inhibited [^{3}H]-guanosine flux to the same degree as excess guanosine (Fig. 1), and 2′-deoxyguanosine [12]. Using [^{3}H]-ara-G, total ara-G transport was essentially equally allocated to *csg* and *es* systems (Fig. 2B, inset). This distribution of transporter phenotypes is similar to that

previously described in NB4 cells for guanosine uptake [12], suggesting that these two substrates directly compete with each other for entry and probably subsequent metabolic activation.

The effectiveness of ara-G as a therapeutic agent, depends on its conversion to ara-GTP [17]. Activity of intracellular enzymes responsible for ara-G activation, dGK and dCK [18–20] and their affinity for ara-G contribute to successful activation. Our estimated transport rates for 50 μM ara-G suggest that transport is not rate-limiting in NB4 cells. Despite this, [^{3}H]-ara-G did not accumulate intracellularly to the same degree as guanosine. At an equivalent concentration of 50 μM guanosine, metabolites concentrate at least 7-fold in 5 min [12], while only a 20% concentration of substrate is observed in the same experiment carried out with ara-G (Fig. 2A). The bi-directional *es* system could be responsible for preventing accumulation of ara-G since it accounted for only 25% of total guanosine [12] but 50% of ara-G transport, providing an exit pathway for ara-G as it entered via the *csg* system. Furthermore, activities of nucleoside-associated enzymes have the potential to influence accumulation and retention by metabolically trapping metabolites. It is possible that the kinetic parameters of such enzymes differ for guanosine and ara-G, e.g. the K_m and/or V_{max}, so that while their rates of inward transport are similar their rates of metabolic conversion and/or efflux may be different. Previous studies determined that metabolism is rate limiting when measuring guanosine transport at 50 μM guanosine. Guanosine is quickly phosphorylated upon entering NB4 cells with the majority of intracellular [^{3}H]-guanosine found in ribonucleotide pools, [GMP] = [GDP] > GTP.[1] Analysis of intracellular [^{3}H]-ara-G metabolites and kinetic analysis of metabolic enzymes may provide clues as to reasons for the differences in overall accumulation of the two metabolites.

Low ara-G accumulation may explain its low potency and poorer ability to induce cell death in NB4 cells than equivalent concentrations of guanosine or other nucleoside analogues. Ara-G exhibits selective cytotoxicity to T-lymphoblasts *in vitro* and in the clinical setting, compared to B-lymphoblasts and other leukemia cell lines [16,17,21–23]. The sensitivity of T-lymphoblasts is thought to be due to their increased ability to activate ara-G to ara-GTP, and decreased ability to eliminate these active products, when compared to other cell types [17,24]. While intracellular accumulation of [^{3}H]-guanosine exceeds accumulation of [^{3}H]-ara-G, the IC_{50} value for ara-G is approximately 10-fold less than for guanosine and suggests that while the uptake mechanisms for the two substrates may be similar, their cytotoxic effects are mediated by different pathways. Both metabolites require transport into the cell to elicit their effects. However, cytotoxicity of ara-GTP requires incorporation into nuclear [25] or mitochondrial [26] DNA while guanosine-induced death is, in part, a result of an imbalance in nucleosides.[2]

Thio-xyl-G proved to be a competitor for *csg* and inhibited the flux of guanosine by about 50%. The IC_{50} value for this analogue in NB4 cells was similar to IC_{50} values obtained for other cancer cell lines, including: CAKI-1 (Renal), DLD-1 (colon), NCI-H23 (lung), SK-MEL-28 (melanoma), SNB-7 (CNS) where values >400 μM were observed (Dr. John Secrist III, Southern Research Institute, Drug Discovery Division, AL). The issue of solubility together with its low potency suggests that clinical application of this analog is not appealing for the treatment of APL.

Thio-ara-G potentiated the flux of guanosine. This analogue may inhibit the efflux of guanosine from the bi-directional equilibrative routes by occupying these sites. Future direct examination of the transport of [^{3}H]-thio-ara-G may help to clarify our speculation. Due to its potency at physiologically relevant concentrations and its H_2O solubility, further biological studies examining its biochemical pharmacology, are warranted.

Relatively minor structural changes in nucleoside analogs can result in profound effects on anti-tumor activity. Future isolation of the cDNA encoding the *csg* transporter protein will lead to the development of specific antibodies for study of transporter distribution, and allow for the functional expression of isolated cDNAs in systems such as *Xenopus laevis*. Such research tools will facilitate the goal of identifying therapeutic agents with a high therapeutic index and possibly a *csg* specific agent.

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