Proton-mediated Liberation of Aldophosphamide from a Nontoxic Prodrug: A Strategy for Tumor-selective Activation of Cytotoxic Drugs

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ABSTRACT

Based on the findings that the pH in malignant tumors can be preferentially decreased by stimulation of their aerobic glycolysis, acid-sensitive prodrugs, which are nearly nontoxic at physiological pH, were synthesized. At lower pH, however, these compounds are cleaved with liberation of a cytotoxic species. The prototypical drug compound 2-hexenopyranoside of aldophosphamide was prepared, which releases aldophosphamide by acidi-catalyzed hydrolysis. Exposure of cultured M1R rat mammary carcinoma cells to this agent at pH 7.4 only resulted in slight toxicity. However, when drug treatment was performed at pH 6.2, the mean pH in malignant tumors of hyperglycemic hosts, the colony-forming fraction of M1R cells decreased to 0.05 and 0.0001 of controls treated at pH 7.4 after exposure for 24 h and 48 h, respectively. The synthesis of the 2-hexenopyranoside of aldophosphamide is described in detail.

INTRODUCTION

Tumor-selective chemotherapy requires the exploitation of phenotypic differences that distinguish malignant cells from normal cells. The ability to convert glucose to lactic acid in the presence of oxygen, although not strictly associated with malignancy (1), is a metabolic characteristic of a large variety of malignant cells (2, 3). There is evidence that by stimulation of tumor cell glycolysis in vivo differential changes can be generated in malignant as compared to normal tissues. In normal unperturbed tissues the rate of lactic acid formation is low; in addition, it does not increase with increasing glucose supply. In contrast, in malignant tumors glucose supply is one of the most important determinants of glucose uptake and, consecutively, lactic acid formation (4, 5). In a previous study we have shown that the concentration of lactic acid in malignant tumors, as measured by changes of pH, can be increased by parenteral administration of glucose (6). In hyperglycemic tumor hosts (BD IX rats) the mean pH of TV1A neurinomas decreased from 6.9 (normoglycemic value) to 6.1. The latter value corresponds to an increase of the hydrogen ion concentration in tumor tissue, as compared to arterial blood (pH 7.4), by a factor of 20. Minimum pH values were measured 2 h after initiation of i.v. glucose infusion and could be maintained for up to 48 h. This acidosis was tumor specific. In brain and kidney the mean hydrogen ion concentration varied by less than 0.1 pH units in response to glucose (6). Similar results in a series of studies on both animal and human tumors have been reported by other investigators (7).

The present study was performed to investigate whether the differential accumulation of acidic metabolites in malignant as compared to normal tissues can be exploited to improve the selectivity of anticancer drugs (8). Specifically, the following question has been addressed: Can cytotoxic agents be liberated from non-toxic prodrugs selectively in malignant tissues by proton catalyzed hydrolysis? There are two prerequisites for this approach: (a) the design of molecular structures which can be cleaved by proton catalyzed hydrolysis at the hydronium ion concentration measured in malignant tumors in vivo; (b) the availability of cytotoxic agents which can reversibly be transformed into nontoxic prodrugs. In addition, the prodrugs should be stable in basic media and water soluble. To our knowledge there are only a few reports concerning the proton-mediated liberation of a cytotoxic drug from an inactive precursor (9). These studies however, were unsuccessful, since the hydronium ion concentration required for the hydrolysis of the prodrugs was well below the range of values which can be generated in malignant tumors in vivo (10).

There are acid-labile moieties known which could be used for blocking cytotoxic compounds to form nontoxic prodrugs. The functionality which best meets with the requirements outlined above is an acetal. In order to obtain a sufficient degree of acid lability we designed a new type of compounds, referred to as acetalglycosides, which consist of a sugar moiety, an aldehyde, and an alcohol (11, 12).

As cytotoxic species, bifunctional aldehydes were chosen. In acetalglycosides these agents are blocked as nonactive derivatives. Free aldehydes, in contrast, are not usually suited for in vivo application since they exhibit high systemic toxicity. In addition, they are rapidly deactivated after i.v. injection by aldehyde-dehydrogenases and by reaction with serum proteins and glutathione (13). In the present investigation aldophosphamide 5 was used as a cytotoxic aldehyde. Free aldophosphamide is an unstable intermediate in cyclophosphamide 1 bioactivation. CP is one of the most effective anticancer drugs with proven activity against a large variety of human cancers. It is “activated” by hepatic mixed function oxidases to give 4-hydroxycyclophosphamide 2, which is in equilibrium with aldehyde mustard 3 (Fig. 1). Aldophosphamide rapidly decomposes with liberation of acrolein 4 and phosphoramide mustard 3. The latter agent, which is considered to be the principal alkylating CP metabolite, reacts directly or after formation of an aziridinum salt with DNA or 3',5'-exonucleases (14, 15).

We have recently shown that the cytotoxic action of various bis-chloroethylyating agents including 4-hydroxycyclophosphamide per se is enhanced at low pH (7). In the present communication we describe the synthesis and in vitro activity of the acid-labile acetalglycoside of 2,3-dideoxy-D-erythro-2-hexenopyranose and aldophosphamide.
Fig. 1. Metabolic activation of cyclophosphamide (1). For details see “Introduction.”

MATERIALS AND METHODS

Melting points were determined with Mettler FP 61 or a Kofler melting-point apparatus, and are uncorrected. Elemental analyses were performed by the Microanalytical Laboratory of the University of Göttingen. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. 1H- and 13C-NMR Spectra were recorded with a Varian XL-200 and a Varian VXR-200 instrument (200 MHz; internal tetramethylsilane). The progress of all reactions was monitored by TLC on silica gel 60 (Merck), Kieselgel 0.04-0.063 and 0.063-0.200 mm (ICN, XL-200 and a Varian VXR-200 instrument (200 MHz; internal tetramethylsilane). The progress of all reactions was monitored by TLC on silica gel (Solvent A) petroleum ether (40-60)/isobutyl methyl ether = 3:2; Solvent B, chloroform/hexane/methanol = 3:2:1; Solvent C, petroleum ether (40-60) = 1:1, Solvent D, acetone/petroleum ether (40-60) = 3:2; Solvent E, chloroform/hexane/methanol = 3:2:1; Solvent F, tert-butyl methyl ether/methanol = 1:1.

Synthesis of HEX-ALD. 3,4,6-Tri-O-acetyl-l,5-anhydro-2-deoxy-D-arabinohex-1-enitol (6, Fig. 2): (5.00 g, 18.4 mmol) was rapidly added with an ice bath. After addition of barium carbonate (1.8 g) the mixture was stirred for 14 min and afterwards immediately cooled to room temperature for 14 min and afterwards immediately cooled to room temperature and made neutral. 1,1,1,3,3,3-Hexamethyldisilazane (2.11 ml, 10.0 mmol) and triethylamine (558 µl, 4.00 mmol) and A',A'-bis(2-chloroethyl) phosphor acid dichloride 12 (338 mg, 1.50 mmol) and the mixture was stirred for 72 h at 40°C to give the intermediate 13 (TLC control, 268 mg, 1.00 mmol; Fig. 3) and 3-(3-methoxybenzyloxy)propanal (315 mg, 0.50 mmol). For workup the reaction mixture was filtered over silica gel (200 µm) with dichloromethane as solvent. Concentration in vacuo and column chromatography on silica gel (Solvent A) afforded 368 mg (82%) of 9 as a colorless oil. Rf = 0.24 (Solvent A); [α]D = +88° (c = 0.5 in CHCl3); IR (film): ν = 1740 cm⁻¹ (C=O), 1240/cm (C-O); 1H-NMR (CDCl3): δ = 1.20 (t, J = 7.0 Hz, 3 H, 2'-H2), 1.90–2.11 (m, 2 H, 2'-H2), 2.04, 2.07, 2.08, 2.09 (4s, 6 H, OAc), 3.41–3.87 (m, 4 H, 1'-H, 3'-H, 2'-H2, 3'-H2, 1'-H, 1'-H, 1'-H, 1'-H2), 4.22 (s, 2 H, PhCH2O), 4.48 (t, J = 5.5 Hz, 0.4 H, l'-H, [1'S]), 4.98 (t, J = 5.5 Hz, 0.6 H, l'-H, [1'S]), 5.16–5.36 (m, 2 H, 1-H, 4-H), 5.64–5.99 (m, 2 H, 2-H, 3-H), 6.82–6.91 (m, 2 H, Ph-H), 7.19–7.29 (m, 2 H, Ph-H).

Required: C 61.05, H 7.13
Found: C 60.69, H 7.04

To a mixture of 9 (680 mg, 1.51 mmol) in dichloromethane (90 ml) and water (5 ml) was added at 20°C 2,3-dichloro-5,6-dicyano-p-benzoquinone (451 mg, 1.99 mmol). After stirring for 4.5 h (TLC) the reaction was quenched by addition of a phosphate buffer (pH 7), the organic layer was removed, and the aqueous layer was extracted with dichloromethane (2 × 50 ml). The combined organic phases were washed (brine, dried (Na2SO4)), and concentrated in vacuo at 0°C. Immediately following, column chromatography afforded 422 mg (84%) of 11 (Fig. 4) as a colorless oil. Ratio of (1'R):(1'S) = 3:2; [α]D = +115.8° (c = 0.5 in CHCl3); IR (film): ν = 3500 (OH), 1740 (C=O), 1240 cm⁻¹ (C-O); 1H-NMR (CDCl3): δ = 1.24 (t, J = 7.0 Hz, 1.8 H, 2'-H2, (1'R)), 1.25 (t, J = 7.0 Hz, 1.2 H, 2'-H2, (1'S)), 1.97 (q, J = 6.0 Hz, 2 H, 2'-H2, (1'R)), 1.90–2.05 (m, 1 H, OH2), 2.10 (s, 6 H, OAc), 3.46–3.99 (m, 4 H, 3'-H, 1'-H, 3'-H, 1'-H), 4.02–4.28 (m, 3 H, 5-H, 6-H), 4.92 (t, J = 5.5 Hz, 0.4 H, l'-H, [1'S]), 5.02 (t, J = 5.5 Hz, 0.6 H, l'-H, [1'S]), 5.24–5.48 (m, 2 H, 1-H, 4-H), 5.73–5.98 (m, 2 H, 2-H, 3-H).

Required: C 54.21, H 7.28
Found: C 53.81, H 7.57

To a solution of 11 (300 mg, 0.90 mmol) in anhydrous benzene (2 ml) and dichloromethane (2 ml) was added via a syringe anhydrous triethylamine (558 µl, 4.00 mmol) and N,N-bis(2-chloroethyl) phosphoric acid dichloride 12 (338 mg, 1.50 mmol) and the mixture was stirred for 72 h at 40°C to give the intermediate 3 (TLC control, Solvent C, Rf = 0.43; Fig. 5). The volatile substances were removed by distillation in vacuo and the residue was dissolved in anhydrous toluene (5 ml); anhydrous ammonia was passed through the solution for 4 h at 20°C (TLC control, solvent D, Rf = 0.24). Concentration in a vacuum and column chromatography on silica gel (Solvent D) afforded 278 mg.
RESULTS

Synthesis of Acid-labile Acetalglycosides of Aldophosphamide
5. The acid lability of acetalglycosides can be modified by appropriate choice of the sugar moiety. Mechanistic studies on the proton-catalyzed hydrolysis of glycosides have shown that, in general, a protonation occurs at the exo-oxygen of the glycoside followed by a cleavage of the glycosyl oxygen bond to give a glycosyl cation (16). Since the formation of this cation is the rate-determining step, any substituent at the sugar moiety which stabilizes the glycosyl cation should enhance the proton-catalyzed hydrolysis in the following order: acetalglycosides of glucopyranose, 2-deoxy-α-D-arabino-hexopyranose, 2,6-dideoxy-α-L-arabino-hexopyranose and 2,3-dideoxy-α-erythro-2-hexopyranose.

For the synthesis of HEX-ALD a newly developed method was used which allows an efficient and selective entry to these compounds (11, 12, 17, 18). The procedure differs from known methods of glycosidation, since the formation of the glycosidic bond is not accomplished by an attack at C-1 of an activated sugar derivative such as acetobromoglucose, but at the exo-oxygen of a protected trimethylsilyl glycoside with retention of the configuration at C-1. For the synthesis of HEX-ALD the trimethylsilyl glycoside δ was used, which can be obtained from the glucal 6 by treatment with water at 100°C to give 7 (19), followed by reaction with chlorotrimethylsilane and hexamethyldisilazane. The yield for the transformation of 6 to 7 could be improved by irradiation with a high pressure mercury lamp to give an overall yield of 60%. The detailed discussion of the mechanism will be published elsewhere.

Treatment of the trimethylsilylglycoside δ with the acetal 9 and the corresponding aldehyde in the presence of a catalytic amount of trimethyltrifluoromethanesulfonate in dichloromethane at −90°C affords the acetal-α-glycoside 10 as a mixture of diastereomers according to C-1’ in 82% yield. For deblocking of the protecting group hydrogenolysis could not be used, since this would also cause a hydrogenation of the C=C bond in the sugar moiety. Therefore the p-methoxybenzyl group was removed by an oxidative cleavage (20) using 2,3-dichloro-5,6-dicyano-1,1-benzoquinone to give 11 in 84% yield. The phosphoramidate mustard group was introduced by reaction of 11 with N,N-bis(2-chloroethyl)phosphoric acid dichloride 12 (21). The primarly formed phosphoric acid monochloride derivative 13 is not stable and was therefore immediately transformed without isolation to the phosphorous diamidate 14 in an overall yield of 58%. The best method for removal of the acetyl groups to 15 was the solvolysis with methanol in the presence of potassium carbonate. The use of sodium methanolate as base resulted mainly in a decomposition of the educt. In the formation of the acetylglycoside 10 the α-glycoside was obtained predominantly, although the starting material δ was used throughout all experiments. Following drug exposure the cells were washed with drug-free culture medium, tryponsonized, and counted. Dilutions of 10^6, 10^5, and 10^4 cells were seeded in triplicate in 60-mm dishes. After incubation in DMEM, pH 7.4, for 12 days the colonies were fixed and stained with Loeffer’s methylene blue. Colonies of more than 1 mm in diameter were counted. As was shown in separate experiments the plating assay exhibited a linear relation between the number of cells seeded and the number of colonies formed in the range of 25–500 cells plated. The plating efficiency of treated cells was normalized to the plating efficiency of untreated controls (60 ± 5%) and the colony-forming fraction was calculated taking into account cell lysis during treatment (7). Results are presented as means of at least three separate experiments.

Fig. 5. Synthesis of (3RS)-3-ethoxy-3-(4,6-di-O-acetyl-2,3-dideoxy-α-D-erythro-2-hexopyranosyloxy)propyl-N,N-bis(2-chloroethyl)phosphorodiamidate (14) (formula weight 535.4).

Fig. 6. Synthesis of (3RS)-3-ethoxy-3-(2,3-dideoxy-α-D-erythro-2-hexopyranosyloxy)propyl-N,N-bis(2-chloroethyl)phosphorodiamidate (15) (formula weight 451.4).
used as an anomeric mixture. A proven explanation cannot be
given so far; however, it may be possible that the α-anomer of
8 reacts faster and the remaining β-anomer may anomerize to
the α-anomer of 8, or that the intermediately formed acetal-β-
glycoside isomerizes to 10. In the formation of 10, in addition,
a second stereogenic center at C-1' is formed. In this case a
control is not yet possible, therefore one obtains a 3:2 mixture
of the two diastereomers which cannot be separated by
chromatography or other methods.

Structure and Chemical Properties of the Synthesized Prod-
ucts. The configuration at C-1 of the 2-hexenopyranoside can-
ot be determined by 1H-NMR spectroscopy, since both the α-
and the β-anomers show only a broad singlet for 1-H. This is
in contrast to, e.g., α- and the β-glucosides where the coupling
constant for 1-H in the α-anomer is J = 3 Hz and for the β-
anomer J = 7.5 Hz. However, the specific rotation of the com-
ounds allows a clear assignment, since the α-anomers show
a strong positive and the β-anomers a weak negative
specific rotation of linear polarized light. The ratio of the
diastereomers at C-1' was determined by 1H- and 13C-NMR
spectroscopy. It has been shown that C-1' in (1'R)-acetals-α-
glycosides absorbs usually at higher field than in the corre-
sponding (1'S)-acetals-α-glycosides (12, 22). For the acetal-α-
glycoside 10 the following chemical shifts for C-1' were found,
δ = 99.30 for the 1'R- and δ = 101.30 for the 1'S-isomer. The
acid catalyzed hydrolysis of the HEX-ALD was measured in
D2O as solvent at different pD ranging from pD 2.7-4.5. The
change in the concentration of HEX-ALD was determined from
the interaction of the signals for the CH2-moiety in 1H-NMR
spectra taken at different time intervals. In this reaction the
active anticancer agent aldophosphamide 5, as well as ethanol
and 3-(1,2-dihydroxyethyl)furan are formed. The half-life of
HEX-ALD at pD 4.5 and 33°C was 13 h.

Cytotoxicity of HEX-ALD on Malignant Cells as Function of
Extracellular pH. The cytotoxic effect of HEX-ALD was eval-
uated in vitro by exposure of M1R rat mammary carcinoma
cells to this agent for 24–72 h. At physiological pHe (7.4) HEX-
ALD at concentrations up to 100 μg/ml exhibited only very
limited toxicity as was measured by colony formation of surviv-
ing cells after treatment for 24 h (Fig. 7). At 100 μg/ml, e.g.,
the surviving fraction of M1R cells was only lowered to 0.5 of
untreated controls.

There was no significant increase of cytotoxicity when ex-
posure was prolonged from 24 to 72 h. For comparison the
survival curve of M1R cells following treatment with 4-hydro-
peroxycyclophosphamide, an agent which hydrolyzes to “acti-
vated” cyclophosphamide at pH 7.4, is also shown in Fig. 7.

The effect of an acidic cellular environment on the cytotoxic
action of HEX-ALD is shown in Fig. 8. M1R cells were exposed
to the drug at either pHe 6.2 or pHe 5.6 for 24 h. At pHe 6.2
there was only moderate enhancement of HEX-ALD activity
as compared to pHe 7.4. However, at pHe 5.6 HEX-ALD
exhibited strong dose-dependent cytotoxicity. For example,
at 3 and 5 μg/ml the colony-forming fraction of M1R cells de-
creased to 1 × 10⁻³ and 8 × 10⁻⁵, respectively. The latter value
corresponds to an enhancement of HEX-ALD cytotoxicity at
pHe 5.6 as compared to pHe 7.4 by a factor of 10⁴.
PROTON-MEDIATED LIBERATION OF CYTOTOXIC DRUGS

In general, acetalglycosides, e.g., of glucose, exhibit a sufficient rate in the proton-catalyzed hydrolysis only at pH <2. Thus, at pH > 4 there is no detectable cleavage of these compounds. However, by introducing a 2,3-double bond into the sugar moiety the transition state of the proton-catalyzed hydrolysis is stabilized and thus the energy of activation is lowered. Therefore, acetalglycosides of 2-hexenopyranose, e.g., HEX-ALD, show a high acid lability. For the synthesis of HEX-ALD we developed a new method, which leads stereoselectively to the α-glycoside. HEX-ALD is water soluble and stable in basic medium allowing its application as aqueous solutions and facilitating its handling. At pH 7.4 HEX-ALD is almost not cleaved. However, at pH 5.6 and at pH 6.2 HEX-ALD is hydrolyzed to give the cytotoxic aldophosphamide as well as 3-(1,2-dihydroxyethyl)furan and ethanol within 24 h and 48 h, respectively.

The survival curves of M1R cells exposed to HEX-ALD at different pHs are consistent with these data. Following treatment at pH 7.4 there was only very limited cytotoxicity even after exposure for 72 h. The maximum HEX-ALD concentration tested exceeds the equitoxic dose of 4-hydroperoxycyclophosphamide ("activated" CP) on M1R cells by a factor of >100. In contrast, at pH 5.6 (10 μg/ml, 24 h) the surviving fraction of M1R cells decreased to less than 10^{-5} of untreated controls. A similar degree of cytotoxicity was observed at pH 6.2, however, only following treatment for 48 h.

From the data presented in Fig. 8 it can be calculated that in order to exert equitoxic effects on M1R cells, at pH 5.6 the concentration of HEX-ALD can be lowered by a factor of 20 as compared to pH 6.2. Assuming first-order kinetics of proton-catalyzed liberation of aldophosphamide one would expect this factor to only be in the order of 4. This discrepancy does not necessarily suggest that the mode of HEX-ALD hydrolysis differs from that delineated above. The additional enhancement of HEX-ALD cytotoxicity over the effect expected solely by a more rapid liberation of HEX-ALD may be due to a proton-mediated potentiation of aldophosphamide toxicity per se. We have previously shown that the cytotoxicity of CP metabolites on M1R cells, in particular that of norphosphamide, less of phosphoramidomustard, is enhanced at low environmental pH (7). Thus, at low pH two activating mechanisms may be operative simultaneously: liberation of aldophosphamide from HEX-ALD by proton-catalyzed hydrolysis and proton-mediated enhancement of aldophosphamide toxicity per se.

In transplanted animal tumors mean pH values as low as 6.2 can be maintained for up to 48 h (6). Both the duration and the magnitude of this pH shift correlate well with the conditions required for HEX-ALD "toxification" in vitro. Irrespective of this correlation we still consider HEX-ALD a prototypic drug. This is mainly due to its relatively long half-life at pH 6.2. The blood glucose required for generation of intratumoral acidosis (mean pH, 6.2) is in the range of 20–25 mm (6). In humans these levels have been maintained solely by i.v. infusion of glucose for up to 24 h (23). However, the potential therapeutic application of acid-labile prodrugs would be greatly facilitated if the duration of hyperglycemia could be limited to less than 8 h. Preliminary results from our laboratory indicate that proton-
sensitive compounds with a half-life of less than 8 h at pH 6.2 can, in principle, be synthesized.

In summary, the results presented here are consistent with the view that a metabolic property of malignant cells, their glycolysis, may be exploited to liberate cytotoxic agents from inactive precursors preferentially in malignant tissues. Experiments to prove this hypothesis in transplanted tumors in vivo are in progress. Also in process is the synthesis of nontoxic prodrugs which can be selectively cleaved by enzymatic hydrolysis at lower pH (24).

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