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pH Dependent general base catalyzed activation rather than isocyanate liberation may explain the superior anticancer efficacy of laromustine compared to related 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine prodrugs

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Abstract

Laromustine (also known as cloretazine, onrigin, VNP40101M, 101M) is a prodrug of 90CE, a short-lived chloroethylating agent with anticancer activity. The short half-life of 90CE necessitates the use of latentiated prodrug forms for *in vivo* treatments. Alkylaminocarbonyl based prodrugs such as laromustine exhibit significantly superior *in vivo* activity in several murine tumor models compared to analogs utilizing acyl, and alkoxycarbonyl latentiating groups. The alkylaminocarbonyl prodrugs possess two exclusive characteristics: (i) they are primarily unmasked by spontaneous base catalyzed elimination; and (ii) they liberate a reactive carbamoylating species. Previous speculations as to the therapeutic superiority of laromustine have focused upon the inhibition of enzymes by carbamoylation. We have investigated the therapeutic interactions of analogs with segregated chloroethylating and carbamoylating activities (singly and in combination) in the *in vivo* murine L1210 leukemia model. The combined treatment with chloroethylating and carbamoylating prodrugs failed to result in any synergism and produced a reduction in the therapeutic efficacy compared to the chloroethylating prodrug alone. Evidence supporting an alternative explanation for the superior tumor selectivity of laromustine is presented that is centered upon the high pH sensitivity of its base catalyzed activation, and the more alkaline intracellular pH values commonly found within tumor cells.

Graphical Abstract

Laromustine is a prodrug of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE), a component with confirmed anticancer activity, and methyl isocyanate, a component of speculated benefit. Laromustine exhibits superior *in vivo* activity to analogs generating only 90CE. However, treatment with combinations of prodrugs that separately generate 90CE or methyl isocyanate

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resulted in antagonism. An alternative non-isocyanate dependent explanation for the superior tumor selectivity of laromustine is presented based upon the pH sensitivity of its activation, and the typically more alkaline intracellular pH of tumor cells.

Keywords

laromustine; sulfonylhydrazine; methyl isocyanate; MGMT; intracellular pH; phosphate; alkylation; crosslinking; targeted chemotherapy

Introduction

1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE) is a short lived ($t_{1/2} \sim 30$ s at 37°C , pH 7.4) weakly acidic ($\text{pK}_a \sim 6.7$) chloroethylating agent that targets the *O*-6 position of DNA guanine to produce an anticancer effect (Fig. 1 panels A, B) (1–4). The short half-life of 90CE precludes its direct use as a therapeutic agent due to insufficient time for distribution. Laromustine (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine, also known as cloretazine, onrigin, VNP40101M, 101M) and related 90CE prodrugs were produced by replacing the *N*-2 proton on 90CE with various masking groups. This prevents the formation of the unstable 90CE anion, essential for the generation of chloroethylating electrophiles, until the masking group is cleaved (Fig. 1 panel A) (1–4). Thus slow cleavage of the masking group from the *N*-2 position results in the liberation of active 90CE after the distribution of the prodrug form has occurred. Acyl, alkoxycarbonyl, and alkylaminocarbonyl 90CE prodrug classes were synthesized and all three classes contained agents capable of exhibiting high activity in *in vivo* murine tumor models. The alkylaminocarbonyl derivatives, exemplified by laromustine, were superior in activity towards several *in vivo* murine tumor models (4), and the rank order of antitumor activity against L1210 leukemia for the most active members of each class was alkoxycarbonyl $\approx$ acyl < alkylaminocarbonyl (4–7). Laromustine exhibited a therapeutic index ($\text{LD}_{50}/\text{ED}_{50}$) against L1210 leukemia in female CD_2F_1 mice of > 8 . This value is approximately 4 times that of the best acyl prodrug 1-acetyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine (90AC) (Fig. 1 panel C) (5,8). The outstanding therapeutic index of laromustine played a major role in its selection for clinical evaluation (4). However the

reasons behind the significant disparity in therapeutic index between the different 90CE prodrug forms have remained obscure.

During its decomposition the 90CE anion produces transient oxophilic chloroethylating species that react with DNA guanine to generate the *O*⁶-(2-chloroethyl)guanine DNA lesion (1,2,9). This lesion undergoes an intramolecular nucleophilic substitution followed by reaction with the the opposing cytosine to form a highly cytotoxic DNA inter-strand cross-link (G-C ethane cross-link) (2–4) (Fig. 1 panel B). These cross-links appears to be responsible for the vast majority of the cytotoxicity of 90CE and its prodrugs towards responsive tumor cell lines (3,10,11). *O*⁶-Alkylguanine-DNA alkyltransferase (MGMT) is the major protein involved in the repair of the initial lesions, preventing the formation of the highly cytotoxic G-C ethane cross-links (11) (Fig. 1 panel B). Murine tumor models that are highly responsive to 90CE prodrugs typically lack detectable MGMT activity, and it is likely that differentials in MGMT activity between tumor and normal cells are the primary determinant of efficacy for agents of this type in most cases (4). A small sub-set of human tumors (of diverse types) have been reported to lack detectable MGMT activity (3,4,10–12), and these sub-sets would be expected to be suitable candidates for treatment with 90CE prodrugs.

During the liberation of 90CE from the alkoxycarbonyl and acyl prodrugs relatively innocuous byproducts are generated from the masking group (simple alcohols/CO₂ and carboxylic acids, respectively); in contrast, the alkylaminocarbonyl prodrugs produce a reactive isocyanate (Fig. 1 Panel A) (13–16). There are at least three different classes of simple alkylating anticancer agents that contain alkylaminocarbonyl moieties, that either obligatorily generate reactive isocyanates during their decomposition (chloroethylnitrosoureas) (17, 18), or this property is possessed by the most therapeutic members of their respective classes (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines (4,8), and 1,3-dialkyltriazenes) (19). This has led to speculation on potential beneficial role(s) of isocyanates in the anticancer activity of these agent classes (13,20–24). Isocyanates are highly reactive towards soft nucleophiles such as thiol groups, and are thus able to inhibit numerous enzymes that contain an essential thiol group(s) as part of their active site in *in vitro* assays (25) by acting as non-specific protein covalent modifiers (NSPCMs) (Fig. 1 Panel A insert). Thus it is not surprising that agents that are the prodrugs of isocyanates have been shown to inhibit multiple thiol dependent enzymes in *in vitro* assays including MGMT and other proteins involved in DNA repair (15,26–29). These isocyanate dependent DNA repair inhibitions could, at least in theory, provide a potential explanation for the superior cytotoxic performance of DNA damaging agents that co-release reactive isocyanates. However, using this argument to account for the greater neoplastic selectivity of laromustine compared to other 90CE prodrugs is more challenging, because such inhibitions would be expected to sensitize both normal and tumor cells to 90CE. Additionally, the lack of detectable MGMT expression in highly responsive tumor models, such as L1210 (wild type) (30), excludes isocyanate inhibition of MGMT as a potential explanation for laromustine's superiority in these cases. Furthermore, many *in vitro* isocyanate dependent inhibitions likely do not accurately reflect the *in vivo* situation. High concentrations of glutathione, in *in vivo* conditions, can strongly temper isocyanate dependent inhibitions by acting as competing targets (15). In *in vitro* cellular studies, due to

the very low cell densities relative to *in vivo* conditions, essential enzyme thiol groups are ineffectively protected from the vast molar excess of isocyanate/isocyanate precursors available within the medium. Under these circumstances the limited low molecular weight thiol pools within the cells are readily titrated and the inhibition of isocyanate-sensitive enzymes heavily favored. Similarly, in studies involving isolated enzymes (in the absence of high concentrations of protective thiols) deceptively strong non-physiological inhibitions can be observed. Owing to the strong tendency of NSPCMs to give misleading *in vitro*-specific positive results they have been classified as PAINS (pan-assay interference compounds) (31). It has therefore been advocated that NSPCMs/PAINS be removed from or ignored in compound libraries due to their tendencies to generate artefactual (or inflated minor-effect) research sidetracks (31). However, to further complicate this picture, some isocyanates, such as chloroethyl isocyanate, can indirectly function as specific protein covalent modifiers due to the tendency of their initial carbamoylation reaction products to participate in transcarbamoylation reactions with particular thiol residues on specific enzymes (32,33). Thus while low concentrations of both methyl isocyanate and chloroethyl isocyanate strongly and equivalently inhibit isolated human glutathione reductase in the absence of competing thiols, only chloroethyl isocyanate (and precursors thereof) retains significant inhibitory activity in whole blood, cell suspensions, and animal models where thiols are abundant (15, 32,33). Additionally, some isocyanate inhibited processes are expected to have negative effects on the anticancer activity of cytotoxic species; such as the reported inhibition of apoptosis via the inactivation of caspases (caspases are cysteine-aspartic proteases (34)) by chloroethyl isocyanate (29). Therefore it remains an open question as to the beneficial role, or otherwise, played by isocyanates in the *in vivo* therapeutic activity of alkylaminocarbonyl based prodrugs of 90CE such as laromustine.

The acyl, alkoxycarbonyl, and alkylaminocarbonyl 90CE prodrug classes also differ in their mode of masking group cleavage. Prodrugs containing acyl and alkoxycarbonyl masking groups do not undergo spontaneous masking group cleavage at any significant rate at close to neutral pH values and are activated by metabolic processes, while the alkylaminocarbonyl masking groups readily undergo spontaneous base catalyzed elimination/cleavage (4–6,15,16) (Fig. 1 panel A). The atypically alkaline intracellular pH (pHi) values of ~ 7.5 to 7.65 commonly found in tumor cells (35,36) (Fig. 1 panel D), including L1210 leukemia (37), compared to those of normal tissue cells (pH value of ~ 7.2) (35) could potentially speed the base catalyzed activation of alkylaminocarbonyl prodrugs.

Herein we explore the therapeutic interactions of carbamoylating and chloroethylating activities in the *in vivo* murine L1210 leukemia model by treatment with 1,2-bis(methylsulfonyl)hydrazines analogs (Fig. 1 panel E) with segregated chloroethylating and carbamoylating activities both singly, and in combination, to model the two activities generated by laromustine. Additionally we examine the pH and base concentration dependency of the spontaneous activation of laromustine. The overall aim of these studies is to illuminate processes behind the therapeutic superiority of laromustine compared to other simple 90CE prodrugs that may be useful in future antineoplastic drug design.

Materials and methods

Chemicals

All chemicals were purchased from the Sigma-Aldrich Chemical Company, St. Louis, MO, except where specified. Hoechst 33258 was obtained from Molecular Probes, Inc., Eugene, OR. Laromustine (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine) and 90AC, (1-acetyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine) were synthesized in this laboratory as previously described (5,7) (structures given Fig. 1 panels A and E). ProMIC 1,2-bis(methylsulfonyl)-1,2-bis(methylaminocarbonyl)hydrazine was synthesized using a procedure similar to that described for laromustine, but substituting 1,2-bis(methylsulfonyl)hydrazine for 90CE (structure given Fig. 1 panel E).

Cultivation of L1210 leukemia

L1210 murine leukemia cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% FBS in air/5% CO₂ at 37° C. The cells were subcultured as required every 2–3 days.

Murine L1210 cell DNA preparation

DNA was isolated using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) using procedures recommended by the manufacturer.

Murine L1210 leukemia studies

Female CD₂F₁ mice (4–5 mice per group) were inoculated with 1×10^5 murine L1210 leukemia cells. Mice were then treated with a single i.p. dose of agent(s) (laromustine, 90AC or ProMIC or combinations thereof) dissolved in 100% dry DMSO (0.001 mL/g of mouse body weight) 24 h post inoculation. Death due to drug toxicity was assigned on the basis of the mouse's appearance, body weight and lack of leukemia cells in the peritoneum at the time of death. The post-inoculation life span was monitored for a total of 60 days. Treated/control (T/C) survival values are the arithmetic mean values only for the mice that died and do not contain a value for long term survivors.

Determination of the effects of pH, and [Pi] on the half-life of laromustine

Initial studies of the dependency of laromustine's half-life upon pH and [Pi] (inorganic phosphate, a mixture of H₂PO₄⁻ and HPO₄²⁻ at biological pH values) were conducted at an elevated temperature of 50°C for experimental convenience. The elevated temperature reduces the experimental time required to follow laromustine decay to near completion by ~8-fold compared to 37°C. During the decay of laromustine H⁺ ions are generated after the rate determining cleavage of the methylaminocarbonyl masking group (Fig. 2 panels A-D). The H⁺ ion liberation permits the real-time tracking of laromustine decay. Laromustine initially produces methyl isocyanate and 90CE, then these two fleeting products breakdown with very short half-life values (< 20 s, and ~6 s at 50°C, pH 7–8, respectively) (1, 38,39) resulting in a net liberation of H⁺ ions (Fig. 2 panels B–D). Thus the relatively slow initial base catalyzed elimination/activation of laromustine controls the overall kinetics of H⁺

liberation. The observed kinetics closely approximate to a first order exponential decay process (Fig. 2 panel E). The half-life values at 50°C, and various pH values were calculated using GraphPad Prism software (Version 3.02) by obtaining the best fit to a one phase exponential decay process. In these 50°C studies, laromustine was used at final concentrations of 5 μ M – 10 μ M added as 0.25–0.5 μ L/mL of a 20 mM stock solution in DMSO. The assay solution contained 1 mM potassium Pi and 10 mg/L phenol red, and experiments were performed with initial pH values between 7.0 and 8.0. The protonometric assay method utilized is based upon the relatively linear change in absorption at 560 nm of phenol red in proportion to small quantities of H⁺ ions generated (or consumed) in a weakly buffered solution of phenol red at pH values close to the pKa of the buffer/indicator components over small pH ranges (maximum total pH < 0.03 units) (1). In these studies the initial pH of the solutions was adjusted versus pH standards at 50°C. To ascertain if the decay of laromustine was subject to general or specific acid/base catalysis the [Pi] was varied between 1 and 32 mM using the fastest previously used decay conditions (pH 8.0 and 50°C). The increased [Pi] strengthens the buffering capacity (decreasing the assay sensitivity) necessitating the use of greater laromustine concentrations in these experiments. Thus laromustine was used at concentrations from 5.0 μ M to 125.0 μ M for assay conditions utilizing 1 to 32 mM Pi.

At physiological temperature (37°C), pH values (7.2–7.6), and [Pi] (1–20 mM) the half-life of laromustine is much longer, this would result in excessively long experiments if the half-life values were to be determined by following laromustine decay to near completion. We therefore calculated the half-life under these conditions from the initial rate of H⁺ liberation utilizing the first order decay equation. To calculate the half-life by this method we need to know the total quantity of H⁺ liberated at reaction completion. This latter value can be easily determined at the appropriate pH values knowing the initial concentration of laromustine and the pKa values of the decomposition products. 90CE produces two moles of strongly acidic species independently of the proportion entering either of its two major decomposition pathways (1,9) (Fig. 2, panels C and D). Thus each mole of 90CE generates essentially two moles of H⁺ and this is largely responsible for the overall H⁺ yield from laromustine. Methyl isocyanate hydrolyzes to produce methylamine (a moderately strong base pKa 10.66) and carbonic acid (apparent pKa 6.34) (40) (Fig. 2, panel B). Thus the H⁺ yield from the carbonic acid almost exactly cancels out the H⁺ consumption of the methylamine in the pH range of interest. This results in small net consumptions of 0.13, 0.08, and 0.05 moles of H⁺ per mole of methyl isocyanate at pH values of 7.2, 7.4, and 7.6, respectively. Thus, the calculated overall molar H⁺ yields from laromustine are 1.87, 1.92, and 1.95 at pH values of 7.2, 7.4, and 7.6, respectively. The H⁺ sensitivities for the change in absorbance at 560 nm of these assay solutions (100 mM KCl, 10 mg/mL phenol red and 1, 5, 10 and 20 mM Pi) were determined using HCl standards for each pH value and [Pi] studied. To obtain sufficiently fast (easily measured) peak initial rate values the reactions were initiated by adding between 0.20 and 2.0 mM laromustine. The highest laromustine concentrations being utilized under conditions where the buffering capacity of the assay solution was the greatest (20 mM Pi, pH 7.2), and the lowest where the buffering capacity of the assay solution was the least (1 mM Pi, pH 7.6).

Determination of the effects of pH on the cross-linking of L1210 DNA by laromustine and 90CE

DNA molecules containing one or more covalent interstrand cross-links rapidly renature upon snap cooling following thermal denaturation, because the cross-links hold the complementary DNA strands in register. Since the dye H33258 forms a highly fluorescent complex with double stranded DNA, but only weakly fluoresces with mispaired/denatured DNA, DNA molecules containing cross-links will yield highly fluorescent complexes following a heat/chill cycle; whereas DNA molecules devoid of such cross-links will not (2,41). L1210 DNA prepared as described above was washed 4× with either pH 7.2 or pH 7.6, 20 mM potassium Pi, 100 mM KCl, 0.1 mM NaN₃, buffer using Amicon Ultra-0.5 mL (Ultracel-10 membrane, NMWL 10,000) Centrifugal Filters. Small aliquots (15 µL) of washed DNA (final concentration ~200 µg DNA/mL) were then mixed with an equal volume (15 µL) of freshly prepared ice cold 2×90CE or 2× laromustine solutions in equivalent buffers and incubated for 10 mins at 37°C. These reaction mixtures were then quenched by a 50-fold dilution with 5 mM Tris-HCl, 1.0 mM EDTA, and 1.0 mM NaN₃ buffer (pH 8.0). This dilution largely blocks but does not completely halt further DNA alkylation from occurring during the workup procedure. Therefore, to correct for any post quenching reaction an additional control for each sample is included in which the initial sample contains no DNA and the DNA is added after the 50-fold dilution step. All samples are then incubated in sealed tubes for 2.5 h at 55 °C, this temperature is sufficient to allow the initial O⁶-(2-chloroethyl)guanine lesions to quickly transition into DNA-DNA interstrand cross-links, but is insufficient to thermally denature the DNA. These samples are then returned to room temperature and Hoechst H33258 fluorescent dye added to give a final concentration of 0.1 µg/mL. The fluorescence was then measured using a Hoefer Scientific Instruments TKO 100 fluorometer (λ_{ex} 365 nm, λ_{em} 460 nm). The mixtures were then heated to 100 °C for 5 min and plunged into a water bath at room temperature for 5 min and the fluorescence immediately measured. The percentage of DNA molecules that were cross-linked (i.e., containing at least one cross-link per molecule) and the number of cross-link moieties per DNA molecule in each sample were then calculated from the change in fluorescence in comparison to DNA not treated with cross-linking agent as previously described (2, 41). To obtain the number of cross-link moieties per DNA molecule occurring during the 10 minute reaction window, the value obtained for samples where the DNA was added after the dilution step is subtracted from the value obtained for samples where the DNA was initially present.

Results and discussion

In vivo activity of 90AC, ProMIC and combinations thereof against L1210 leukemia

The *in vivo* activity of combinations of chloroethylating (90AC) and carbamoylating (ProMIC) bis(sulfonyl)hydrazine prodrugs were evaluated against L1210 leukemia in mice. The wild type L1210 murine leukemia cell line used in this study lacks detectable MGMT expression (42) and thus is highly responsive to prodrugs of 90CE (8). In these experiments mice bearing L1210 leukemia were treated with either a single dose of a prodrug that generated methyl isocyanate (carbamoylating activity) or one that generated 90CE (chloroethylating activity), or combinations of these agents to deliver both activities to the same animal via independent prodrugs. It can be seen in Fig. 3, panels A and B that

treatment with 90AC alone resulted in a maximum therapeutic effect at 40 mg/Kg where 3 out of the 4 mice became long term survivors, and no toxic deaths were observed until a dose of 60 mg/Kg was reached. These findings are similar to earlier 90AC single dose studies in our laboratory where 40 mg/Kg was found to yield the greatest therapeutic effect (producing up to 5/5 long term survivors) with deaths due to 90AC toxicity again being observed at doses of 60 mg/Kg (Fig. 1 panel C). In contrast ProMIC as a single agent exhibited no observable anti-leukemic activity or overt signs of toxicity at doses up to 60 mg/Kg (Fig. 3, panel A). Nevertheless co-treatment with both agents resulted in reduced efficacy compared to 90AC alone due to an apparent antagonism of the therapeutic actions (Fig. 3, panel B) and an increase in toxicity to the mice from the prodrug combination (Fig. 3, panel C). If the broader therapeutic range observed with laromustine compared to 90AC (Fig. 1, panel C), had been largely the consequence of laromustine being a dual prodrug of synergistic 90CE and methyl isocyanate, it may have been expected that co-treatment with 90AC and ProMIC may have, at least partially, recapitulated the superior activity of laromustine at some dose combinations.

Bis(sulfonyl)hydrazines with segregated carbamoylating and chloroethylating activities have been used in an analogous manner to successfully dissect the contributions of these activities towards various processes such as DNA cross-linking/nicking, enzyme inhibition, and cytotoxicity in a number of *in vitro* studies (13,15,16,20,21,26–28). It is realized that these *in vivo* experiments involve many additional variables that can greatly complicate interpretation compared to *in vitro* studies. However, the strongly negative rather than positive consequences of *in vivo* 90CE/methylisocyanate co-liberation do not support the contention that the dual prodrug nature of laromustine is likely the direct basis for its higher therapeutic index. These *in vivo* cytotoxicity observations are somewhat reminiscent of earlier *in vitro* studies where the cytotoxic effects of agents with carbamoylating and chloroethylating activities on wild type L1210 leukemic cells, and transfectants expressing MGMT, were studied (30). In these earlier experiments it was observed that the cytotoxicity of 90CE was highly dependent upon the MGMT activity, with MGMT transfected L1210 cells having approximately a 22-fold greater IC₉₀ concentration, while the cytotoxicity of a methylisocyanate liberating prodrug was independent of the MGMT activity (30). As expected the simultaneous exposure to carbamoylating (MGMT independent toxicity) and chloroethylating (MGMT dependent toxicity) activities resulted in a net cytotoxicity with a lower MGMT dependency than observed with the chloroethylating activity alone. In the *in vivo* L1210 studies the therapeutic selectivity of 90AC is primarily a consequence of the MGMT activity differential between wild type MGMT negative L1210 leukemia and MGMT expressing normal murine tissues. Thus the addition of a non MGMT-dependent non tumor-selective toxicophore would similarly be expected to reduce the overall tumor selectivity, and this effect is observed in the *in vivo* experimental outcome. These studies suggest that the therapeutic superiority against L1210 leukemia, is a consequence of some other distinctive property of laromustine rather than its combined carbamoylating and chloroethylating activities.

Effects of pH, and [Pi] concentration on the half-life of laromustine

Unlike the stable (metabolically activated) acyl and alkoxycarbonyl prodrugs the alkylaminocarbonyl prodrugs readily undergo spontaneous masking group cleavage/activation in neutral buffers (13,15,16). Since neoplastic cells typically have higher pH_i values than normal cells (35,36,43) we conducted a study of the effects of pH on the spontaneous activation of laromustine. The activation of laromustine was initially investigated over a pH range of 7.0 – 8.0 in a 1 mM potassium Pi buffer. A higher than physiological temperature (50 °C) was used in preliminary experiments because this reduced the decay time by ~ 8-fold. Under such conditions the decay of laromustine could be followed to essential completion over conveniently short time intervals. The spontaneous decomposition of laromustine closely approximated to first order decay kinetics (Fig. 2, panel E) with the half-life values being strongly dependent upon the pH. The half-life of laromustine at pH 8.0 was found to be ~ 8-fold shorter than at pH 7.0, and was ~2.4-fold shorter at pH 7.6 (neoplastic cell pH_i) than at pH 7.2 (normal cell pH_i) (Fig. 4, panel A). There are two major classes of acid/base catalyzed reaction (a) specific acid/base catalyzed, where the rate of reaction is solely dependent upon the ambient pH, and (b) general acid/based catalyzed, where the rate of reaction is also dependent upon the concentrations of other species (in addition to [H⁺] and [OH⁻]) that can participate in H⁺ ion transfer reactions at the studied pH, such as buffer components (44). In general acid/based catalyzed reactions the rate possesses additional pH dependent variables reflecting the change in the proportions/concentrations of the buffer's acidic and conjugate base forms in response to the ambient pH (44). General acid/based catalysis is usually observed when proton transfer is involved in the rate limiting reaction step, and is most dominant when the pH of the solution is near the pK_a of the catalytically active acid/base buffer component (44). Anions of strong acids such as Cl⁻ are inactive as general base catalysts at neutral pH values as they have extremely low proton affinities (low basicity) under these conditions. Specific and general acid/base catalysis are easily distinguished; in specific acid/base catalysis the rate of reaction is dependent upon the pH but is independent of the buffer concentration, whereas in general acid/base catalysis the rate of reaction is dependent upon the buffer concentration at constant pH values (44). To determine if specific or general base catalysis was involved in the activation of laromustine we measured the effect of varying the [Pi] from 1 – 32 mM on its half-life. The conditions utilized above which resulted in the fastest activation rate (pH 8.0, 50 °C) were utilized for the initial experiments. The half-life of laromustine was found to be ~ 2.7-fold shorter at 32 mM Pi than at 1 mM Pi indicating that this activation was subject to general base catalysis (Fig. 4, panel B). The observed half-life dependencies upon both pH and [Pi] have potentially important pharmacological consequences. Therefore the half-life of laromustine was mapped at 37 °C over physiologically and neoplastically relevant pH and [Pi] ranges (Fig. 5). Owing to the significantly slower rate of reaction at 37 °C the half-life values were calculated from initial rate measurements, rather than by following the decay to near completion. As expected, slower but similar fold laromustine half-life dependencies upon pH and [Pi] were observed at 37 °C (Fig. 5). This activation rate response to pH and to a lesser extent [Pi] could provide a simple alternative explanation for the therapeutic edge of laromustine versus other 90CE prodrugs. Laromustine being a small, neutral, and permeable molecule (7,8) should rapidly equilibrate throughout all tissues, but as a result of the increased laromustine activation rates (>2-fold) within tumor cells/tissues deliver a greater

90CE exposure compared to that experienced by normal tissue cells. The short half-life of 90CE and its largely anionic nature at tumor cell pHi values (pKa 90CE ~ 6.7) would then restrict its extensive equilibration/redistribution, and help confine its activity within a close proximity to its site of activation. This action would result in a tumor cell biased delivery of cytotoxic stress. Pi and phosphomonoesters occur at high total combined intracellular concentrations and have pKa values close to neutrality making them the most important intracellular buffers (9,45), and low molecular weight general acid/base catalysis. Thus the concentrations of phosphates would be expected to be important players in addition to pH affecting the biological half-life of laromustine. Tumor cells may possess somewhat greater levels of Pi and some phosphoesters compared to normal cells, as it has been reported that their high glycolytic rate is associated with an increased rate of Pi uptake and higher levels of polyphosphates (46–48). This could modestly augment the relative laromustine activation rates within tumor cells. Bicarbonate is the most important extracellular buffer but it is the conjugate base of a strong acid (true pKa ~ 3.5 (49,50)) with little proton affinity and therefore would not significantly add to the general base catalyzed activation of laromustine. The ‘apparent pKa’ of carbonic acid is usually given as 6.35 (50) this value arises because the combined concentrations of dissolved CO₂ and H₂CO₃ are used in place of the actual concentration of H₂CO₃ in its calculation. The CO₂ + H₂O ↔ H₂CO₃ equilibrium lays heavily to the left in favor of dissolved CO₂ and the true concentration of H₂CO₃ is very much lower than the combined value. Thus the acidity of H₂CO₃ is far greater, and the basicity of HCO₃⁻ is much lower than its apparent pKa would imply (50). The relatively low [Pi] (and soluble monoesters thereof) in plasma (~1 mM) compared to intracellular levels (>10 mM) (45,51) should result in the spontaneous activation half-life of laromustine being nearly equivalent within plasma and normal cells, despite the plasma being ~ 0.2 pH units more alkaline (pH 7.4 versus 7.2). In our simple buffers (Fig. 5) of corresponding [Pi] and pH values the spontaneous laromustine half-life was in the 30–40 min range, this is close to the ~ 30 min plasma half-life determined in patients (52–54,). The presence of other minor biological buffering species/general base catalysts should further contribute to a shortening of laromustine’s half-life. Thus the initial biological transformation of laromustine is for the most part attributable to its spontaneous activation as a result of the ambient pH and the presence of non-enzymatic general base catalysts/buffering components. A high proportion of the total laromustine activation rate would have to be due to spontaneous base catalyzed elimination for a pH dependent tumor cell targeting mechanism to function efficiently.

The hypoxia activated 1,2-bis(methylsulfonyl)-1-(alkyl)hydrazine prodrugs KS119 and KS900 provide a precedent for the targeting benefits of intracellular liberation of short-lived 1,2-bis(methylsulfonyl)-1-(alkyl)hydrazines. KS119 and KS900 are both largely activated as a result of the intracellular reductive cleavage of a nitrophenyl(ethoxy)carbonyl masking group from the N-2 position of 1,2-bis(methylsulfonyl)-1-(alkyl)hydrazines selectively under hypoxic conditions (55,56). KS119 and KS900 exhibit profoundly greater cytotoxicity in *in vitro* experiments under hypoxic compared to normoxic conditions, and are also far more potent than their non-latentiated universally active counterparts which lack reductive intracellular activation (56,57). While the pH dependent laromustine activation rate differential between tumor/normal cells would be much smaller than the hypoxic/normoxic

activation rate differentials of KS119 and KS900 (56–58) a similar albeit smaller degree of targeted cytotoxicity should still be observed.

Effects of pH on DNA cross-linking

To verify that the increased rates of laromustine activation are reflected in a proportional increase in the rate of DNA cross-linking, the *in vitro* cross-linking of isolated L1210 DNA was measured after a 10 min exposure to either 400 μ M laromustine or 0 – 400 μ M 90CE at 37°C, in 20 mM potassium Pi, 100 mM KCl, 0.1 mM NaN₃, buffer at pH values of 7.2 and 7.6. This exposure duration results in only partial laromustine reaction, but total reaction of 90CE. The 90CE experiments were performed for two reasons (i) to ascertain if there was a significant difference in the cross-linking of DNA by 90CE itself between these two pH environments, and (ii) to examine the proportionality of the yields of cross-link moieties/DNA molecule versus 90CE concentration. It can be seen in the insert panel in Fig. 6 that there was no significant difference between the cross-linking efficiency of 90CE in this buffer between these two pH values. Additionally the yields of cross-link moieties/DNA molecule showed a relatively linear dependence upon the 90CE concentration. It can be seen in Fig. 6 that laromustine in the 10 min reaction window produced a ~2.4-fold increase in the yields of DNA crosslink moieties at pH 7.6 versus pH 7.2. This ratio is in reasonable agreement with the expected ratio for a 10 minute reaction window based upon the half-life of laromustine under these conditions, and a linear dependency of the yields of cross-link moieties/DNA molecule versus 90CE concentration.

The presence of 20 mM Pi has been previously shown to result in a modest decrease in the yield of DNA cross-links produced by 90CE compared to the absence of Pi. This is owing to a decreased yield of the chloroethylating species due to Pi acting as a Brønsted-Lowry base catalyzing the pathway shown in Fig. 2, panel D (9). However, it is unlikely that differences in the total concentrations of Pi and phosphomonoesters between normal and neoplastic cells, unless truly exceptional, would result in any large decrease in the cross-linking yields from 90CE between these two environments. It should be noted that any detrimental effects on the DNA cross-link yields from 90CE as a consequence of Pi/phosphomonoester concentration differentials between normal and tumor cells would not affect the relative standing of laromustine compared to the acyl or alkoxycarbonyl based 90CE prodrugs, as all would be equivalently compromised with respect to their activity at the level of 90CE.

Laromustine targeting implications

The primary cytotoxic selectivity of 90CE prodrugs appears to be dependent upon the absence of MGMT or its low level expression (4,21,30,42), unfortunately this only seems to occur in a small sub-set of neoplasms. In the case of laromustine an additional high pH_i targeting mechanism that results in greater rates of 90CE delivery to tumor cells is likely superimposed upon the primary cytotoxic selectivity. The alkaline pH_i responsible for this weaker secondary cytotoxic targeting mechanism is a common cancer cell feature and is associated with the ubiquitous Warburg effect (36) (the tendency of cancer cells to use a high flux through glycolytic/anaerobic energy pathways irrespective of the availability of oxygen) (Fig. 1 panel D). The switching to a high pH_i seems to be at least in part responsible for producing the increases in glycolytic flux via activating highly pH-sensitive rate limiting

glycolytic enzymes such as phosphofructokinase 1 (35). Thus most MGMT deficient tumor cells are expected to also possess elevated pH_i values. The degree of selectivity resulting solely from pH_i differentials is unlikely to be of a sufficient magnitude, at least at the level possessed by laromustine, to elicit a large selective anticancer effect per se. However, when superimposed upon the selectivity resulting from a large MGMT differential a significant boost in therapeutic index is likely.

In order for the pH dependent targeting to function efficiently it is necessary for the active components liberated to exert their effects only locally (by possessing short half-lives) to prevent equilibration with and action on normal tissues, as this would blur the targeting bias. The half-life values that would be optimal for treating leukemias would probably be shorter than those required for solid tumors because the escape of active material from individual leukemia cells would be largely to body fluids and normal tissue cells. Whereas in solid tumor tissues where tumor cells are closely associated, more escape could be tolerated as it would result in a compensating bystander effect on neighboring tumor cells. The short half-life of methyl isocyanate especially in the thiol rich intracellular environments means that both the carbamoylating and chloroethylating activities of laromustine would be expected to exhibit tumor cell pH biased delivery. Since the *in vivo* co-treatment experiments (Fig. 3) indicated that carbamoylating activity may antagonize the therapeutic actions of the chloroethylating activity, the benefits of isocyanate generation per se may not be positive and analogs that liberate either self-immolative isocyanates or utilize alternative masking groups that are also subject to general base catalyzed activation may be preferable.

90CE targeting as a result of increased laromustine activation at elevated tumor cell pH_i could provide an explanation for earlier puzzling *in vitro* observations (21). In these studies greater quantities of DNA cross-links were measured with laromustine using the comet assay in wild type MGMT negative L1210 cells than generated by equimolar concentrations of 90CE. In these experiments a carbamoylating-only analog failed to exhibit any DNA cross-linking activity (as expected), and thus was unable to account for the enhanced DNA cross-linking levels occurring with laromustine.

Implications for improved anticancer prodrug designs

There are at least four potential ways in which the effects of the differing pH (and P_i /phosphomonoester concentration) environments between normal and tumor cells could be exploited in the development of improved laromustine analogs: (i) using masking groups with a more pronounced pH dependency for their base catalyzed cleavage, (ii) liberation of a self-immolating isocyanate or the use of a non-isocyanate-forming base cleaved masking group, (iii) using laromustine analogs liberating bis(sulfonyl)hydrazines (BSHs) with shorter half-lives and/or lower pK_a values (higher proportion in a lower permeability anionic form) to more precisely confine their cytotoxic action to the high pH_i environment, (iv) utilizing BSHs which minimize the increased flux through the thiophilic electrophile generating pathway in response to elevated P_i /phosphomonoester concentrations.

The latentiation of existing or novel anticancer agents by the generation of prodrug forms that require a simple base catalyzed activation step may be a viable strategy to further enhance their tumor selectivity. The application of this strategy would likely be most

efficacious in cases where the active agent has a relatively short biological half-life or possesses low tissue permeability so as to minimize re-equilibration of the active agent between tumor and normal tissues.

It is interesting to speculate if an advantage derived from base catalyzed activation could also be responsible for providing some known anticancer agents with a therapeutic edge. For example, the prodrug 1-(2-chloroethyl)-3-acyl-3-methyltriazene (CMM) (19) possesses a latentating methylaminocarbonyl moiety that is almost certainly susceptible to base catalyzed activation. CMM, like laromustine, also generates a short-lived chloroethylating species. Thus with respect to both activation and activity CMM is probably a close functional homologue to laromustine, and like laromustine, CMM exhibited significantly superior *in vivo* activity against several murine tumor models compared to triazene analogs using other latentating groups (19).

Unlike pHi, values the extracellular pH values (pHe) of solid tumors are typically more acidic than those of normal tissues with an average pHe value of ~ 6.9 compared to ~ 7.4 for normal tissues. This is largely a result of the high glycolytic flux/lactate output of tumor cells (35), coupled with the aberrant microvascular structure of solid tumors resulting in poor lactate clearance (58,59). However the extracellular space comprises a relatively small fraction of the overall tumor tissue volume typically, but not always < 10% (60). Thus the slower activation of laromustine in the relatively small extracellular volume would not offset a > 2-fold rate of intracellular activation. Furthermore, a slower activation rate of laromustine in the acidic extra-cellular environment would help minimize wasteful extracellular reaction and aid in bystander kill.

Conclusions and future directions—The antagonistic rather than agonistic effects on the therapeutic efficacy of 90AC (90CE prodrug) by co-treatment with ProMIC (methyl isocyanate prodrug) implies that the dual prodrug nature of laromustine (generating both 90CE and methyl isocyanate) is likely not responsible for its superior *in vivo* therapeutic activity. However, the high dependency of laromustine's masking group cleavage rate upon general base catalyzed activation, provides a potential explanation for its greater tumor selectivity. The atypically alkaline pHi values of tumor compared to normal cells (35) would be expected to result in a tumor cell biased delivery of cytotoxic stress under *in vivo* conditions. A > 2-fold increase in 90CE liberation rate from laromustine would be anticipated in tumor versus normal cells. This effect would superimpose an additional tumor targeting mechanism upon its primary selectivity based upon the absence of MGMT expression. The recognition of these sensitivities could permit the identification of highly responsive patient sub-populations and aid in the development of personalized therapies. For example AML patients whose leukemic blasts expressed little or no MGMT activity, could also be evaluated for elevated pHi values. This could be measured directly with fluorescent probes or inferred based upon the expression of elevated levels of proteins involved in hydrogen ion extrusion such as the monocarboxylic acid transporters 1 and 4. Since cells lacking both MGMT expression and homology directed repair (HDR) have been reported to be hypersensitive to laromustine (61), HDR competence could represent an additional sensitivity marker.

Based upon the concept of pH_i targeting it should be possible to design improved second generation laromustine analogs. Furthermore, it may be possible to exploit the elevated pH_i values of tumor cells as a secondary therapeutic target to enhance the therapeutic index of existing or novel anticancer agents by the incorporation of a latentating group that is subject to base catalyzed cleavage.

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Abbreviations

90AC

(1-acetyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine)

90CE

1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine

BSH

bis(sulfonyl)hydrazine

G-C ethane cross-link

1-(*N*^β-cytosinyl),-2-(*N*^l-guaninyl)ethane

Hoechst 33258

2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi(1H-benzimidazole) trihydrochloride

KS119

1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine

KS900

1,2-bis(methylsulfonyl)-1-(methyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine

Laromustine

(1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine)

pH_e

extracellular pH

pH_i

intracellular pH

Pi

inorganic phosphate (mixture of H₂PO₄⁻ and HPO₄²⁻)

ProMIC

1,2-bis(methylsulfonyl)-1,2-bis(methylaminocarbonyl)hydrazine

References

1. Penketh PG, Shyam K, Sartorelli AC. Studies on the Mechanism of Decomposition and Structural Factors Affecting the Aqueous Stability of 1,2-Bis(sulfonyl)-1-alkylhydrazines. *J Med Chem.* 1994; 37:2912–2917. [PubMed: 8071939]
2. Penketh PG, Baumann RP, Ishiguro K, Shyam K, Seow HA, Sartorelli AC. Lethality to leukemia cell lines of DNA interstrand cross-links generated by Cloretazine derived alkylating species. *Leukemia Res.* 2008; 32:1546–1553. [PubMed: 18479747]
3. Ishiguro K, Zhu YL, Shyam K, Penketh PG, Baumann RP, Sartorelli AC. Quantitative relationship between guanine O⁶-alkyl lesions produced by OnriginTM and tumor resistance by O⁶-alkylguanine-DNA alkyltransferase. *Biochem Pharmacol.* 2010; 80:1317–1325. [PubMed: 20654586]
4. Shyam K, Penketh PG, Baumann RP, Finch RA, Zhu R, Zhu YL, Sartorelli AC. Antitumor Sulfonylhydrazines: Design, Structure-Activity Relationships, Resistance Mechanisms, and Strategies for Improving Therapeutic Utility. *J Med Chem.* 2015; 58:3639–71. [PubMed: 25612194]
5. Shyam K, Penketh PG, Divo AA, Loomis RH, Rose WC, Sartorelli AC. Synthesis and evaluation of 1-acyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazines as antineoplastic agents. *J Med Chem.* 1993; 36:3496–3502. [PubMed: 8246218]
6. Shyam K, Penketh PG, Loomis RH, Sartorelli AC. Thiolysable prodrugs of 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine with antineoplastic activity. *Eur J Med Chem.* 1998; 33:609–615.
7. Shyam K, Penketh PG, Loomis RH, Rose WC, Sartorelli AC. Antitumor 2-Aminocarbonyl-1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazines. *J Med Chem.* 1996; 39:796–801. [PubMed: 8576923]
8. Finch RA, Shyam K, Penketh PG, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-(methylamino)carbonylhydrazine (101M): A Novel Sulfonylhydrazine Prodrug with Broad-Spectrum Antineoplastic Activity. *Cancer Res.* 2001; 61:3033–3038. [PubMed: 11306484]
9. Penketh PG, Shyam K, Zhu R, Baumann RP, Ishiguro K, Sartorelli AC. Influence of Phosphate and Phosphoesters on the Decomposition Pathway of 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (90CE), the Active Anticancer Moiety Generated by Laromustine, KS119, and KS119W. *Chem Res Toxicol.* 2014; 27:818–833. [PubMed: 24618018]
10. Bodell WJ, Aida T, Berger MS, Rosenblum ML. Repair of O⁶-(2-chloroethyl)guanine mediates the biological effects of chloroethylnitrosoureas. *Environ Health Perspect.* 1985; 62:119–126. [PubMed: 4085418]
11. Gerson SL. Clinical relevance of MGMT in the treatment of cancer. *J Clin Oncol.* 2002; 20:2388–2399. [PubMed: 11981013]
12. Christmann M, Verbeek B, Roos WP, Kaina B. O⁶-Methylguanine-DNA methyltransferase (MGMT) in normal tissues and tumors: enzyme activity, promoter methylation and immunohistochemistry. *Biochim Biophys Acta.* 2011; 1816:179–180. [PubMed: 21745538]
13. Penketh PG, Shyam K, Baumann RP, Remack JS, Brent TP, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (VNP40101M): I. Direct Inhibition of O⁶-Alkylguanine-DNA Alkyltransferase (AGT) by Electrophilic Species Generated by Decomposition. *Cancer Chemother Pharmacol.* 2004; 53:279–287. [PubMed: 14704831]
14. Baumann RP, Shyam K, Penketh PG, Remack JS, Brent TP, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(methylamino)carbonyl]hydrazine (VNP40101M): II. Role of O⁶-Alkylguanine-DNA Alkyltransferase in Cytotoxicity. *Cancer Chemother Pharmacol.* 2004; 53:288–295. [PubMed: 14685775]
15. Rice KP, Penketh PG, Shyam K, Sartorelli AC. Differential Inhibition of Cellular Glutathione Reductase Activity by Isocyanates Generated from the Antitumor Prodrugs CloretazineTM and BCNU. *Biochem Pharmacol.* 2005; 69:1463–1472. [PubMed: 15857610]

16. Penketh PG, Shyam K, Sartorelli AC. Comparison of DNA lesions produced by tumor-inhibitory 1,2-bis(sulfonyl)hydrazines and chloroethylnitrosoureas. *Biochem Pharmacol.* 2000; 59:283–91. [PubMed: 10609557]
17. Brubaker WF Jr, Zhao HP, Prusoff WH. Measurement of carbamoylating activity of nitrosoureas and isocyanates by a novel high-pressure liquid chromatography assay. *Biochem Pharmacol.* 1986; 35:2359–65. [PubMed: 3729992]
18. Ludlum DB. The chloroethylnitrosoureas: sensitivity and resistance to cancer chemotherapy at the molecular level. *Cancer Invest.* 1997; 15:588–598. [PubMed: 9412665]
19. Smith RH, Scudiero DA, Michejda CJ. 1,3-Dialkyl-3-acyltriazines, a novel class of antineoplastic alkylating agents. *J Med Chem.* 1990; 33:2579–2583. [PubMed: 2391696]
20. Baumann RP, Seow HA, Shyam K, Penketh PG, Sartorelli AC. The antineoplastic efficacy of the prodrug Cloretazine is produced by the synergistic interaction of carbamoylating and alkylating products of its activation. *Clinical Cancer Therapeutics.* 2005; 15:313–25.
21. Ishiguro K, Seow HA, Penketh PG, Shyam K, Sartorelli AC. Mode of Action of the Chloroethylating and Carbamoylating Moieties of the Prodrug Cloretazine. *Mol Cancer Ther.* 2006; 5:1–8. [PubMed: 16432157]
22. Gibson NW, Hickman JA. The role of isocyanates in the toxicity of antitumor haloalkylnitrosoureas. *Biochem Pharmacol.* 1982; 31:2795–2800. [PubMed: 7138575]
23. Newman RA, Farquhar D. Release of methyl isocyanate from the antitumor agent caracemide (NSC-253272). *Invest New Drugs.* 1987; 5:267–271. [PubMed: 3667162]
24. Slatter JG, Davis MR, Han DH, Pearson PG, Baillie TA. Studies on the metabolic fate of caracemide, an experimental antitumor agent, in the rat. Evidence for the release of methyl isocyanate *in vivo*. *Chem Res Toxicol.* 1993; 6:335–340. [PubMed: 8318655]
25. Brown WE, Green AH, Cedel TE, Cairns J. Biochemistry of Protein-Isocyanate Interactions: A Comparison of the Effects of Aryl vs. Alkyl Isocyanates. *Environmental Health Perspectives.* 1987; 72:5–11. [PubMed: 3622443]
26. Ji W, Yang M, Praggastis A, Li Y, Zhou HJ, He Y, Ghazvinian R, Cincotta DJ, Rice KP, Min W. Carbamoylating Activity Associated with the Activation of the Antitumor Agent Laromustine Inhibits Angiogenesis by Inducing ASK1-Dependent Endothelial Cell Death. *PLOS ONE.* 2014; 9:E103224. [PubMed: 25068797]
27. Rice KP, Klinkerch EJ, Gerber SA, Schleicher TR, Kraus TJ, Buros CM. Thioredoxin reductase is inhibited by the carbamoylating activity of the anticancer sulfonylhydrazine drug laromustine. *Mol Cell Biochem.* 2012; 370:199–207. [PubMed: 22864532]
28. Frederick AM, Davis ML, Rice KP. Inhibition of human DNA polymerase β activity by the anticancer prodrug Cloretazine. *Biochem Biophys Res Commun.* 2009; 378:419–23. [PubMed: 19026985]
29. Peták I, Mihalik R, Bauer PI, Süli-Vargha H, Sebestyén A, Kopper L. BCNU is a caspase-mediated inhibitor of drug-induced apoptosis. *Cancer Res.* 1998; 58:614–8. [PubMed: 9485009]
30. Ishiguro K, Shyam K, Penketh PG, Sartorelli AC. Role of O6-alkylguanine-DNA alkyltransferase in the cytotoxic activity of cloretazine. *Mol Cancer Ther.* 2005; 4:1755–63. [PubMed: 16275997]
31. Baell J, Walters MA. Chemistry: Chemical con artists foil drug discovery. *Nature.* 2014; 513:481–483. [PubMed: 25254460]
32. Davis MR, Kassahun K, Jochheim CM, Brandt KM, Baillie TA. Glutathione and N-acetylcysteine conjugates of 2-chloroethyl isocyanate. Identification as metabolites of *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea in the rat and inhibitory properties toward glutathione reductase *in vitro*. *Chem Res Toxicol.* 1993; 6:376–83. [PubMed: 8318660]
33. Kassahun K, Jochheim CM, Baillie TA. Effect of carbamate thioester derivatives of methyl- and 2-chloroethyl isocyanate on glutathione levels and glutathione reductase activity in isolated rat hepatocytes. *Biochem Pharmacol.* 1994; 48:587–94. [PubMed: 8068046]
34. Chang HY, Yang X. Proteases for Cell Suicide: Functions and Regulation of Caspases. *Microbiol Mol Biol Rev.* 2000; 64:821–846. [PubMed: 11104820]
35. Webb BA, Chimenti M, Jacobson MP, Barber DL. Dysregulated pH: a perfect storm for cancer progression. *Nat Rev Cancer.* 2011; 11:671–7. [PubMed: 21833026]

36. Calderon-Montano J, Burgos-Moron E, Perez-Guerrero C, Salvador J, Robles A, Lopez-Lazaro M. Role of the Intracellular pH in the Metabolic Switch between Oxidative Phosphorylation and Aerobic Glycolysis - Relevance to Cancer. *WebmedCentral Cancer*. 2011; 2(3):WMC001716.
37. Kuin A, Aalders M, Lamfers M, van Zuidam DJ, Essers M, Beijnen JH, Smets LA. Potentiation of anti-cancer drug activity at low intratumoral pH induced by the mitochondrial inhibitor m-iodobenzylguanidine (MIBG) and its analogue benzylguanidine (BG). *Br J Cancer*. 1999; 79:793–801. [PubMed: 10070871]
38. Pearson PG, Slatter JG, Rashed MS, Han DH, Baillie TA. Carbamoylation of peptides and proteins in vitro by *S*-(*N*-methylcarbamoyl)glutathione and *S*-(*N*-methylcarbamoyl)cysteine, two electrophilic *S*-linked conjugates of methyl isocyanate. *Chem Res Toxicol*. 1991; 4:436–444. [PubMed: 1912331]
39. Castro EA, Moodie RB, Sansom PJ. The Kinetics of Hydrolysis of Methyl and Phenyl Isocyanates. *J Chem Soc Perkin Trans II*. 1985; 5:737–742.
40. Jencks, WP., Regenstein, J. *Handbook of Biochemistry and Molecular Biology*. 4. Lundblad, RL., MacDonald, FM., editors. CRC Press; 2010. p. 595-635.
41. Penketh PG, Shyam K, Sartorelli AC. Fluorometric Assay for the Determination of DNA-DNA Cross-links Utilizing Hoechst 33258 at Neutral pH values. *Anal Biochem*. 1997; 252:210–213. [PubMed: 9324963]
42. Ishiguro K, Shyam K, Penketh PG, Sartorelli AC. Development of an *O*⁶-alkylguanine-DNA alkyltransferase assay based on covalent transfer of the benzyl moiety from [benzene-3H] *O*⁶-benzylguanine to the protein. *Anal Biochem*. 2008; 383:44–51. [PubMed: 18783719]
43. Reshkin SJ, Greco MR, Cardone RA. Role of pHi, and proton transporters in oncogene-driven neoplastic transformation. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 2014; 369(1638):20130100.doi: 10.1098/rstb.2013.0100
44. Ault A. General Acid and General Base Catalysis. *Journal of Chemical Education*. 2007; 84:38–39.
45. Segawa H, Hanazaki A, Miyamoto K. Intracellular and extracellular functions of phosphorus compound in the body. *Clin Calcium*. 2016; 26:187–91. [PubMed: 26813497]
46. Schulman J, Falkenheim M, Gray SJ. The phosphorus turnover of carcinoma of the human stomach as measured with radioactive phosphorus. *J Clin Invest*. 1949; 28:66–72. [PubMed: 16695662]
47. Schneider WC, Klug HL. Phosphorus Compounds in Animal Tissues IV. The Distribution of Nucleic Acids and Other Phosphorus-Containing Compounds in Normal and Malignant Tissues. *Cancer Research*. 1946; 6:691–694. [PubMed: 20276076]
48. Evans FE, Kaplan NO. ³¹P nuclear magnetic resonance studies of HeLa cells. *Proc Natl Acad Sci U S A*. 1977; 74:4909–4913. [PubMed: 270724]
49. Pocker Y, Bjorkquist DW. Stopped-flow studies of carbon dioxide hydration and bicarbonate dehydration in H₂O and D₂O acid–base and metal ion catalysis. *J Am Chem Soc*. 1977; 99:6537–6543.
50. Pines D, Ditzkovich J, Mukra T, Miller Y, Kiefer PM, Daschakraborty S, Hynes JT, Pines E. How acidic is carbonic acid? *J Phys Chem B*. 2016; 120:2440–51. [PubMed: 26862781]
51. Bansal, VK. Serum Inorganic Phosphorus. In: Walker, HK, Hall, WD., Hurst, JW., editors. *Clinical Methods: The History, Physical, and Laboratory Examinations*. 3. Vol. Chapter 198. Boston: Butterworths; 1990.
52. Giles F, Thomas D, Garcia-Manero G, Faderl S, Cortes J, Verstovsek S, Ferrajoli A, Jeha S, Beran M, Koller C, Andreeff M, Cahill A, Clairmont C, Sznol M, Kantarjian H. A Phase I and pharmacokinetic study of VNP40101M, a novel sulfonylhydrazine alkylating agent, in patients with refractory leukemia. *Clin Cancer Res*. 2004; 10:2908–17. [PubMed: 15131024]
53. Giles F, Thomas D, Garcia-Manero G, Faderl S, Cortes J, Verstovsek S, Ferrajoli A, Jeha S, Beran M, Koller C, Andreeff M, Cahill A, Clairmont C, Sznol M, Kantarjian H. A Phase I and pharmacokinetic study of VNP40101M, a novel sulfonylhydrazine alkylating agent, in patients with refractory leukemia. *Clin Cancer Res*. 2004; 10:2908–17. [PubMed: 15131024]
54. Gururangan S, Turner CD, Stewar CF, O'Shaughnessy M, Kocak M, Poussaint TY, Phillips PC, Goldman S, Packer R, Pollack IF, Blaney SM, Karsten V, Gerson SL, Boyett JM, Friedman HS, Kun LE. Phase I Trial of VNP40101M (Clotretazine) in Children with Recurrent Brain Tumors: A

- Pediatric Brain Tumor Consortium Study. Clin Cancer Res. 2008; 14:1124–30. [PubMed: 18281546]
55. Baumann RP, Penketh PG, Ishiguro K, Shyam K, Zhu YL, Sartorelli AC. Reductive activation of the prodrug 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119) selectively occurs in oxygen-deficient cells and overcomes O6-alkylguanine-DNA alkyltransferase mediated KS119 tumor cell resistance. Biochem Pharmacol. 2010; 79:1553–61. [PubMed: 20005211]
56. Baumann RP, Ishiguro K, Penketh PG, Shyam K, Zhu R, Sartorelli AC. KS900: A hypoxia-directed, reductively activated methylating antitumor prodrug that selectively ablates O⁶-alkylguanine-DNA alkyltransferase in neoplastic cells. Biochem Pharmacol. 2011; 81:1201–10. [PubMed: 21396917]
57. Seow HA, Penketh PG, Shyam K, Rockwell S, Sartorelli AC. 1,2-Bis(methylsulfonyl)-1-(2-chloroethyl)-2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine: an anticancer agent targeting hypoxic cells. Proc Natl Acad Sci USA. 2005; 102:9282–7. [PubMed: 15964988]
58. Leach RM, Treacher DF. Oxygen transport-2. Tissue hypoxia. BMJ. 1998; 317:1370–1373. [PubMed: 9812940]
59. Awwad HK, el Naggat M, Mocktar N, Barsoum M. Inter-capillary distance measurement as an indicator of hypoxia in carcinoma of the cervix uteri. Int J Radiat Oncol Biol Phys. 1986; 12:1329–33. [PubMed: 3759554]
60. Jain RK. Determinants of tumor blood flow: a review. Cancer Res. 1988; 48:2641–58. [PubMed: 3282647]
61. Rockwell S, Liu Y, Seow HA, Ishiguro K, Baumann RP, Penketh PG, Shyam K, Akintujoye OM, Glazer PM, Sartorelli AC. Preclinical evaluation of Laromustine for use in combination with radiation therapy in the treatment of solid tumors. Int J Radiat Biol. 2012; 88:277–285. [PubMed: 22111842]

Fig. 1. Introductory graphic

Panel A, The generation of 90CE from acyl, alkoxy carbonyl, and alkylaminocarbonyl prodrugs. Prodrugs with acyl or alkoxy carbonyl *N*-2 substitutions are activated metabolically, whereas aminocarbonyl *N*-2 substituents are primarily activated by spontaneous base catalyzed hydrolysis. The alkylaminocarbonyl prodrugs also produce a reactive isocyanate during 90CE generation that is capable of inhibiting enzymes with essential active site thiol(s) or reacting with non-protein sulfhydryls such as glutathione. 90CE undergoes a rapid elimination reaction to produce a fleeting oxophilic chloroethylating species that can react with *O*-6 position of DNA guanine. **Panel B,** Reaction sequence leading to the formation of a highly toxic DNA interstrand cross-link following the *O*-6 chloroethylation of DNA guanine, and the points where the repair protein MGMT can block cross-link formation. **Panel C,** Greater therapeutic index (lethal dose 50%/effective dose 50%, LD₅₀/ED₅₀) of a single *i.p.* dose of the alkylaminocarbonyl based 90CE prodrug laromustine compared to the acyl based prodrug 1-acetyl-1,2-bis(methylsulfonyl)-2-(2-chloroethyl)hydrazine (90AC) towards L1210 leukemia in female CD₂F₁ mice (pooled historical data 5–14 mice per data point (8)). Deaths at the higher dosage levels are due to drug toxicity. The approximate ED₅₀ and LD₅₀ values are indicated. **Panel D,** Cancer cells frequently utilize aerobic glycolysis (the Warburg effect) as their major energy producing pathway even in the presence of sufficient oxygen for normal aerobic glucose oxidation. This results in the production of only two ATP molecules per glucose molecule versus ~36 via normal oxidative glucose catabolism. The lower ATP yield is compensated for by having a higher pathway flux. To permit this increased glucose flux

and manage the high production of lactic and pyruvic acids cancer cells express high levels/activities of glucose transporters and acid extruders (monocarboxylic acid transporters 1 and 4, and proton export systems (35,36)). This results in atypically high intracellular pH values in cancer cells, and this further promotes glycolysis due to the high pH activation of several glycolytic enzymes (35,36). For cancer cells the extracellular pH can be quite variable, for a leukemia cell in the circulation the extracellular pH would be ~ 7.4, but in solid tumors due to the inefficient clearance of exported acids the extracellular pH can be much more acidic (35). **Panel E**, Structures of bis(sulfonyl)hydrazine prodrugs possessing chloroethylating activity only (90AC), carbamoylating activity only (ProMIC), and combined chloroethylating and carbamoylating activities (laromustine).

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Fig. 2. Yields of H⁺ from laromustine during decomposition at near neutral pH values

Panel A, Laromustine generates two very short-lived species, methyl isocyanate and 90CE, as a consequence of its rate limiting base catalyzed activation (7, 13–16). These react further resulting in net H⁺ generation. **Panel B**. The decomposition of methyl isocyanate to generate methylamine (moderately strong base pK_a = 10.66) and carbon dioxide/carbonic acid (acidic species apparent pK_a = 6.34) is almost H⁺ silent in the pH range of interest because under these conditions methylamine is fully protonated, but carbonic acid is not quite fully dissociated. This results in small consumptions of 0.13, 0.08, and 0.05 moles of H⁺ per mole of methyl isocyanate at pH values of 7.2, 7.4, and 7.6, respectively. **Panels C and D**, The decomposition of 90CE via its two major decomposition routes, these pathways are initially identical but branch after the rate determining methyl sulfinat elimination step (1,9). Both pathways result in the net generation of 2.0 moles of H⁺ (both CH₃SO₂H and HCl are fully ionized) per mole of 90CE. Therefore the distribution of reaction flux between these two pathways does not influence the calculation of laromustine loss based upon H⁺ production. Using the Henderson-Hasselbalch equation and the above pK_a values the following stoichiometries for moles of H⁺ generated per mole of laromustine can be calculated: 1.87, 1.92, and 1.95 at pH values of 7.2, 7.4 and 7.6, respectively. **Panel E**. Typical curve for a laromustine decomposition experiment, obtained by following the loss in absorbance of phenol red at 560 nm as a result of H⁺ generation. The observed decomposition closely approximates to a first order decay process. The curve shown in red is a best fit single phase exponential decay curve fitted to the experimental data points shown in blue.

Fig. 3. The effects of dose combinations of 90AC and ProMIC on the survival of L1210 leukemia infected mice

Female CD₂F₁ mice (minimum 4 per data) were infected with L1210 leukemia (10⁵ cells/mouse) on day 0 and were then treated with single *i.p.* doses of 90AC (90CE prodrug) and ProMIC (methyl isocyanate prodrug) and combinations thereof. Post inoculation life span was monitored for 60 days, death due to drug toxicity was assigned primarily on the absence of leukemia cells in the peritoneum at the time of death. Treated/control (T/C) survival values are calculated only for the mice that died and exclude long term survivors (LTS).

Panel A. Tabulated LTS, drug toxicity deaths (DTD) and % T/C data for 90AC/ProMIC combination study. **Panel B.** Plot of the percentage of LTS versus 90AC dosage at various ProMIC dose levels. **Panel C.** Plot of the percentage of DTD versus 90AC dosage at various ProMIC dose levels.

Fig. 4.

Panel A. The effects of pH on the half-life of laromustine at 50 °C. The half-life values were obtained by following the loss in absorbance of phenol red at 560 nm, as a result of H^+ generation, and fitting the data to a first order decay process. The half-life values are the means of at least 3 separate experimental determinations $\pm$ SE. **Panel B.** The effects of $[Pi]$ on the half-life of laromustine at pH 8.0 and 50 °C. A temperature of 50 °C was utilized in these experiments (and elevated pH in the panel B experiments) for experimental convenience as this permits the determination of the half-life values by following the decay to essential completion within a relatively short time interval, and using a first order decay curve fit. The half-life values are the means of at least 3 separate experiments $\pm$ SE.

Fig. 5. Half-life of laromustine at 37 °C, physiological/neoplastic disease relevant pH values (7.2 to 7.6), and [Pi] ranges. Due to the slower rate of decay of laromustine at 37 °C the half-life values were calculated from the peak initial rates of H^+ generation using the following stoichiometries for moles of H^+ per mole of laromustine: 1.87, 1.92, and 1.95 for pH values of 7.2, 7.4 and 7.6, respectively. The plotted values are the means of at least 3 experiments $\pm$ SE.

Fig. 6.

Comparison of the relative number of cross-linking moieties generated per test DNA (L1210) molecule as a consequence of an initial 10 minute reaction window with either 400 μ M laromustine or 200 μ M 90CE in 20 mM potassium Pi, 100 mM KCl, 0.1 mM NaN_3 at pH values of 7.2 and 7.6 and 37 °C. The 10 minute reaction window results in only partial laromustine reaction, but total reaction for the much short-lived 90CE. The insert panel shows the relative number of cross-linking moieties generated per test DNA (L1210) molecule versus 90CE concentration (0–400 μ M) in 20 mM potassium Pi, 100 mM KCl, 0.1 mM NaN_3 at pH values of 7.2 and 7.6 and 37 °C. The data in the insert indicates that the reaction of 90CE with DNA is approximately equivalent at both pH values and approximately proportional to the 90CE concentration. All values are the means of at least 3 experiments $\pm$ SE.