

Lipid Nucleoside Conjugates for the Treatment of Cancer

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Abstract: Phospholipid nucleoside conjugates and nucleosides with chemical additions to the hydroxyl and amino moieties have been used since the 1970s in order to increase the biological activity of the parent compound. Previous investigators have found that adding lipid moieties to ara-C or chemically linking ara-C to a phospholipid creates a prodrug that exhibits superior cytotoxic activity compared to ara-C alone when used in animal tumor models. The novel ara-C molecules reveal different pharmacological profiles from the parent compound such as decreased catabolism by cytidine deaminase, increased plasma half-life, and release of nucleoside monophosphate, a reaction that bypasses the rate limiting initial nucleoside phosphorylation. Additionally, these compounds were able to penetrate the blood-brain barrier and were active against tumor cells implanted i.c. The purpose of this review is to briefly cover the history and successes of previous investigators who have synthesized and tested these phospholipid ara-C conjugates, to discuss recent phospholipid ara-C and fludarabine conjugates, and to discuss the synthetic design and synthesis of a novel phospholipid gemcitabine conjugate. These phospholipid nucleoside conjugates possess the potential to have superior anti-neoplastic cytotoxicity profiles with fewer side effects than the parent nucleoside and merit further investigation.

Key Words: Synthetic phospholipids, lipid-drug conjugates, antimetabolites, ara-C, gemcitabine, fludarabine

INTRODUCTION

Antitumor antimetabolites have been used for many years for the treatment of different cancers. Commonly used antimetabolites include gemcitabine (2', 2'-difluorodeoxycytidine, dFdC), cytarabine (1-β-D-arabinofuranosylcytosine, ara-C), and fludarabine (9-β-D-arabinofuranosyl-2-fluoro-adenine, F-ara-A). Although these agents demonstrate clinical efficacy, there are limitations to using these drugs due to resistance mechanisms. Resistance of some cells to ara-C and gemcitabine has been attributed to decreased phosphorylation of ara-C or gemcitabine by deoxycytidine kinase [1, 2] or increased degradation of ara-C or gemcitabine by cytidine deaminase [3-6]. Resistance mechanisms ultimately lead to decreased amounts of active drug at the desired target site, and patients who express the above mentioned resistance mechanism phenotypes have lower survival rates. An example of this correlation can be seen in patients with acute myeloid leukemia (AML) who were treated with ara-C and developed resistance due to decreased deoxycytidine kinase [7]. Therefore, there is urgent need for compounds that will bypass mechanisms of resistance.

In order to address this need, many groups have chemically linked different lipids and phospholipids with antimetabolites agents such as ara-C, gemcitabine, and fludarabine. The purpose of these "conjugates" or "prodrugs" is to increase the efficacy of active compounds and bypass key resistance mechanisms. This review will explore some of the lipid nucleoside conjugates that have been synthesized for

cancer treatment. While there are many groups that have used this type of technology, the drugs will be limited to ara-C, gemcitabine, and fludarabine, and the chemical structures can be seen in Fig. (1).

ARA-C

Ara-C is a deoxycytidine analog that has shown efficacy in several types of hematological cancers such as leukemia and lymphoma [8, 9, 10, 11] but it is not as effective in solid tumors [12]. The drug is transported across cell membranes *via* a facilitated diffusion nucleoside transporter [13-15]. Once inside the cell, ara-C is phosphorylated by deoxycytidine kinase to yield ara-C monophosphate (ara-CMP), which is further phosphorylated to its active form, ara-CTP (ara-C triphosphate) [16-18]. The initial phosphorylation of ara-C is the rate-limiting step in the activation of this drug [14, 15, 19, 20], and the active form of ara-C is a potent inhibitor of DNA synthesis that is incorporated into the growing DNA chain causing DNA chain termination and DNA strand breaks [21-23]. One limitation in the use of ara-C is that it has a short plasma half-life (biphasic disappearance: first $t_{1/2}$ = 10 minutes, second $t_{1/2}$ = 1 to 2 hours) because it is deaminated to uracil arabinoside by cytidine deaminase which is found in the highest concentrations in the intestine, liver, and kidneys [24]. Due to this enzyme, ara-C is not orally available and must be administered intravenously.

GEMCITABINE

Gemcitabine is also a deoxycytidine analog [25] that shows some activity against leukemias [26] but more importantly against different solid tumors including ovarian [27], pancreatic [28], colorectal [29], lung [30, 31], head and

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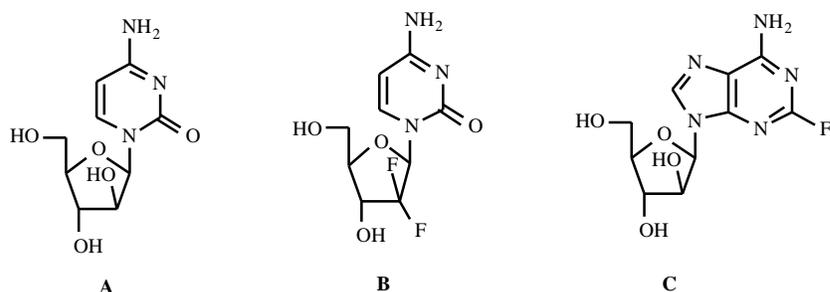


Fig. (1). The chemical structure of ara-C (A), gemcitabine (B), and fludarabine (C).

neck [32], urothelial [33], breast [34], and renal [35] cancers. Like ara-C, gemcitabine is translocated across the cellular membrane by a nucleoside transporter [36] and is phosphorylated by deoxycytidine kinase to gemcitabine monophosphate [37]. The drug is further phosphorylated to the active form gemcitabine triphosphate, which can be incorporated into DNA and inhibit DNA polymerase, ultimately leading to apoptosis [25, 38, 39]. Gemcitabine may have other mechanisms of actions [25, 40, 41], including inhibition of ribonucleotide reductase which further inhibits DNA synthesis [42].

FLUDARABINE

Fludarabine is an deoxyadenosine analog that is transported across the cell membrane and phosphorylated by deoxycytidine kinase, similar to ara-C [43]. The drug must be phosphorylated to the triphosphate form in order to be incorporated into DNA where fludarabine will render its cytotoxic effects [44]. Data have shown that the extent to which fludarabine is incorporated into DNA relates directly to its cytotoxicity [45]. Fludarabine is currently used for the treatment of cancers such as chronic lymphocytic leukemia [46] and lymphoma [47]. As with ara-C and gemcitabine, this drug is limited clinically due to resistance mechanisms

[48]. Previous investigators have shown that an increase in ribonucleotide reductase activity or an increase in a nuclear exonuclease activity are attributed to some resistance mechanisms [49, 50].

RATIONALE FOR PRODRUG MOLECULES

One method for bypassing resistance mechanisms as well as increasing bioavailability is to use prodrugs to carry drugs to their desired site of action. A prodrug is an inactive pharmacological derivative of an active parent compound, which undergoes a spontaneous or enzymatic transformation resulting in the release of a compound that is active as a drug or can be further metabolized into an active drug [51]. Some of the reasons for the design of these prodrugs are to increase/decrease absorption, or increase the amount of drug at the target site [52, 53]. By attaching a nucleoside analog to a lipid carrier, the drug may enter the cell in a unique fashion and no longer require the nucleoside transporter to move the chemotherapeutic agent into the intracellular space as seen in Fig. (2).

Another reason for the synthetic design of the lipid prodrug approach is to decrease metabolism of the nucleoside analogs in the gastrointestinal tract by phospholipase A₂ (PLA₂) and other lipases that degrade ester-linked lipid

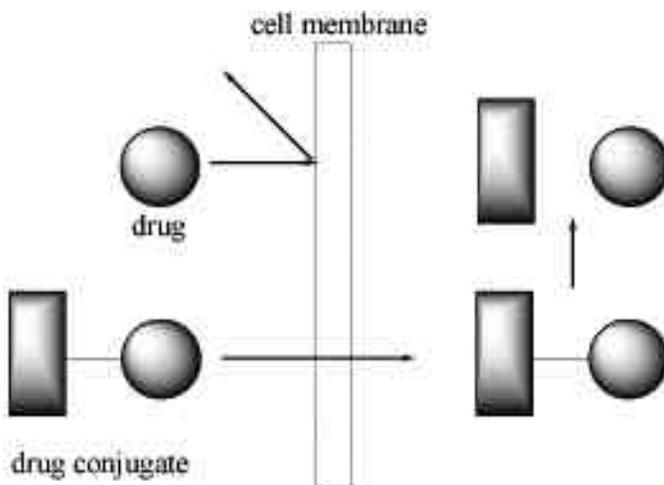


Fig. (2). Adapted and modified from Lambert [54]. The picture depicts the rationale for using lipid prodrugs for increased delivery of pharmacologic agents to cancer cells. Since the pharmacologic agent is coupled to an adduct (lipid, phospholipid, etc.), it can enter the cell through an altered mechanism (e.g. diffusion) releasing the free drug within the cell.

molecules [54]. Therefore, when one is designing a lipid or phospholipid drug carrier, the specific type of linkages (ester vs. ether) for the aliphatic hydrocarbon chains are important in determining whether the new prodrugs will be orally bioavailable. By making these compounds more lipophilic through the use of carbon chains of differing lengths, the prodrugs may be preferentially absorbed by the gastrointestinal lymphatics rather than entering the portal vein and degraded by first-pass metabolism in the liver [55]. Furthermore, the number of carbons in the aliphatic side chains of the lipid may also play a role in increased or decreased cytotoxicity which is discussed below.

MODIFICATION OF ARA-C

In the early 1970s, Wechter *et al.* at the Upjohn Company in Kalamazoo, Michigan were some of the first investigators to modify ara-C with the goal of bypassing metabolism of ara-C to ara-U by cytidine deaminase [56, 57]. This group attached different lipid moieties to the 2', 3' and 5' hydroxyl groups (-OH) of ara-C and tested water solubility, hydrolysis in plasma (human and mouse), partition coefficients, and survival of tumor burdened animals injected with a single (i.v.) dose of the modified ara-C prodrugs. Their results suggested that they could predict biological activity based on water solubility and enzyme hydrolytic rates. The results of the lead compounds can be seen in Table 1. The percent increase in life span (% ILS) was defined as follows: % ILS = ((test group mean day of death/control group mean day of death) x 100) - 100 [56]. Wechter *et al.* considered all % ILS values of 25 or greater to be significant. Table 1 lists those compounds with a % ILS greater than 200, of which there are several.

Table 1. Lead Compounds (% ILS > 200) from Wechter *et al.* [56]

Compound	% ILS single dose of ~200 mg/kg
ara-C	< 25
5' - Palmitate	> 300
5' - Laurate	> 300
5' - Adamantoate	> 300
5' - Stearate	200 - 300
3' - Stearate	200 - 300
5' - 2-(p-isobutylphenyl)propionate	200 - 300
5' - Benzoate	200 - 300
5' -p-Methoxybenzoate	200 - 300

Hamamura *et al.* also conducted similar experiments to those discussed above by chemically linking different saturated and unsaturated lipids to ara-C [58]. When HeLa (cervical carcinoma) cells were incubated with the different compounds, the ED₅₀ values ranged from 0.05 - 0.38 µg/ml. However, when the compounds were tested *in vivo* using a

murine L1210 leukemia model and dosing i.p. once with 200, 500, or 1000 mg/kg, the shorter chain (C₂-C₁₀) saturated and unsaturated lipids did not significantly increase the life span of these mice. Furthermore, at higher doses the compounds were toxic to the animals (500 and 1000 mg/kg). In contrast, the longer chain (C₁₈-C₂₂) saturated and unsaturated alkyl carbon chains attached to ara-C demonstrated enhanced antitumor activity and little host toxicity even at the higher doses. These results were interpreted by the group as a possible increase in cellular penetration or a change in tissue distribution [58]. The complete answer is unknown and merits further investigation.

PHOSPHOLIPID ARA-C CONJUGATES

In the late 1970s and early 1980s, groups began to investigate cytotoxicity after either alkyl chain additions to ara-C or conjugation of ara-C to phospholipids [59, 60]. Raetz *et al.* used natural or synthetic phospholipids to chemically modify ara-C. The chemical structure of the lead compound, ara-CDP-DL-dipalmitin, can be seen in Fig. (3). Their results show that leukemia bearing mice had increased life-spans of 24, 32, and 37% after dosing at 20, 40, and 50 mg/kg, respectively, thus providing evidence that the lipid ara-C conjugate had greater biological activity than that of ara-C given alone. Additionally, in order to understand the metabolism of these compounds, Raetz *et al.* performed *in vitro* cell-free assays by adding their conjugate molecules to several phospholipid enzymes including phosphatidylglycerophosphate synthetase (rat liver mitochondria) and two other bacterial enzymes. Their results proposed that these conjugates released the ara-C monophosphate which bypasses the rate-limiting step in the activation of this drug. However, the mechanism by which the phosphorylated drug is released from the lipid carrier is not completely understood. It may involve an esterase-like enzyme activity. In conclusion, by conjugating ara-C to phospholipids, Raetz *et al.* were able to increase the efficacy of ara-C and bypass the rate-limiting step in the activation of ara-C through the release of ara-C monophosphate.

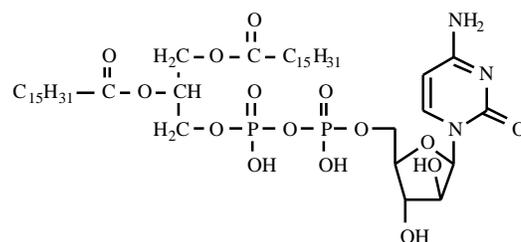


Fig. (3). The chemical structure of ara-CDP-DL-dipalmitin as published by Raetz *et al.* [59].

Since Raetz *et al.* used a racemic mixture of the D- and L-isomers of dipalmitin, MacCoss *et al.* developed a new chemical synthesis for this conjugate as well as isolated a single enantiomer (L-isomer) of the lipid molecule [60]. The results of their experiments determined that by isolating the natural L-isomer the ara-C conjugate did not demonstrate enhancement of cell kill. In an additional study, Matsushita *et al.* demonstrated that ara-C attached to the L-isomer form

of dipalmitoylphosphatidic acid was not subject to deamination by cytidine deaminase from murine kidney while cytidine was significantly deaminated. Ara-C was partially deaminated, but not to the extent of cytidine and more so than the phospholipid conjugate of ara-C [61]. These studies also demonstrated the advantage of the lipid conjugate (% ILS = 260) to ara-C (% ILS = 32) alone in leukemic mice. When ara-C is dosed over several days versus one injection, the drug is more effective. It is interesting to note, however, that the only 45-day survival was seen in the group that was dosed with the ara-C conjugate with an i.m. route of administration.

In some later studies, Ryu *et al.* developed more synthetic schemes for using diacylglycerols with different aliphatic chain lengths attached to ara-C monophosphate or ara-C diphosphate [62]. The new synthetic scheme for the synthesis of a conjugate with only one phosphate required the protection of ara-C using levulinic acid protecting groups. After protection, ara-C was linked to the phospholipid carrier by a 1, 3-dicyclohexyl-carbodiimide (DCC) reaction, and the protecting groups were removed. These compounds demonstrated promising *in vitro* and *in vivo* results (Table 2). In the murine myeloma cells, the lipids that were comprised of longer, saturated fatty acids demonstrated greater antiproliferative activity, but the effect of chain length on the L1210 cell line was not as clear. In contrast to the *in vitro* experiments, the *in vivo* data clearly suggest that the phospholipid ara-C conjugates are better than ara-C alone. The best increase in % ILS (188) was observed in the group of animals administered ara-CDP-L-dipalmitin at a dose of 40 mg/kg/day for 5 days. This group alone had a 30-day survivor post treatment.

In further animal studies using these conjugates, Hong *et al.* sought to determine the active range of doses for ara-CDP-L-dipalmitin and found that one dose of 300 mg/kg/day produced greater than 543% ILS in five out of six of the forty-five day DBA/J2 mice survivors [63]. Clearly, these results suggest further that the addition of phospholipids with aliphatic groups of different chain lengths can change the biological efficacy of ara-C to different degrees. These investigators also used the phospholipid and free ara-C as a dose together, and the results indicated that the treatment was only as effective as ara-C alone. Therefore, the covalent

bond between the phospholipid and ara-C is necessary for the increased efficacy observed *in vivo*. This increase in biological activity could be due to altered routes of entry and changes in tissue distribution for ara-C. An interesting note is that when mice were implanted with L1210 leukemia cells i.c., the conjugate with the best cytotoxic activity was the ara-CDP-L-dipalmitin isomer (% ILS > 374) with 4/6 survivors. The % ILS for ara-C was between 44 and 72. The % ILS for ara-CDP-D-dipalmitin and a racemic mixture were between 150 and 190 with no survivors.

OXY- AND THIOETHER PHOSPHOLIPID CONJUGATES

One of the major concerns of the agents discussed above is that they are subject to degradation by lipases and other esterases that degrade ester linked lipid molecules [54]. With this consideration in mind, Hong *et al.* synthesized oxyether phospholipids with different chain lengths and attached them to ara-C [64]. The most significant finding was the use of the compound seen in Fig. (4A) (% ILS = 293 in i.p. implanted, L1210 leukemic mice). Other compounds demonstrated enhanced efficacy in these animals compared to ara-C alone. In i.p. implanted, P388 leukemic mice, all phospholipid conjugates tested except one demonstrated a 3-fold change in % ILS and many groups of mice had four or more forty-five day survivors. These studies also proved that the linkage between the phospholipid and ara-C is necessary in order to observe biological efficacy since ara-C and the carrier given together were no more effective than ara-C alone.

When Hong *et al.* implanted L1210 leukemia cells i.c. into mice, the results showed that the conjugate molecule, namely the molecule in Fig. (4A), was superior to ara-C in this particular dosing schedule (ara-C, % ILS = 100, using 100 mg/kg/day for nine days and ara-CDP-*rac*-1-O-hexadecyl-2-O-palmitoylglycerol, % ILS = 229, using 500 mg/kg/day in a single dose). The conjugate's ability to increase survival especially with cells implanted i.c. could demonstrate a new way to treat central nervous system (CNS) tumors because compounds that are active against i.c. implanted tumors are demonstrating the ability of these conjugates to cross the blood-brain barrier. Hong *et al.* conclude from their results that among possible explanations for why these conjugates are more biologically active

Table 2. Selected *In Vitro* and *In Vivo* Results from Ryu *et al.* [62]

Compound	Murine myeloma cells (ED ₅₀ = μM)	L1210 ^c cells (ED ₅₀ = μM)	% ILS (dose is 40 mg/kg/day for 5 days except ara-CMP-L-dipalmitin which is 50 mg/kg/day for 5 days)
ara-C	0.99	0.1	65
ara-CDP-L-dipalmitin	65	9.6	188
ara-CDP-L-distearin	195	20	167
ara-CDP-L-dimyristin	15	16	153
ara-CDP-L-diolein	62	27	153
ara-CMP-L-dipalmitin		13.4	75

^cL1210 are murine lymphoid leukemia cells. Not all data are shown from Ryu *et al.* [62].

compared to ara-C alone include the following: (1) resistance to hydrolysis by cytidine deaminase, (2) rapid interaction with serum lipoproteins (better plasma transport), (3) rapid uptake by cells, (4) effects on lipid biosynthesis, and (5) hydrolysis of the conjugates to release ara-CMP [64]. Lastly, the carrier molecules themselves may have antitumor effects after the conjugates are hydrolyzed in the cytoplasm.

Hong *et al.* also synthesized a series of thioether lipids that demonstrated antitumor activity *in vivo*: ara-CDP-*-*palmitoyl-DL-thiochimyl alcohol (ara-CDP-DL-PTCA) and ara-CDP-*-*palmitoyl-DL-thiobatyl alcohol (ara-CDP-DL-PTBA), see Fig. (4B and 4C), respectively [65]. A brief summary of some of the key findings can be seen in Table 3. Both ether lipids demonstrated better biological advantage compared to ara-C, and the ara-CDP-DL-PTBA compound demonstrated biological activity in animals implanted *i.c.* which further supports the idea that these conjugates may be active against CNS tumors. In addition to these biological activity assays, Hong *et al.* also tested different routes of administration (*i.p.*, *i.v.*, *i.m.*, and *s.c.*). They observed that the best route of administration for these compounds was *i.p.* compared to the other modalities of drug delivery (ara-CDP-DL-PTBA, % ILS = 294, using a single dose of 400 mg/kg and ara-CDP-L-dipalmitin, % ILS = 264, using a single dose of 300 mg/kg).

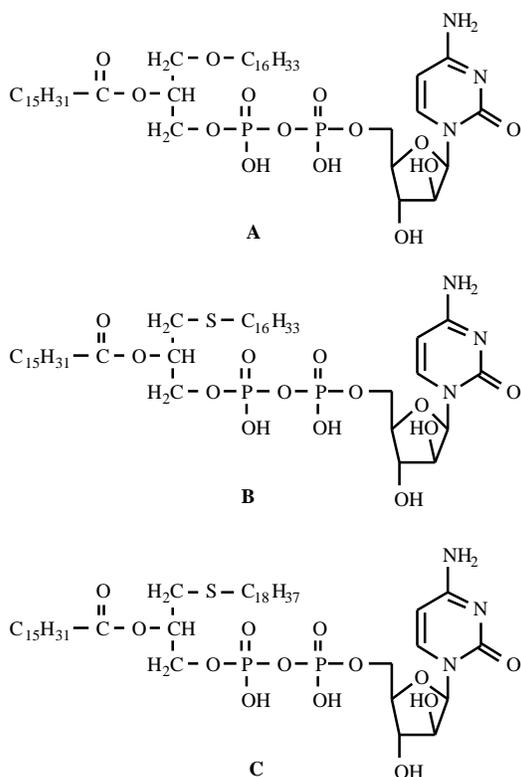


Fig. (4). The chemical structures of (A) ara-CDP-*rac*-1-O-hexadecyl-2-O-palmitoylglycerol [64] (B) ara-CDP-*-*palmitoyl-DL-thiochimyl alcohol [65] and (C) ara-CDP-*-*palmitoyl-DL-thiobatyl alcohol [65].

These two conjugates were also tested in mice that were implanted with an ara-C resistant lymphoid leukemia [66].

Their results show that the ara-CDP-DL-PTCA and the ara-CDP-DL-PTBA were both active against the *i.p.* implanted resistant cells (% ILS > 310 for both with a dose of 60-400 mg/kg/day on day 1, days 1, 5, 9, or days 1-5). Hong *et al.* also altered the lipid backbone to use an oxyether linkage at the C2, and used a methyl, ethyl, or a hexadecyl carbon chain. These oxyether phospholipid conjugates were less active than the compounds with an ester group at C2. Further tests also proved that racemic mixtures of their compounds were equally as active as the enantiomerically pure compounds tested [67]. These results give support to key ideas. First, these conjugates are able to bypass resistance mechanisms as seen in the ara-C resistant model. Second, the chain length and use of ether and ester bonds may be important for the cytotoxicity of these conjugate molecules, and the isolation of racemates is not important.

Other groups synthesized oxy- and thioether phospholipids alone and evaluated cytotoxicity against different tumor cell lines [68]. Morris-Natschke *et al.* synthesized five phospholipids (ET-16S-OMe, ET-16S-OEt, ET-18S-OMe, ET-16S-OEt, and ET-16SO₂-OEt) and tested them against a leukemia cell line (HL-60) and two ovarian cancer lines (BG-1 and BG-3) using trypan blue exclusion and clonogenic assays. These compounds did not have a nucleoside conjugated to the phospholipid. Since ET-18-OMe is active in different tumor cell lines, this molecule was used as the positive control [69]. All compounds were equally as active in the HL-60 cell line as the ET-18-OMe. However, in the BG-1 and BG-3 cells, ET-16S-OEt was more active than the positive control ET-18-OMe. These data suggest that the carrier molecule could in fact have activity against tumor cell growth. Therefore, by conjugating the most cytotoxic phospholipid with ara-C, one could potentially develop a powerful new treatment for cancer. Surles *et al.* also provided new synthetic pathways in order to synthesize multigram quantities of these lipids for biological activity [70].

One question that many investigators have about these phospholipid conjugates is whether the compounds are chemically stable in a solution of chloroform:methanol:H₂O (2:3:1). Hong *et al.* determined that the ara-CDP-DL-PTBA (discussed above) samples stored at 3-4°C were stable for over six months while the samples stored at room temperature (25°C) for three months began to hydrolyze (3 - 11%) into the phosphatidic acid and the ara-C monophosphate [71]. Furthermore, the activity of these compounds, when tested *in vivo* in a murine leukemia model, remained constant when stored at 3-4°C. In other studies, Hong *et al.* describe the importance of micellization to improve biological activity [72]. Taken together, these results suggest that proper storage of these compounds is important as well as the ability of these conjugates to form micelles in order to achieve biological activity.

More recently, Brachwitz *et al.* synthesized a series of ara-C-diphosphate lipid conjugates [73]. The *in vitro* results of the compounds tested showed that the conjugates were slightly less active than ara-C alone. In contrast, the *in vivo* results suggest that the conjugates are equally or more active than ara-C given alone, and furthermore, the conjugate molecules were orally bioavailable and had fewer side effects (less decrease in white blood cell numbers) than ara-

Table 3. Selected *In Vivo* Results for Thioether Lipid Conjugates [65]

Compound	Dose (mg/kg/day)	Dose schedule (days)	ILS
Antitumor activity against i.p. inoculated L1210 leukemic mice			
ara-C	200	5	129
ara-CDP-DL-PTCA	400	1	293
	80	5	379
ara-CDP-DL-PTBA	400	1	307
	80	5	371
Antitumor activity against i.c. inoculated L1210 leukemic mice			
ara-C	100	9	100
ara-CDP-DL-PTBA	400	1	220
	300	1	180
	60	5	187
	50	9	179
Antitumor activity against i.p. inoculated P388 leukemic mice			
ara-C	200	5	109
ara-CDP-DL-PTBA	500	1	>329
	60	5	>255

C. Brachwitz *et al.* also synthesized a series of fludarabine compounds that were active in a number of tumor cell lines (see Fig. (5) and Table 4) [74]. They observed that depending on the cell type and lipid moiety, the drug had different effects. For example, the fludarabine was active in the two leukemia cell lines (Daudi and HL-60) while not as active in the solid tumor and one nontumorigenic human mammary epithelial cell line (H 184). However, the conjugate molecules were cytotoxic to some of the other cell lines. As identified by other investigators, Brachwitz, *et al.* confirmed that the type of lipid moiety used does play a role in how cytotoxic the lipid conjugates may be. Furthermore, by attaching an antitumor agent to a lipid carrier molecule, the drug is modified in its pharmacologic properties and may become more active against different tumor cells.

PHOSPHOLIPID GEMCITABINE AND ARA-C CONJUGATES

Previous investigators have had many successes with phospholipid/nucleoside analog conjugates; however, there are still improvements that can be made to these compounds [59, 60, 62-64, 73]. In our laboratory, we focused on the synthesis of a thioether phospholipid (1-*S*-dodecyl-2-*O*-decyl-thioglycerol-3-phosphatidic acid) conjugated to either gemcitabine or ara-C [75]. We chose the thioether phospholipid carrier for two reasons. First, the thioether linkages at the C1 position and oxyether at the C2 position will not be subject to metabolism by lipases thereby making these conjugates orally bioavailable [76]. Second, a similar phospholipid AZT conjugate has demonstrated continuing

success both *in vitro* as an antiviral agent and *in vivo* for the treatment of HIV in humans [77]. The metabolism of the conjugate molecule was observed to produce AZT monophosphate [78, 79]. With respect to the antitumor activity of these lipid conjugates upon metabolic activation of the compound intracellularly, the released nucleoside (ara-C and gemcitabine) monophosphate serves two purposes: a) it bypasses the rate-limiting step, phosphorylation by deoxycytidine kinase and b) the phosphate "locks" the nucleotide within the cell. The mechanism for this cleavage is unknown, but may be carried out by a phospholipase-C-like enzyme. Furthermore, the lipid moiety is potentially an inhibitor of protein kinase C, an enzyme that, among other activities, stimulates cellular proliferation [80, 81]. This dual property of the phospholipid/nucleoside analog conjugates would attack multiple targets in order to induce apoptosis and ultimately cell death.

These key observations are of importance given the fact that by attaching a phospholipid molecule to gemcitabine or ara-C, the conjugate molecule may bypass some resistance mechanisms such as decreased uptake, altered enzymes for activation, or active efflux. These mechanisms are often associated with refractory tumor cells. Previous research has shown that by attaching ara-C to phospholipids, the bioavailability profile is altered. Our initial findings have shown that gemcitabine conjugated to 1-*S*-dodecyl-2-*O*-decyl-thioglycerol-3-phosphatidic acid produces a drug that exhibits greater cytotoxicity compared to a conjugate of 1-*S*-dodecyl-2-*O*-decyl-thioglycerol-3-phosphatidic acid and ara-C in all cell lines tested. Further, the phospholipid/gemcit-

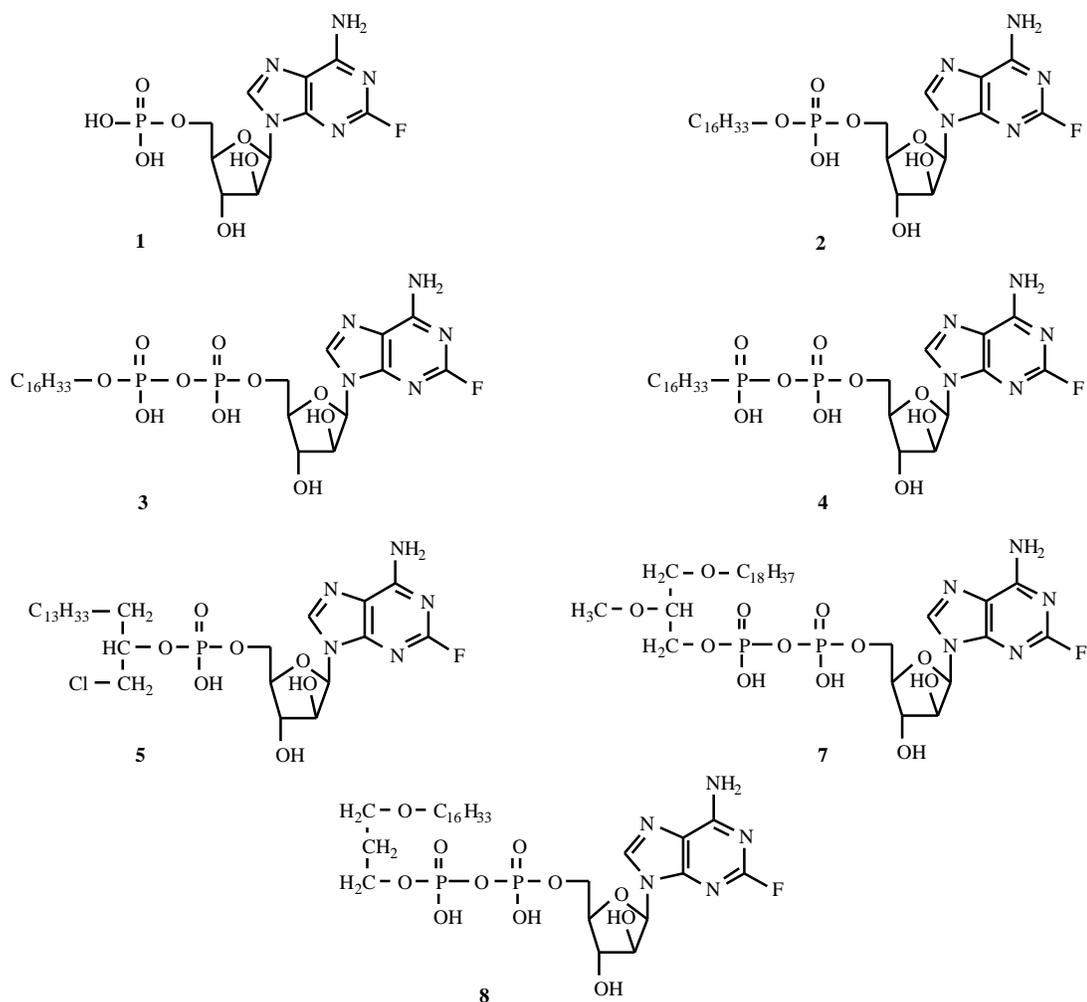


Fig. (5). The chemical structures of the lipid fludarabine conjugates [74]. Chemical structure number one is ara-C.

Table 4. Selected *In Vitro* IC₅₀ Results for Fludarabine Conjugates [74]. For compound references, see Figure 5. All values are micromolar (μM). If no value is listed, the IC₅₀ is greater than fludarabine

Cell Line	Conjugate or Fludarabine						
	1 (fludarabine)	2	3	4	5	7	8
H 184	16.0	--	--	8.3	--	--	13.5
MaTu	97.3	11.5	36.0	7.0	19.3	10.3	39.7
MCF 7	42.2	6.6	--	--	--	--	--
Daudi	10.3	--	--	--	--	7.0	7.0
HL-60	2.8	--	--	--	--	--	--

abine conjugate has been able to bypass resistance mechanisms such as altered transport and active efflux (Alexander *et al.* Cancer Chemotherapy and Pharmacology, in press).

NEW SYNTHETIC TECHNIQUES

Our synthetic technique has taken a new approach to attaching nucleoside analogs to phospholipid backbones.

Previously, Ryu *et al.* used levulinic acid to protect the hydroxyl groups and amino groups on ara-C [62]. The protected ara-C was then conjugated to the phospholipid by using dicyclohexylcarbodiimide (DCC) and the reaction only yielded about twenty-two percent. In our labs using this technique, the yield from the coupling reaction was even lower, and the products were extremely difficult to purify. Another problem with this synthetic procedure using the levulinic acid as a protecting group, is that we had difficulty determining by NMR, exactly how many protecting groups had actually attached to the molecule (ara-C or gemcitabine).

Mavromoustakos *et al.* provided an alternative method for constructing a phospholipid-AZT conjugate that afforded a seventy-two percent yield [82]. While this new procedure is promising, the reagents needed to execute this procedure are very costly. Additionally, AZT does not need the protecting groups like ara-C or gemcitabine. Therefore, the synthesis is not as straightforward, and the yields could be lower.

With these considerations in mind, we chose a new conjugation method which combines a series of previously published reports which in the end were not as costly and afforded excellent yields [75]. Instead of using levulinic acid to protect ara-C or gemcitabine, we used benzoic anhydride to first protect the amino group [83]. These peaks were readily identifiable using NMR. The 5'-OH group was protected using tert-butyldimethylsilyl (TBDMS) chloride, and the remaining -OH groups were protected using acetic anhydride as previously described for attaching fludarabine to different lipid carrier molecules [62, 74]. Also, the TBDMS and acetic anhydride peaks were easily detected using NMR. The TBDMS was removed as previously published, and we used 2, 4, 6-triisopropylbenzene-sulfonyl chloride (TPS) for the conjugation reaction instead of DCC [62, 74]. Using these methods, we achieved reaction yields of about eighty-three percent. Furthermore, the reaction products are easily purified and identified using NMR. In summary, our new synthetic scheme greatly increased reaction yields of highly purified conjugate molecules.

Another advantage of our synthetic scheme is that it can be applied to other nucleoside analogs besides ara-C, gemcitabine, and fludarabine. Many biologically active compounds with a free -OH group could potentially be attached to a lipid carrier molecule for better absorption and more biological activity. Additionally, previous reports have not used a p.o. route of administration due to the ester linkages at the C1 or C2 position of the glycerolipid and their potential cleavage within the GI tract [54, 76]. With our technology, ether linked phospholipids will not be subject to metabolism in the GI tract thus allowing oral administration.

In conclusion, the use of phospholipid-ara-C conjugates for cancer treatment has been pursued by many investigators. We have studied a series of phospholipid conjugates that may prove to be orally available, have a different pharmacokinetic profile, bypass resistance mechanisms, and be more biologically active compared to the native antitumor agent itself. This technology is applicable to a number of other drugs that have poor bioavailability or are subject to lower biological activity due to tumor cell resistance mechanisms. The application of phospholipid conjugates as

new strategies for cancer chemotherapy merits further investigation.

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ABBREVIATIONS

AML	= Acute myeloid leukemia
ara-C	= Cytarabine
ara-CDP-DL-dipalmitin	= Ara-C diphosphosphate dipalmitin, D and L isomers
ara-CDP-DL-PTCA	= Ara-CDP- -palmitoyl-DL-thiochimyl alcohol
ara-CDP-DL-PTBA	= Ara-CDP- -palmitoyl-DL-thiobatyl alcohol
ara-CMP	= Ara-C monophosphate
ara-CTP	= Ara-C triphosphate
ara-U	= Ara-uridine
AZT	= 3'-azido-2', 3'-dideoxythymidine
BG-1 and BG-3	= Human ovarian cancer cell lines
Daudi	= Human leukemia cell line
dFdC	= Gemcitabine
DCC	= 1, 3-dicyclohexyl-carbodiimide
DNA	= Deoxyribonucleic acid
ED ₅₀	= Effective dose 50%
ET-16S-OMe	= 1-S-hexadecyl-2-O-methyl-rac-glycero-4-phosphocholine
ET-16S-OEt	= 1-S-hexadecyl-2-O-ethyl-rac-glycero-4-phosphocholine
ET-16SO ₂ -OEt	= 1-SO ₂ -hexadecyl-2-O-ethyl-rac-glycero-4-phosphocholine
ET-18-OMe	= 1-O-octadecyl-2-O-methyl-rac-glycero-4-phosphocholine
ET-18S-OMe	= 1-S-octadecyl-2-O-methyl-rac-glycero-4-phosphocholine
ET-16S-OEt	= 1-S-octadecyl-2-O-ethyl-rac-glycero-4-phosphocholine
F-ara-A	= Fludarabine

H 184	= Nontumorigenic human mammary epithelial cell line
HeLa	= Human cervical carcinoma cell line
HIV	= Human immunodeficiency virus
HL-60	= Human leukemia cell line
i.c.	= Intracranial
% ILS	= Percent increase in life span, defined as ((test group mean day of death/control group mean day of death) x 100) – 100
i.m.	= Intramuscular
i.p.	= Intraperitoneal
i.v.	= Intravenous
MaTu	= Human breast cancer cell line
MCF 6	= Human breast cancer cell line
NMR	= Nuclear magnetic resonance
-OH	= Hydroxyl group
PLA ₂	= Phospholipase A ₂
p.o.	= Oral route of administration
s.c.	= Subcutaneous
t _{1/2}	= Half-life
TBDMS	= Tert-butyl dimethylsilyl
TPS	= 2, 4, 6-triisopropylbenzene-sulfonyl chloride

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