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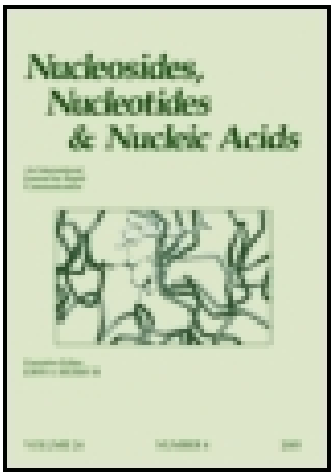

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Preparation of Oligomeric 2′-Deoxy-5-Fluorouridylate of Defined Length and Backbone Composition: A Novel Pro-Drug form of the Potent Anti-Cancer Drug 2′-Deoxy-5-Fluorouridylate

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NUCLEOSIDES & NUCLEOTIDES, 14(1&2), 243-253 (1995)

PREPARATION OF OLIGOMERIC 2'-DEOXY-5-FLUOROURIDYLATE OF DEFINED LENGTH AND BACKBONE COMPOSITION: A NOVEL PRO-DRUG FORM OF THE POTENT ANTI-CANCER DRUG 2'-DEOXY-5-FLUOROURIDYLATE

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Abstract: A recursive protection scheme for FdUMP is employed by synthesizing oligonucleotides consisting of only FdU. 3'-O-exonuclease action releases FdUMP and a shortened oligonucleotide from which further exonuclease action releases more FdUMP. Such oligonucleotides are more cytotoxic to H4IIe, mouse fibroblast, and metastatic mouse tumor cells than are equivalent concentrations of FdU monomer.

INTRODUCTION

The fluorinated pyrimidine nucleoside analogues are potent anti-cancer agents believed to act primarily by their conversion to 2'-deoxy-5-fluorouridylate (FdUMP)[1]. FdUMP is a potent and nearly irreversible inhibitor of thymidylate synthase (TS), the enzyme that converts dUMP to dTMP, the enzymatic step that is rate limiting for DNA synthesis[2]. Although not directly inhibitory of TS, other fluorinated pyrimidine compounds such as 5-fluorouracil (5-FU) that are metabolically converted to FdUMP are preferentially administered to cancer patients in the clinic. Direct administration of FdUMP is ineffective because small, charged molecules penetrate cellular membranes poorly compared with neutral species. FdUMP, or its alternative forms, remain one of the most frequently utilized anti-cancer compounds in the clinic although their initial synthesis in 1957[3] pre-dates much of our current understanding of carcinogenesis.

Regardless of their mechanism of action, administration of fluoropyrimidines as nucleosides or nucleoside bases is plagued by their rapid clearance from the body and the neuro- and cardiotoxicity of ß-fluoroalanine, the chief degradatory product of 5-fluorouracil. A typical half-life for 5-FU in humans is 15 minutes[4]. 5-FU is commonly administered either as an intravenous bolus

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of 10-15 mg/kg[5] or constant intravenous infusion of 20-30 mg/kg/24 h over a five day period[6]. The apparent total body clearance is dependent on the method of administration and ranges from 1 - 6 l/min[7]. Although the anabolic metabolic pathways are important for the anti-cancer activity of 5-FU, the drug is excreted by catabolic metabolism. Most of the drug is catabolized and excreted within 4 h of an intravenous bolus[8]. The major catabolite of 5-FU is 2-fluoro-ß-alanine (FBAL). FBAL may be retained over long periods in the central nervous system and heart. The optimization of chemotherapy regimens involving 5-fluorouridine to minimize the production of FBAL is an important objective in anti-cancer research. In the present study, the effectiveness of oligomeric 2'-deoxy-5-fluorouridylate[9] as a cytotoxic agent in cell culture is explored (Figure 1). Advantages to this pro-drug approach include the improved cellular uptake properties of oligonucleotides compared to nucleotides and nucleosides and the reduced number of steps required to produce FdUMP once the homo-oligomer enters the cell. The present study shows that these advantages are demonstrable in cell culture and are likely to occur in-vivo.

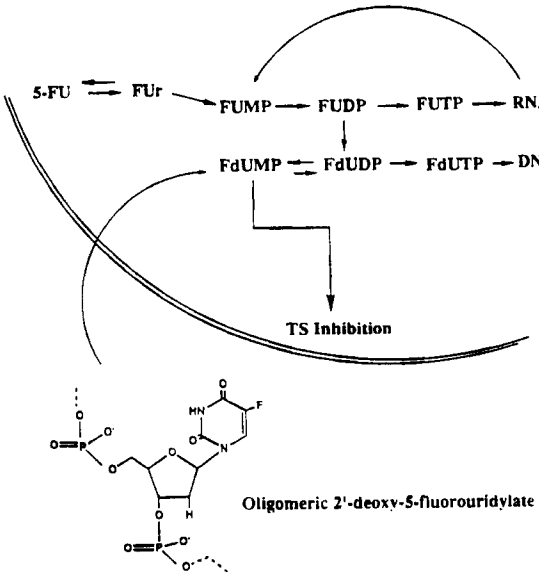

Figure 1 - The structure of oligomeric 2'-deoxy-5-fluorouridylate. These oligomeric compounds gain entry into the cell at least partially in oligomeric form. They are degraded by intracellular 3'-O-exonuclease activity to yield FdUMP, the potent inhibitor of thymidylate synthase. In contrast to the oligomers, monomeric 5-FU requires several steps of metabolic activation to yield FdUMP.

A variety of pro-drug forms of FdUMP have been developed previously to address the issues of cellular uptake, sustained release, and transdermal or intestinal uptake that are problematic for the target compound. Nucleoside analogues that are ultimately metabolized to FdUMP include Tegafur [(1-(2-tetrahydrofuryl)-5- fluorouracil], Ftorafur [R,S-1-(tetrahydro-2-furanyl)-5- fluorouracil] and a variety of 5-fluorocytidine derivatives[10]. A variety of polymeric forms of 5-fluorouridine have also been prepared to provide sustained release of 5-FU and ultimately FdUMP. 5-Fluorouracil has been prepared as a conjugate of chito-oligosaccharides[11] and also as a conjugate of poly(ethylene glycol)[12]. These polymeric forms

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were designed to provide macromolecular drugs with reduced side-effects and strong anti-tumor activity and showed good biological activity and low toxicity in animal models. The chief advantages of oligomeric 5-fluorouridine compounds that are oligonucleotides compared to other possible polymeric structures is that the protection of FdUMP is recursive with enzymatic release of FdUMP releasing a protecting group that is itself oligomeric 2'-deoxy-5-fluorouridylate. Enzymatic hydrolysis proceeds until the oligomer is converted completely into FdUMP with release of no residual groups that could interfere with FdUMP. Oligomeric 2'-deoxy-5-fluorouridylate retain the advantages that make other polymers useful, such as increased bioavailability, and are readily taken up by cells, perhaps through a facilitated mechanism.

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Experimental Section

Synthesis and Purification of Oligodeoxyribonucleotides. 5-Fluorodeoxyuridine was purchased from Sigma. The 5'-O-4,4'-dimethoxytrityl derivative was prepared from the nucleoside by reaction with the dimethoxytrityl chloride in pyridine and purified by column chromatography on silica gel. The 3'-O-phosphoramidite was prepared from the dimethoxytrityl derivative by reaction with 2-cyanoethyl N,N diisopropyl phosphonamidic chloride in THF and purified by column chromatography. Products were analyzed by ^{1}H, ^{13}C, ^{31}P NMR and EI-MS and found to analyze correctly. DNA synthesis was performed on an Applied Biosystems 380B DNA synthesizer. The synthesis conditions were essentially the same as the standard 10 μmole cycles except the columns were custom packed with 15 μmoles instead of 10 μmoles, and any void volume in the column was filled with uncoated controlled pore glass (CPG) to eliminate channeling[13]. After synthesis, the oligonucleotide is cleaved from the CPG support by treatment with 28% ammonium hydroxide (90 min, room temperature). The ammonium solution is then heated in a sealed tube at 55° overnight to remove the protecting groups. The crude material is then desalted on Sephadex G-25 prior to purification by HPLC. Aliquots of 200-250 ODU are then purified by HPLC on a Waters Protein-Pak DEAE-5PW anion exchange column (22.5 mm x 150 mm). A gradient from 0-0.15M sodium perchlorate (90 min) at 5 ml/min is used to elute the product. Pure fractions are combined, lyophilized, and desalted on Sephadex G-25.

Stability of Oligomeric 5-fluorouridine Compounds. H4IIe cells (9 x 10^6) were split and 3 x 10^6 cells were suspended in 490 μL of RPMI media in each of three 6 mL polypropylene tubes. To each tube was added 10 μL of a 0.1 M solution of either FUrd or oligomeric 2'-deoxy-5-fluorouridylate of length 8 or 16. The final concentration of FUrd compound in each tune was 2.0 mM. Control tubes consisted of the compounds at the same concentration in RPMI media alone. Samples (4 μL) were pipetted from each tube at 1, 3, 5, 10, 20, 40, 60, 120, and 240 min. Samples were analyzed by thin layer chromatography on Whatman flexible TLC plates (Al Sil G/UV) using 1:1 chloroform:methanol as the mobile phase. Mobilities of components were assessed by visualizing the plates under UV light and recording the RF values. Semi-quantitative analysis of the concentration of metabolites was done by visually inspecting the intensity of the spots under UV light. The RF values for all components observed in the experimental samples were compared with the RF values of the control samples. All experiments were performed in duplicate.

Cytotoxicity of Oligomeric 5-fluorouridine Compounds. Cells were maintained in RPMI media (Sigma) containing 10% fetal calf serum (GIBCO) and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The oligomeric 2'-deoxy-5-fluorouridylate compounds were added for from one to six days. The cytotoxicity of oligomeric 5-fluorouridine compounds was determined by plating H4IIeor other cells at a density of 1 x 10^4 cells per well in a 96 well microtiter plate. Cells were grown for 24 hours under the same conditions described above for the cellular uptake studies. Culture medium containing oligomeric 5-fluorouridine compounds at concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10 nM were added. Cell viability was determined by measuring the uptake and reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a formazon product that is insoluble in the cell. Formazon production was measured spectrophotometrically at 540 nm by using a Molecular Devices V-max plate reader. The LD_{50} for each of the oligomeric 5-fluorouridine compounds was determined from plots of the optical density at 540 nm versus the log concentration of oligomeric 5-fluorouridine compound. The cytotoxicity of the oligomeric 5-fluorouridine compound of length six at concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10 nM was evaluated in five cell lines in addition to H4IIe cells. Two cell lines (AMRC1.7 and AMRC1.12) derive from H4IIe cells and

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are deficient in oligonucleotide transport. The three other cell lines are from mice and are 3T3 (mouse fibroblast) and both a metastatic and highly metastatic mouse tumor cell line (168, 410).

Results

To assess the cytotoxicty of oligomeric 2'-deoxy-5-fluorouridylate, a number of these compounds were tested for their ability to inhibit the viability of H4IIe cells. The monomer 5-fluorodeoxyuridine (FdU), which is of known potency, was used as a control. Two variables were assessed in these cell culture studies. The first is the length of oligomeric 2'-deoxy-5-fluorouridylate and the second is the type of phosphate backbone present in these compounds. Studies with oligomeric 2'-deoxy-5-fluorouridylate of length 6, 8, 12, 16, and 20 were undertaken with both phosphodiester and phosphorothioate backbones prepared for each length. Dose response curves obtained with several of these compounds against H4IIe cells using the MTT assay are shown in Figure 2. The dose response curves clearly show that the homo-oligomers with phosphodiester backbones are cytotoxic at lower doses than is monomeric FdU. The cytotoxicity of the oligomeric 2'-deoxy-5-fluorouridylate expressed as an LD_{50} dose depends on both the length and the type of phosphate backbone. The $(FdU)_6$ compound with a phosphodiester backbone has an LD_{50} of less than one-tenth that of FdU. Thus, the oligomeric compounds are more effective than FdU even on a per residue basis, i.e., when corrected for the multiplicity of units per oligomer. The increased efficiency per residue for the phosphodiester series depends on the length of the oligomeric 2'-deoxy-5-fluorouridylate. For the phosphodiester series lengths less than or greater than 16 are less effective on a per residue basis than is $(FdU)_{16}$. The data are summarized in Table 1.

The cytotoxicity of monomeric deoxyuridine (dU) and oligomeric 2'-deoxy-uridylate of length eight (dU_8) were also assessed using these same cells and assay methods and no toxicity was observed at concentrations up to 10 μM. Thus, contaminants from synthesis that might persist following purification are not potential explanations for these observations concerning cytotoxicity. In contrast to the results obtained with oligonucleotides having phosphodiester backbones, oligomeric 2'-deoxy-5-fluorouridylate with phosphorothioate backbones are ineffective cytotoxic agents. The LD_{50} for oligomeric 2'-deoxy-5-fluorouridylate with phosphorothioate backbones are 10–30% that of

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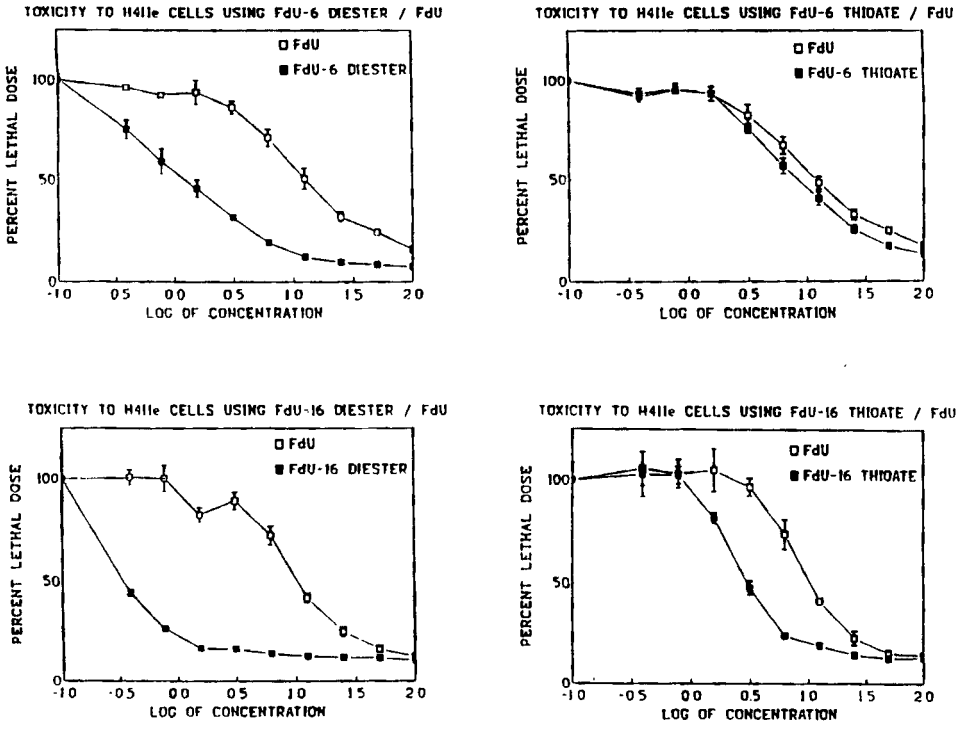

Figure 2 – Dose response curves that correlate the log concentration (nM) for the oligomeric 2'-deoxy-5-fluorouridylate with their cytotoxicity towards H4IIe cells as monitored by an MTT assay. The data for oligomeric compounds of length 6 and 16 with both phosphodiester and phosphorothioate backbones are displayed.

Table 1 The LD_{50} values towards H4IIE cells for homo-oligomeric FdUMP compounds of various lengths with either phosphodiester or phosphorothioate backbones. The $FdU/n(FdU)_n$ ratio corrects for the oligomeric nature of these compounds and reflects the per-residue cytotoxicity.

	PHOSPHODIESTER			PHOSPHOROTHIOATE		
	LD_{50}(nM)	r^2	$FdU/n(FdU_N)$	LD_{50}(nM)	r^2	$FdU/n(FdU_N)$
FdU	8.51	--	1.0	--	--	--
Fdu_6	0.77	0.999	1.8	7.05	0.994	0.20
FdU_8	0.48	0.999	2.2	3.87	0.999	0.27
FdU_{12}	0.28	0.999	2.5	3.13	0.999	0.23
FdU_{16}	0.18	0.999	3.0	2.56	0.997	0.21
Fdu_{20}	0.29	0.954	1.5	2.88	0.987	0.15

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monomeric FdU when corrected for the multiplicity of FdU in the oligomer. The phosphorothioates are all less than 10% as effective as the phosphodiesters of the same length. The diminished biological response of the phosphorothioate oligonucleotides indicates that oligonucleotide hydrolysis is important for the increased effectiveness of the oligomers compared with FdU. The data for the oligomeric 2'-deoxy-5-fluorouridylate having phosphorothioate backbones is also included in Table 1.

One possibility for the cytotoxicity of the oligomeric 2'-deoxy-5-fluorouridylate compounds is that extracellular degradation releases monomeric 2'-deoxy-5-fluorouridylate and that this monomeric form is taken up by the cells. To determine the extent of extracellular degradation of the oligomeric 2'-deoxy-5-fluorouridylate compounds the time course of degradation was followed by TLC analysis of the culture medium. Monomeric FdU has an $R_f = 0.83$ with the solvent system used (1:1 chloroform:methanol). The intensity of monomeric FdU decreased and fell to about one-half its original intensity over about 20 minutes. No monomeric FdU was evident after 120 min. Oligomeric 2'-deoxy-5-fluorouridylate of length eight has an initial $R_f = 0.51$ with the solvent system used. Material at this R_f value was visible after 240 minutes incubation in approximately one-half the initial intensity. At incubation times of 40 minutes or longer a compound of higher R_f is apparent that gradually increases in intensity. The final intensity of the compound of higher R_f is similar to that of starting material ($R_f = 0.51$) after 240 minutes. The oligomeric 2'-deoxy-5-fluorouridylate of length 16 had an intial R_f similar to that of the oligomer of length eight. The 16mer compound retained approximately one-half the initial intensity after 240 minutes. The monomeric and oligomeric compounds showed no changes in this TLC assay after a 240 minute incubation in the absence of H4IIe cells. These results demonstrate that extracellular degradation of the oligomeric compounds is not responsible for their enhanced cytotoxic potency relative to monomeric FdU.

The cytotoxicity of FdU is believed to occur mainly by its conversion to FdUMP and the inhibition of TS resulting in thymidineless cell death[14]. To explore whether the mechanism of action for the oligomeric 2'-deoxy-5-fluorouridylate compounds having phosphodiester backbones is similar to monomeric FdU and involves induction of a thymidineless state, the effects of added thymidine on cell viability in the presence of FdU and $(FdU)_8$ were examined[15]. Cells remain viable in the

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presence of added thymidine at otherwise lethal doses of either FdU or $(FdU)_8$. This is consistent with TS inhibition being an important operative mechanism for both monomeric and oligomeric 2'-deoxy-5-fluorouridylate. This is consistent with monomeric and oligomeric 2'-deoxy-5-fluorouridylate each being converted to a common intermediate, e.g., FdUMP. The data do not rule out that oligomeric 2'-deoxy-5-fluorouridylate interferes with TS in oligomeric form, but this is unlikely based on the results obtained with oligomeric 2'-deoxy-5-fluorouridylate containing phosphorothioate backbones. These data suggest FdUMP is produced intracellularly by 3'-O-exonuclease activity on oligomeric 2'-deoxy-5-fluorouridylate containing a phosphodiester backbone.

The cytotoxicity of the oligomeric 2'-deoxy-5-fluorouridylate compound of length six at concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10 nM was evaluated in five cell lines in addition to H4IIe cells. Two cell lines (AMRC1.7 and AMRC1.12) derive from H4IIe cells and are deficient in oligonucleotide transport. The three other cell lines are from mice and are 3T3 (mouse fibroblast) and both a metastatic and highly metastatic mouse tumor cell line (168, 410). The results are summarized in Table 2. The relative potency of the oligomeric 5-fluorouridine compound of length six is highest in H4IIe cells and is precipitously declined in the cell lines deficient in oligonucleotide transport. The relative potency of the oligomeric 5-fluorouridine compound of length six in the mouse derived cell lines is increased significantly compared to monomeric FdU. The results establish that the relative potency of the oligomeric 5-fluorouridine compounds is cell line specific and is proportional on the cell lines propensity to take up oligonucleotides.

Discussion

The administration of FdUMP precursors remains a central component of many chemotherapy regimens. Any improvements in the delivery of FdUMP that decrease the dose required for an effective anti-tumor response could be beneficial since the chief degradatory product of 5-FU, FBAL, is highly toxic. The present work utilizes a recursive protection scheme to deliver FdUMP as an oligomer of itself. The cytotoxicity of oligomeric 2'-deoxy-5-fluorouridylate with a

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Table 2 - Relative Potency of (FdU)6 towards Six Cell Lines

Cell Line	LD50 $FdU/(FdU)_n$	Relative Potency LD50/n
H4IIe	14.70	1.80
AMRC1.7	7.12	0.89
AMRC1.12	5.84	0.73
3T3 Mouse Fibroblast	9.84	1.23
168 Metastatic mouse cell	9.60	1.20
410 Highly metastatic mouse cell	10.72	1.34

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phosphodiester backbone is greater per residue than monomeric FdU. This suggests that either the oligomer gains entry to the cell more easily than does the monomer or that its conversion to FdUMP is more facile. The greatest difference between oligomeric 2'-deoxy-5-fluorouridylate and monomeric FdU on a per-residue basis is observed for the 16mer, indicating that this length most efficiently gains entry into the cell or is most efficiently acted on by 3'-O-exonucleases in the cell. The data presented do not preclude that oligomeric 2'-deoxy-5-fluorouridylate may have biological activity as an intact oligomer, for instance by binding the poly-adenylated cap sequence of mRNAs or by directly binding to and inhibiting TS while in oligomeric form, they strongly suggest that hydrolysis of the backbone to liberate FdUMP is important. This is evident as the nuclease resistant phosphorothioate backbone demonstrates diminished potency relative to the phosphodiester.

The effectiveness of oligomeric 2'-deoxy-5-fluorouridylate compounds against solid tumors in-vivo is impossible to predict at this time. The oligomeric nature of these compounds could be advantageous for their delivery from a pharmacokinetic standpoint. Pharmacokinetic studies on oligonucleotides have[16] revealed elimination half-lives from 7 to 57 hours for phosphorothioate oligonucleotides intravenously injected into mice. The half-life of oligomeric 2'-deoxy-5-fluorouridylate will most likely be several hours. Since the half-life of 5-FU is about 15 minutes in humans, the oligomeric 2'-deoxy-5-fluorouridylate are very likely to have a significantly longer residence time. This should result in a substantial reduction in dose required to produce an equivalent plasma concentration. A substantial improvement in the therapeutic index of FdUMP anti-metabolite is possible with this approach. Delivery of oligomeric 2'-deoxy-5-fluorouridylate 5-fluorouridine could result in drugs that are more selective for cancer cells by derivitization with

directing moieties, e.g. antibody fragments. Such an approach is less feasible with monomeric compounds. While many differences exist between administration of monomeric FdU and oligomeric 2'-deoxy-5-fluorouridylate a common target, TS, implies that the advances made in combination chemotherapy involving 5-FU will still be useful with these oligomeric forms. In particular, leucovorin (folinic acid) is expected to potentiate oligomeric 2'-deoxy-5-fluorouridylate by increasing 5,10-CH_2-tetrahydrofolate levels. This will result in a greater preponderance of stable ternary complexes involving FdUMP derived from oligomeric 2'-deoxy-5-fluorouridylate, TS and 5,10-CH_2FAH_4.

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