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Perspective

Lipoproteins as Potential Site-Specific Delivery Systems for Diagnostic and Therapeutic Agents

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Introduction

Ever since the pioneering studies of Paul Ehrlich,[1] medicinal chemists and pharmacologists have been interested in the site-specific delivery of drugs—the "magic bullet" concept. This concept is based on the premise that the selective delivery of drugs to tissues where they exert their pharmacological effects will not only enhance the desired therapeutic result but also minimize the occurrence of unrelated responses or toxic side effects. To achieve this laudable goal, researchers have employed a variety of strategies,[2,3] including drug design (e.g., prodrugs[4]), carrier molecules (e.g., antibodies[5]), and incorporation of drugs into macromolecules (e.g., liposomes[6]). While these approaches have met with varying degrees of success with therapeutic agents (i.e., anticancer agents[7]), site-specific delivery concepts have been particularly rewarding in the field of organ imaging,[8] where only tracer doses of a radiodiagnostic are required to obtain the desired diagnostic information.

In all of these studies, the plasma lipoproteins have been largely overlooked as possible site-specific delivery vehicles. Most pharmacology texts recount the importance of plasma proteins, such as albumin, to the ultimate disposition of drug molecules, but surprisingly few studies have analyzed the possible role of plasma lipoproteins in the transport and distribution of drugs. The involvement of lipoproteins in pharmacokinetics becomes even more pertinent in light of recent findings[9] that show that these macromolecules are taken up into specific cells by receptor-mediated processes. The present article will discuss the nature of plasma lipoproteins, review what is known about their involvement in drug disposition, and outline ways in which these macromolecules may be employed for the site-specific delivery of drugs and diagnostic agents.

Types of Lipoproteins and Their Chemical Composition

Plasma lipoproteins are lipid–protein complexes that are responsible for the transport of water-insoluble lipids in the circulation. Structurally, these complexes consist of an apolar core surrounded by a phospholipid monolayer (Figure 1).[10,11] Triglycerides and cholesteryl esters are the major components of the lipophilic core, while free cholesterol and proteins, known as apoproteins, are associated with the phospholipid membrane.

On the basis of their physical characteristics and chemical composition, lipoproteins are classified into four major families called chylomicrons (CM), very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). The densities at which these various lipoproteins are isolated from the plasma by preparative ultracentrifugation are shown in Table I. As the density of these complexes increases, their lipid content and size decreases. CM and VLDL are the largest and lightest of the lipoproteins and are the major carriers for triglyceride. LDL and HDL, on the other hand, contain cholesterol as the predominant lipid. In man, LDL cholesterol contributes about two-thirds of the circulating plasma cholesterol. In rats and dogs, however, cholesterol is primarily transported to tissues as a component of the HDL complex.

In addition to these differences in lipid composition, the plasma lipoproteins also differ with respect to their apoprotein composition (Table I). For example, CM contain 5–10% Apo-B, 60–70% Apo-C and small amounts of other apoproteins, whereas VLDL contain a larger proportion

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Table I. Physicochemical Properties of Human Plasma Lipoproteins[a]

lipoprotein class	density, g/mL	diameter, Å	major lipid, %					major apoproteins, %				
			TG	CH	CE	PL	P	A	B	C	D	E
chylomicrons	0.95	750-5000	86	1	5	7	2		5-10	60-70		
VLDL	0.95-1.006	300-750	50	7	13	20	10		37	49		12
LDL	1.019-1.063	170-250	8	10	30	30	22		80	40		
HDL	1.063-1.210	70-120	8	4	12	24	52	90-95		5-10	1-2	

[a] Abbreviations are as follows: TG, triglyceride; CH, cholesterol; CE, cholesteryl ester; PL, phospholipid; P, protein; A, Apo-AI and -AII; B, Apo-B; C, Apo-CI, -CII, and -CIII; D, Apo-D; E, Apo-E.

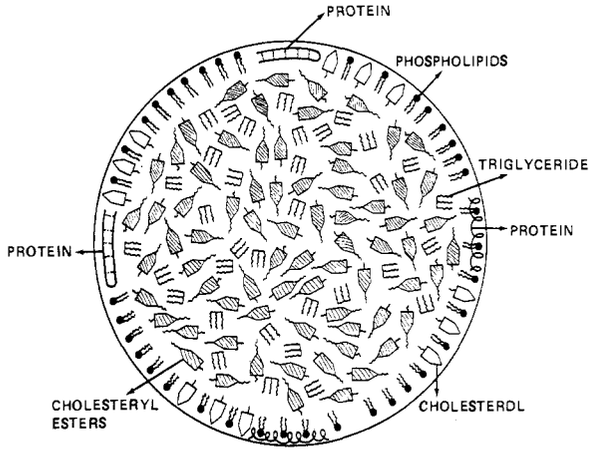

Figure 1. A general structure for lipoproteins.

of Apo-B (37%). Apo-B is also found in high concentration in LDL (>80%) but is virtually absent in HDL. Instead, approximately 90% of the protein associated with HDL consists of Apo-AI and Apo-AII. It is these apoproteins that ultimately determine the function and fate of each class of lipoprotein.

Biosynthesis and Metabolism of Lipoproteins

The biosynthesis and metabolic fate of lipoproteins have been the subject of a number of excellent reviews.[9,11-15] The emphasis here will be to describe those aspects of lipoprotein metabolism that are most relevant to the topic of drug delivery. This is best accomplished by examining each of the lipoprotein classes individually.

CM are responsible for the transport of dietary or exogenous lipids in the circulation. Dietary lipids are absorbed from the intestinal tract and incorporated into CM synthesized in intestinal mucosal cells (Figure 2). These CM are then secreted into and transported through the lymphatics until they reach the circulation via the thoracic duct. Once in the circulation, they are rapidly metabolized by peripheral tissue lipoprotein lipase. This enzyme is activated by Apo-C and is responsible for the hydrolysis of triglycerides contained in CM. This process reduces the size of CM and liberates free fatty acids for utilization in cell metabolism. These restructured CM now contain cholesteryl esters as the main lipid and Apo-B and Apo-E as the major apoproteins. These smaller CM, called CM remnants, are cleared very rapidly from the circulation by the liver ($T_{1/2}$ = ~5 min).[16] Moreover, there is evidence

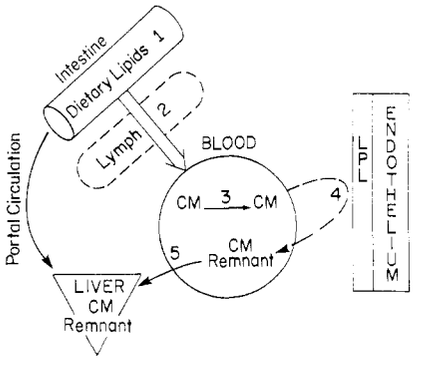

Figure 2. Sequence of biosynthesis and metabolism of chylomicrons: step 1, absorption of dietary lipids; step 2, transport of CM to blood via lymphatics; step 3, transport of CM to extrahepatic tissues; step 4, formation of CM remnant via lipoprotein lipase (LPL); step 5, uptake of CM remant by the liver.

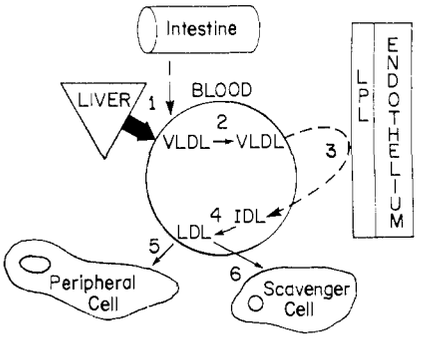

Figure 3. Sequence of biosynthesis and metabolism of VLDL and LDL: step 1, biosynthesis of VLDL in liver and intestine; step 2, transport of VLDL to extrahepatic tissues; step 3, formation of remnant particle (IDL) via lipoprotein lipase (LPL); step 4, remodeling of remnant and LDL formation; step 5, high-affinity uptake of LDL into peripheral cells; step 6, low-affinity uptake of LDL into scavenger cells.

that the uptake by liver cells involves a receptor-mediated process that recognizes both Apo-B and Apo-E.[17]

In contrast to CM, VLDL are responsible for the transport and delivery of endogenously synthesized triglycerides and cholesteryl esters to extrahepatic sites (Figure 3). Synthesized primarily in the liver, VLDL also enter the circulation and become a substrate for lipoprotein lipase. However, lipoprotein lipase utilizes VLDL much less efficiently than CM, and as a result the plasma half-life is much longer (1–3 h).[18] As lipolysis occurs, VLDL become smaller and form remnants known as intermediate density lipoproteins (IDL) (Figure 3). The metabolic fate of IDL differs in rats and humans. In the rat, IDL are rapidly taken up by the liver where they undergo lysozomal degradation.[19,20] In man, IDL serves as the precursor of

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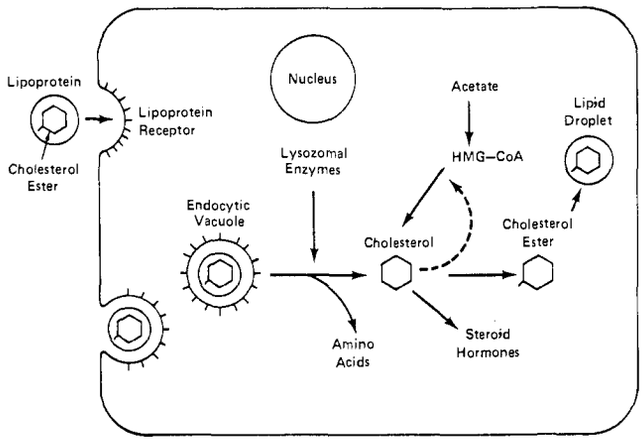

Figure 4. Lipoprotein receptor-mediated process.

LDL, which is the major carrier of cholesterol to extrahepatic tissues.

The elegant studies of Goldstein and Brown[9] have shown that extrahepatic cells acquire their cholesterol by an LDL receptor-mediated process. This process (Figure 4) involves the binding of LDL to specific, high-affinity binding sites located on the cell membrane surface. Once binding has occurred, this LDL–receptor complex is rapidly internalized by endocytosis and subsequently digested by lysozomal enzymes to liberate free cholesterol. This cholesterol is not only utilized as an important structural component for cell membrane synthesis but also serves to regulate the following intracellular processes: (a) the stimulation of the formation of cholesteryl ester, by catalyzing and serving as a substrate for acyl-CoA:cholesterol acyltransferase (ACAT); (b) the intracellular concentration of cholesterol, by suppressing β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting step in cholesterogenesis; and (c) the reduction of the rate of LDL–receptor synthesis in order to decrease the amount of LDL cholesterol being taken up by the cell.

An additional pathway for LDL metabolism involves a lower affinity process associated with scavenger cells or macrophages of the reticuloendothelial system (Figure 3). In man, 33–65% of the plasma LDL is degraded by the high-affinity receptor-mediated process, and the remainder is handled by the scavenger cells. Overall, these degradation processes are much slower than those associated with the larger CM and VLDL and result in a plasma half-life of about 3–4 days for LDL.

In man, HDL have the longest plasma half-life of all the lipoproteins (5–6 days). Moreover, their biosynthesis and metabolism are the most complex. Although initially formed in the liver and intestine, they rapidly become modified in the circulation by interaction with the other lipoproteins. An important distinction of HDL is that they are the major carrier for Apo-AI and Apo-AII. The latter is an essential determinant for lecithin:cholesteryl acyltransferase (LCAT), which serves to esterify HDL cholesterol. The resulting cholesteryl esters are then transferred to VLDL and IDL via a plasma exchange protein. It has been proposed that this may be a mechanism for removing cholesterol from cells.[21]

HDL have been shown to bind to cells, such as fibroblasts, at specific sites that are separate from those that bind LDL.[22] Moreover, the rate of uptake of HDL by extrahepatic cells is much less than that observed for LDL, except in those species, such as the rat, where HDL represent the major cellular source of cholesterol. In the rat, HDL appear to play a role similar to that of LDL in man. For example, in rat steroid-secreting tissues (adrenal and gonads), rat HDL are internalized via a specific receptor-mediated process[23] in a manner similar to that observed for LDL in human fetal adrenals.[24]

Lipoproteins as Transport Systems for Drugs, Diagnostic Agents, and Xenobiotics

The actual role of lipoproteins in the transport and delivery of pharmacological agents to specific sites of action has not been well documented. Nonetheless, the possible importance of lipoprotein binding or interaction with certain lipophilic compounds has been recognized. For example, Rudman et al.[25] have demonstrated that compounds with partition coefficients ≥ 11 were sequestered in the lipoprotein fraction of rabbit, chicken, and human lipemic serum. These compounds included diphenylhydantoin, bishydroxycoumarin, estradiol, testosterone, fatty acids, pentobarbital, and aldosterone. Similarly, equilibrium gel filtration techniques have shown that reserpine binding to lipoproteins was much greater in hyperlipoproteinemic patients than in normal subjects.[26] Furthermore, recent studies have indicated that the interaction between lipoproteins and a variety of lipophilic compounds (i.e., insecticides,[27,28] testosterone undecanoate,[29] Δ^9-tetrahydrocannabinol,[30] benzo[*a*]pyrene,[31] and chloroethylnitrosoureas[32]) involves a partitioning phenomenon whereby these lipophilic agents are dissolved into the hydrophobic core of lipoproteins. It is these and similar observations that lend credence to the possibility that plasma lipoproteins may play a significant role in the transport of nonpolar, lipophilic compounds.

As these observations were being made, others were noting that many lipophilic substances when given orally were being absorbed via the intestinal lymphatics mainly in association with CM. For example, virtually all vitamin A is esterified in mucosal cells during absorption, and the apolar retinyl esters become incorporated into the CM core.[33,34] These esters remain with the CM remnant during metabolism and are taken up by the liver. Vitamin D_3 is absorbed in a similar manner, but in this case, when CM are subjected to lipolysis catalyzed by lipoprotein lipase, some of the vitamin appears to leave the CM during

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remnant formation.[35] During this process, the vitamin D_3 becomes associated with a specific vitamin D_3 binding protein in plasma. It has been proposed that this transfer of vitamin D_3 from CM to the vitamin D_3 binding protein in plasma may be important in the targeting of this vitamin to peripheral tissues.

The notion that lipoproteins could actually affect the therapeutic efficacy of drugs was first described by Van Der Vies.[36] This study showed that testosterone undecanoate was 1.85 times more active when orally administered in oil than in lipid-free tablets. Moreover, this enhancement of biological activity correlated well with the increased incorporation of the drug into CM following its oral administration in oil. Since CM reach the circulation via the lymphatics, first pass metabolism by the liver is avoided. In this way, more drug is able to reach the target tissues.

Other groups have begun to package agents into lipoproteins prior to administration and examine the effect of such packaging on metabolism and tissue distribution of the incorporated agents. Although ingested polycyclic aromatic hydrocarbons have been reported to enter the circulation from the intestines incorporated in CM,[37] little is known about the fate of these carcinogen-bearing CM. Vauhkonen and co-workers[38] incorporated [^{3}H]benzo[*a*]pyrene into mature rat CM in vitro and administered this preparation intravenously to female Sprague–Dawley rats. They found that within 0.5 min approximately 50% of the [^{3}H]benzo[*a*]pyrene was associated with serum albumin and most of the remainder with the fraction composed of CM, VLDL, and CM remnants. With time, radioactivity became distributed into other serum components, especially LDL and HDL. At 60 min, the highest levels of tissue radioactivity were noted in lung, liver, and kidney. The implications of these findings to the carcinogenicity of benzo[*a*]pyrene were discussed.

Lipoproteins have been implicated in the transport of other xenobiotics. For example, oxidation products of cholesterol have been shown not only to be potent inhibitors of cholesterol biosynthesis in cells[39] but also to be highly toxic to cultured aortic smooth-muscle cells of the rabbit. One of the most abundant of these oxidation products, 25-hydroxycholesterol, has been shown by Peng and co-workers[40] to be transported in the circulation largely in association with LDL (55.1%), VLDL (34.7%), and, to a smaller extent, HDL (10.2%) following oral administration to squirrel monkeys. On the basis of the known direct correlation between elevated plasma levels of VLDL and LDL and the incidence of atherosclerosis, these workers have hypothesized that angiotoxic oxidation products of cholesterol are transported to and incorporated into vascular tissue through the agency of LDL. Support for this new hypothesis for atherogenesis must await further studies.

As stated earlier, the liver plays a major role in the metabolism of CM remnants. Since cholesteryl esters are a major constituent of CM remnants, incorporation of polyiodinated esters of cholesterol into CM complexes could give rise to potential hepatographic agents. Preliminary studies have indicated that iopanoic acid, an established cholecystographic agent, when esterified with cholesterol can be incorporated into CM remnants.[41] Administration of remnant-incorporated cholesteryl iopanoate to rats led to a marked increase in the amount of ester reaching the liver within 0.5 h. Approximately 87% of cholesteryl iopanoate was present in the liver following administration in CM as opposed to about 31% when injected in saline. Although these results suggest a potential use of CM as carriers of radiopharmaceuticals for liver imaging, the practicality of CM remnants as carriers of pharmacological concentrations of drugs remains to be ascertained.

As previously stated, LDL is the major cholesterol carrier in man, while in the rat and dog HDL serves this function. Since most tissues receive their cholesterol from these circulating macromolecules via a specific-receptor mediated process, it is intriguing to consider the possible application of lipoproteins as vehicles for the delivery of hydrophobic molecules to specific cells and tissues. However, relatively little work has been done in this area. Krieger et al.[42] have demonstrated that cholesteryl esters can be extracted from LDL with heptane and replaced with two fluorescent probes, 3-(oleoyloxy)-3-(pyrenylmethyl)-23,24-dinor-5-cholen-22-oate and dioleylfluorescein. Both fluorescent lipoprotein preparations were shown to be internalized into cultured human fibroblasts via the receptor-mediated endocytosis of LDL. However, such a process does not appear to occur for all compounds that become associated with LDL. For instance, it has been observed that the carcinogen benzo[*a*]pyrene preferentially associates with human VLDL and LDL.[31,38] However, the entry of benzo[*a*]pyrene into cultured human fibroblasts incubated with LDL-incorporated benzo[*a*]pyrene was found to be independent of specific receptor binding and internalization of the LDL–receptor complex.[43]

On the other hand, selective delivery and uptake of various compounds into specific tissues of the rat have been demonstrated with HDL. The Gram-negative bacterial lipopolysaccharide (LPS), when injected iv into the rat, binds very avidly to HDL.[44] Whole animal tissue distribution studies following the administration of the LPS–HDL complex indicated HDL-mediated uptake of LPS by the adrenal gland. The importance of LPS–HDL binding for determining the extent of LPS uptake by the adrenal gland during bacterial sepsis was implicated.

Radioiodinated derivatives of cholesterol have proven to be useful imaging agents for the diagnosis of a variety of adrenal disorders in humans. It has recently been noted, however, that adrenal visualization was absent in a patient with Cushing's disease who had marked hyperlipidemia.[45] Correction of the hyperlipidemia, followed by subsequent adrenal imaging, resulted in visualization of the adrenals. Studies by Valk et al.[46] provided additional evidence to

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support an inverse relationship between serum cholesterol levels and adrenal uptake of radioiodinated cholesterol derivatives. These results indicated that the expanded pool of serum lipoprotein cholesterol effectively diluted the radiotracer concentration available to the steroid-secreting tissues.

According to current concepts, tissues responsible for the biosynthesis of steroid hormones derive their cholesterol from three possible sources, namely, (1) circulating plasma, (2) hydrolysis of intracellular cholesterol esters, and (3) intracellular biosynthesis (Figure 4). Most animal studies to date indicate that the adrenal cortex relies mainly upon the circulating plasma pool as its source of cholesterol.[23,47] Once this source becomes depleted, as occurs upon treatment of animals with 4-aminopyrazolo-[3,4-*d*]pyrimidine (4-APP), the other two pathways are called upon to meet the metabolic needs of the cell for cholesterol.

Gwynne et al.[48] showed that the uptake of cholesterol into rat adrenal slices was two to three times greater from HDL than LDL and that this uptake was stimulated by the administration of ACTH. Andersen and Dietschy[23] subsequently demonstrated that infusion of HDL, but not LDL, into 4-APP-treated rats suppressed intracellular cholesterol synthesis and increased cholesterol ester content of the adrenals, ovaries, and testes. Conversely, Kovanen et al.[49] demonstrated that membranes from bovine adrenal cortex and ovarian corpus luteum have binding sites for LDL but not HDL. Mouse adrenal gland, on the other hand, appears to have selective uptake processes for both HDL and LDL.[50]

Several years ago, the possible utilization of plasma lipoproteins as site-specific delivery vehicles for radiopharmaceuticals was proposed.[51-53] Prior incorporation of 19-radioiodinated cholesterol into rat HDL failed to enhance adrenal uptake of the tracer over that observed when saline was used as the vehicle for administration. In this instance, the specific activity of the HDL preparation becomes diluted by the circulating plasma HDL. However, when the animals were pretreated with 4-APP to lower the endogenous level of circulating HDL, a fourfold increase in adrenal radioactivity was observed. This finding was consistent with the above-mentioned studies with radioiodinated HDL, which showed rat adrenal cortex to contain high affinity and low capacity receptors for HDL.

Similarly, incorporation of 19-radioiodinated cholesteryl oleate into rat HDL produced a fivefold increase in adrenal uptake and an almost fourfold enhancement in ovarian accumulation of radioactivity at 0.5 h.[54] In this instance, prior treatment with 4-APP was not required to show this effect. Moreover, the uptake in ovary was found to greatly exceed that in the adrenal cortex at 24 h. At this time, there is no obvious explanation for either of these two findings. However, the apparent ability of lipid-lowering agents other than 4-APP to modulate the receptor-mediated uptake of agents by steroid-secreting cells is intriguing and requires further study. The potential use of lipoproteins for the delivery of anticancer agents to neoplastic cells is supported by the studies by Gal and coworkers.[55,56] Based on the knowledge that replicating cancer cells continuously require large amounts of cholesterol for the synthesis of cell membranes, this group studied a number of different types of cancer cells in monolayer culture. They found neoplastic cells to metabolize LDL at higher rates than nonneoplastic cells. In epidermoid cervical carcinoma (EC-50) cells, for example, LDL was metabolized at a rate 15 to 20 times greater than that of normal cells, such as fetal adrenal tissue in organ culture. On the basis of these studies, a model was suggested for the treatment of cancer cells by cytotoxic drugs incorporated into LDL.

In support of this view, others have noted an effect of serum lipoprotein concentrations on the efficacy of chloroethylnitrosoureas. These anticancer drugs undergo chemical reactions in vitro and in vivo to form active alkylating agents. Weinkam and co-workers[32] have demonstrated that lipoproteins can stabilize chloroethylnitrosoureas from base-catalyzed degradation. According to their model, this stabilizing effect involves the partitioning of lipophilic chloroethylnitrosoureas into the hydrophobic core of the lipoprotein where the compound is chemically stable and protected from chemical degradation reactions. As a result, just as in the case with radioiodinated cholesterol and adrenal imaging, variations in serum lipoprotein levels may be a major determinant of interindividual variations in therapeutic response to lipophilic nitrosourea anticancer agents.

Summary

Despite the paucity of literature dealing with the effect of plasma lipoproteins upon drug disposition, evidence is accumulating that shows that these macromolecular complexes can play important roles in the absorption and transport of lipid-soluble agents. Moreover, preliminary studies have demonstrated that radiotracers can be directed to specific tissues by prior incorporation into the hydrophobic core of specific lipoproteins.

Although these studies offer encouragement for the possible use of lipoproteins in the site-specific delivery of radiopharmaceuticals that are used in tracer doses, the use of lipoproteins in the transport of drugs at pharmacological concentrations represents a much greater challenge. Nothing is known at this time about the saturation kinetics of drug incorporation into lipoproteins or partially delipidated lipoproteins. Nor is there any assurance that drug-laden lipoproteins will participate in receptor-mediated uptake processes similar to native lipoproteins. Moreover, receptor-mediated uptake of lipoproteins is a saturable process and may not permit attainment of sufficient drug concentrations within cells. It could be argued, however, that receptor-mediated uptake could be enhanced by prior treatment with hypocholesterolemic drugs as has been shown for cholestyramine.[57,58]

In any event, the possible use of lipoproteins for the

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site-specific delivery of intravenously administered radiodiagnostics or highly potent drugs (e.g., anticancer agents) appears promising. Only the results of ongoing studies will determine the practicality of this approach.

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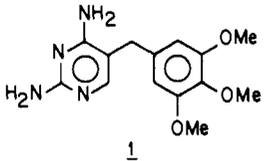
Communications to the Editor

Receptor-Based Design of Dihydrofolate Reductase Inhibitors: Comparison of Crystallographically Determined Enzyme Binding with Enzyme Affinity in a Series of Carboxy-Substituted Trimethoprim Analogues

Sir:

The biochemical basis for the chemotherapeutic effect of trimethoprim (TMP, **1**), a widely used antibacterial



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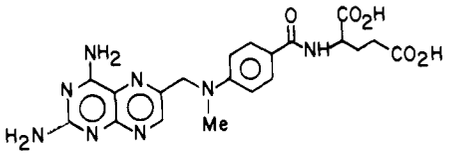

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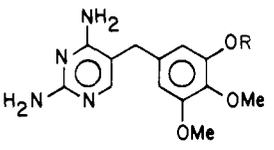
agent,[1] is the specific potent inhibition of dihydrofolate reductase (DHFR) in a broad spectrum of bacteria.[2] Determination of the three-dimensional molecular structure of DHFR, as defined by X-ray crystallography, led to the challenge of using this information to design analogues of TMP. X-ray studies have been reported by Matthews et al. on the binary complex of *Escherichia coli* DHFR and methotrexate (MTX, **2**),[3] the ternary complex of *Lactobacillus casei* DHFR, NADPH, and MTX,[4] and chicken liver DHFR in ternary complex with NADPH and a series of inhibitors including TMP.[5] In addition, one of our laboratories recently reported the crystal structures of *E. coli* DHFR in binary complex with TMP[6] and two closely related analogues.[7]

We report here the design, synthesis, DHFR affinity, and X-ray crystallographic binding analysis of a series of 3′-carboxyalkoxy analogues of TMP. At the time this work began only the unrefined structure of *E. coli* DHFR–MTX was available; the design of these compounds was therefore based solely on that structure. The goal of this design effort was not only to provide TMP analogues with higher affinity for *E. coli* DHFR but also to gain information on

Table I. Affinity Constants from TMP and Compounds **3–13** for *E. coli* DHFR



compd	R	rel binary K_D[a]	$K_i \times 10^9$,[b] M
1 (TMP)	CH_3	1.0	1.3
3	CH_2CO_2H	1.2	2.6
4	$(CH_2)_2CO_2H$	0.29	0.37
5	$(CH_2)_3CO_2H$	0.15	0.035
6	$(CH_2)_4CO_2H$	0.13	0.066
7	$(CH_2)_5CO_2H$	0.063	0.024
8	$(CH_2)_6CO_2H$	0.13	0.050
9	$CH_2CO_2CH_3$		11.0
10	$(CH_2)_3CO_2CH_2CH_3$		0.47
11	$(CH_2)_4CO_2CH_3$		0.76
12	$(CH_2)_5CO_2CH_3$		0.86
13	$(CH_2)_6CO_2CH_3$		1.9

[a] Relative K_D values are derived from competition experiments with MTX by spectrophotometric analysis and are normalized to the value of TMP such that a value less than one indicates higher affinity than that of TMP. Values of multiple determinations agreed within ±20%. This constant measures dissociation of inhibitor from the enzyme-inhibitor binary complex. [b] K_i values for compounds **1** and **3–8** were determined with the Henderson analysis (Henderson, P. J. F. *Biochem. J.* **1973**, *135*, 101). For the weaker binding compounds **9–13**, K_i values were calculated with Cha's equation for competitive inhibitors (Cha, S. *Biochem. Pharmacol.* **1975**, *24*, 2177). For each method, values of multiple determinations agreed within ±15%. Equivalent K_i values were obtained from these two methods for TMP and several closely related analogues. This constant effectively measures dissociation of the inhibitor from the enzyme-NADPH-inhibitor ternary complex.

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