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Perspective

S-Adenosyl-L-methionine-Dependent Macromolecule Methyltransferases: Potential Targets for the Design of Chemotherapeutic Agents

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Since *S*-adenosylmethionine (AdoMet)[1b] (Figure 1) was discovered in 1952 by Cantoni,[2] it has been shown to be one of nature's most versatile molecules. AdoMet serves directly as the methyl donor for numerous methyltransferases and as a precursor to decarboxylated AdoMet.[3,4] Decarboxylated AdoMet is the aminopropyl donor for the aminopropyltransferases, which are involved in the biosynthesis of the polyamines spermidine and spermine.[5,6] AdoMet has also been shown to be a 3-amino-3-carboxypropyl donor in tRNA modification[7] and as an adenosyl donor in the modification of the enzyme pyruvate formate-lyase[8] (Figure 1).

Of these group transfer reactions, the one that has received the greatest attention is that of methyl transfer.[3,4] In the intervening 28 years since Cantoni's original discovery,[2] many AdoMet-dependent methyltransferases have been identified. Since many of these methylations are involved in crucial biochemical processes, the enzymes involved have attracted the attention of medicinal chemists as potential targets for the design of chemotherapeutic agents. To date, most of the methyltransferases studied

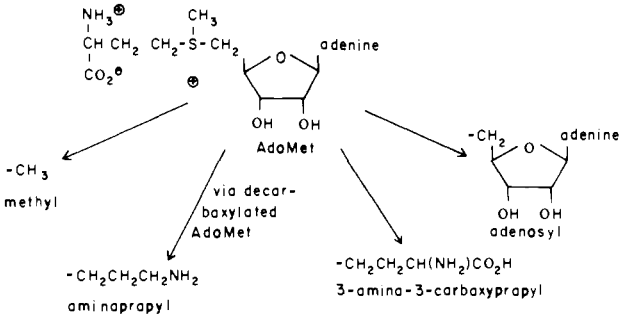

Figure 1. Group transfer potential of *S*-adenosylmethionine.

by medicinal chemists have been those that catalyze the methylation of "small molecules", where either the substrate or the product of the reaction has a well-defined physiological function. Examples would include catechol *O*-methyltransferase, which catalyzes the transfer of a methyl group from AdoMet to a catechol substrate and represents the primary route of extraneuronal inactivation of endogenous catecholamines;[9,10] histamine *N*-methyltransferase, which catalyzes the methyl transfer to histamine and represents the major histamine catabolism pathway in mammals;[10,11] and phenylethanolamine *N*-methyltransferase, which catalyzes the methylation of norepinephrine to epinephrine and represents the biosynthetic pathway to this important hormone–neurotransmitter.[12] The above list is not intended to be all-comprehensive. Numerous other small-molecule methyltransferases are known and have been studied by medicinal chemists.[3,4]

A second group of AdoMet-dependent methyltransferases are those that catalyze the methylation of above would also suggest that protein carboxymethylation

(1) (a) The author's work in this area has been supported by Research Grants from the National Institute of Neurological and Communicative Disorders and Stroke. (b) Abbreviations used are: adenosyltransferase, ATP:L-methionine adenosyltransferase (EC 2.5.1.6); AdoHcy, *S*-adenosyl-L-homocysteine; AdoHcy hydrolase, *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1); AdoHcy nucleosidase, *S*-adenosylhomocysteine nucleosidase (EC 3.2.2.9); AdoMet, *S*-adenosyl-L-methionine; 3-deaza-SIBA, 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine; Gm, 2′-*O*-methylguanosine; hnRNA, heterogeneous nuclear RNA; MAP, methyl acceptor protein; m^7G, 7-methylguanosine; m^6A, 6-methyladenosine; Nm, 2′-*O*-methylnucleoside; PCM, protein carboxy-*O*-methyltransferase (EC 2.1.1.24); SIBA, 5′-deoxy-5′-(isobutylthio)adenosine.

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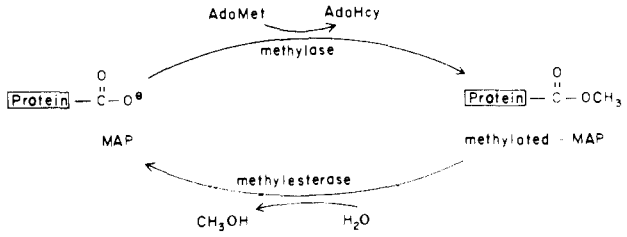

Figure 2. Protein carboxymethylation and demethylation. MAP = methyl acceptor protein.

macromolecules such as the proteins, nucleic acids, and lipids.[3,4] Until recently, it was impossible to assign exact physiological functions to many of these macromolecule methylations. However, it is now readily apparent that AdoMet-dependent methylations play a role in post-translational modification of proteins, in posttranscriptional modification of nucleic acids, and in regulating membrane function through modification of membrane phospholipids. AdoMet-dependent methylations are rapidly emerging as a universal mechanism for regulating the activity of macromolecules in both procaryotes and eucaryotes. In this capacity, methylation would appear to be analogous to phosphorylations, except much broader in scope.

In order to familiarize medicinal chemists with this important and rapidly developing area, I have in this article reviewed three specific, well-defined types of macromolecule methylations. These include mRNA methylation, protein carboxymethylation, and phospholipid methylation. These methylations are well characterized and have been shown to have dramatic effects on important biological processes such as viral replication (mRNA methylation), bacterial and leucocyte chemotaxis (phospholipid and protein carboxymethylations), ligand–receptor or receptor–adenylate cyclase interactions (phospholipid methylations), and neurosecretory processes (protein carboxymethylation). All of these physiological processes are of obvious interest to medicinal chemists. Because some of these macromolecular methylations might be attractive target sites for drug design, I have also reviewed the approaches which are currently being used to develop inhibitors for these macromolecule methyltransferases.

AdoMet-Dependent Macromolecule Methyltransferases

Protein Carboxymethylation. Posttranslational methylation of proteins is a biochemical reaction common in both eucaryotes and procaryotes.[3,4,13] Lysyl, arginyl, histidyl, aspartyl, and glutamyl residues on proteins have been shown to undergo methylation by enzymes that use AdoMet as a methyl donor. Perhaps of most interest to medicinal chemists would be protein carboxyl-*O*-methyltransferase (PCM, EC 2.1.1.24), which catalyzes the transfer of a methyl group from AdoMet to aspartyl or glutamyl residues on proteins, yielding protein carboxyl methyl esters (Figure 2). This enzyme is also referred to in the literature as protein methylase II.

In 1965, Axelrod and Daly[14] reported the presence in bovine posterior pituitary of an enzyme activity which catalyzed the apparent transfer of a methyl group from AdoMet to water with the formation of methanol. In 1973, Morin and Liss[15] and Kim[16] independently established that this methanol-forming enzyme described by Axelrod and Daly[14] was actually PCM. Subsequently, PCM has been shown to be present in many tissues, including brain, testes, thymus, spleen, kidney, heart, muscle, lung, liver, and erythrocytes.[17,18] The highest PCM activity is found in brain, whereas the pituitary contains the highest level of endogenous substrates for PCM. These endogenous substrates are referred to as methyl acceptor proteins (MAPs). PCM is primarily cytoplasmic and it has been purified from various mammalian tissues including calf thymus,[19,20] calf brain,[21] rat erythrocyte,[18] bovine pituitary,[22] and ox brain.[23] A similar enzyme has also been identified in bacteria.[24-26] The substrate specificity of mammalian PCMs are quite broad, which is in sharp contrast to the bacterial enzyme (*S. typhimurium*) that methylates only 65 000-dalton proteins, which are involved in the chemotaxic response mechanism.[27]

An important feature of the protein carboxymethylation is the apparent reversible nature of this protein modification (Figure 2). This reversibility is in sharp contrast to the N-methylation of lysyl, arginyl, or histidyl residues on proteins.[13] Being a readily reversible process, the protein carboxymethylation can be considered as an "on–off" mechanism for regulating a protein's activity. This type of "on–off" mechanism mediated by protein carboxymethylation has been shown to be a crucial component of bacterial chemotaxis.[28] The reversibility in the bacterial system is provided by a specific methylesterase[27] which hydrolyzes methylated MAP to regenerate MAP. One interesting and perhaps important difference between the methylated MAPs from bacterial vs. mammalian systems is that the mammalian methylated MAPs more rapidly undergoes nonenzymatic hydrolysis of the methyl ester. To date, a mammalian methylesterase has not been identified, but it seems reasonable to predict the existence of such an enzyme. Therefore, protein methylation and demethylation catalyzed by PCM and methylesterase, respectively, as shown in Figure 2, may be a general "on–off" biological mechanism which is analogous to protein phosphorylation and dephosphorylation catalyzed by protein kinase and phosphatase, respectively.

The most clearly defined example of protein carboxymethylation serving as an "on–off" mechanism is found in the mechanism of bacterial chemotaxis.[27,28] Bacterial chemotaxis can be viewed as a simple sensor–response system that consists of three components: receptors which detect attractant or repellent chemicals in the cell's environment; a signal transduction system which translates the information from the chemoreceptors; and finally the response apparatus which are basically the flagella. The receptors which exist on the cell periphery have been isolated and characterized.[29] These receptors stereo-

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specifically bind the chemoeffector molecules and produce a conformational change in the protein, which is in turn transmitted to the signal transduction system. The transduction system consists in part of methyl-accepting chemotactic proteins, which are methylated by PCM, in direct response to the interaction of a chemoattractant with the receptors. Chemorepellents have the opposite effect, producing a decrease in steady-state levels of methylated methyl-accepting chemotaxic proteins through the increased activity of the methylesterase.[25] In turn, these changes in the methylation control the direction of flagellar rotation,[30-33] which alters tumbling frequency,[33,34] and thus directs the cell's migration.[35] Through the use of *E. coli* mutants, three types of methyl-accepting chemotactic proteins have been identified and shown to be the signal transduction system for the chemoeffector receptors.[36-38] The PCM responsible for the methylation of the methyl-accepting chemotactic proteins in *S. typhimurium* has been partially purified,[24] as has the corresponding methylesterase.[27,29] The bacterial PCM uses AdoMet as a methyl donor and it shows strict substrate specificity, methylating only the 65 000-dalton membrane proteins.[39] Therefore, strong evidence exists to indicate that protein carboxymethylation catalyzed by an AdoMet-dependent PCM plays a crucial role in the chemoeffector response of bacteria.

In the case of eucaryotes, the physiological role of protein carboxymethylation is less well defined. However, it seems likely that this methylation may play a variety of important roles in different tissues. I have attempted to summarize in the following paragraphs the current information available on protein carboxymethylation in eucaryotic systems.

One type of eucaryotic behavior which shows obvious similarities to bacterial chemotaxis is that of leucocyte chemotaxis. Several groups have examined the possibility that protein carboxymethylation might play a role in leucocyte chemotaxis.[40-43] O'Dea et al.[40] have shown that the addition of a chemoattractant to leucocytes causes an increase in the level of carboxymethylation[40] and that both the behavioral response and the increase in carboxymethylation can be prevented by the prior addition of an attractant antagonist.[43] Pike et al.[41] and Chiang et al.[42] showed that exposure of leucocytes to compounds that inhibit the carboxymethylation reaction produces a simultaneous block in the chemotactic behavioral response, providing indirect evidence for a role of methylation in leucocyte chemotaxis. It would appear, however, that further studies are needed before a firm role for protein carboxymethylation in leucocyte chemotaxis is established.

An extremely interesting observation is that spermatids have a very high level of PCM and MAPs.[44,45] These results suggest that protein carboxymethylation may be involved in sperm motility in analogy with bacterial and leucocyte chemotaxis.[45]

Both PCM and MAPs have also been found in erythrocytes.[18,46-48] Erythrocyte PCM is primarily cytoplasmic,[46] similar to the leucocyte enzyme.[47] In contrast, erythrocyte MAPs are associated almost exclusively with the red cell membranes.[46,48] An exact function for erythrocyte protein carboxymethylation has not yet been identified.

Protein carboxymethylation has been proposed to play a crucial role in the mechanism of neurosecretion.[49] The evidence in support of this hypothesis includes the observation that neural and endocrine tissues in general have very high levels of PCM and MAPs.[21,50,51] Secretory tissues that have protein carboxymethylation capability include the adrenal gland, which synthesizes and releases epinephrine,[49,52] the parotid gland, which synthesizes and releases amylase,[53] and the pituitary gland, which synthesizes and releases various peptide hormones.[54]

In the adrenal medulla, PCM was found to be primarily cytoplasmic,[49,52] whereas the MAPs were associated with both the membranes[49,52,55] and the soluble content[55] of the chromaffin vesicles. The observation that protein carboxymethylation causes an apparent release of membrane protein from the chromaffin granule fraction,[49,55] as well as the neutralization of negative charges on proteins following carboxymethylation, led Diliberto et al.[49] to propose that PCM may mediate stimulus–secretion coupling in the medulla by decreasing the electrostatic repulsion between granule and plasma membranes and exposing granule membrane lipids by removal of surface proteins, thus facilitating membrane–membrane fusion and exocytosis. Further evidence for this hypothesis has come from the observation that splanchnic nerve stimulation during [^{3}H]methionine perfusion of the isolated adrenal gland results in an increase in protein carboxymethylation.[43,56]

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been partially identified as two proteins of apparent molecular weight 32000 and 55000.[57] The MAP of the soluble contents of the granule was shown to be chromogranin A,[55] which is the main constituent of the granule vesicles and involved in the sequestration of the hormone epinephrine. It is of interest that MAPs in the posterior pituitary[58] and platelets[47] are also proteins known to function in the sequestration of secretory material in storage granules.

Another secretory tissue that shows high protein carboxymethylation capacity is the parotid gland. Strittmatter and co-workers[53] have described a rapid and reversible stimulation of PCM and an increase in MAP capacity of the parotid gland both in vitro and in vivo following stimulation by the β-adrenergic agonist isoproterenol. This effect corresponds rather closely to the time course of amylase secretion in this tissue and is β-receptor specific. The increase in PCM activity and MAP capacity observed in this system is independent of protein synthesis, and the increase in MAP capacity is due to an increase in the methylation of only two parotid proteins.[53]

The posterior pituitary has also been shown to have a high level of PCM and MAPs. Diliberto and Axelrod[54] were able to identify the anterior pituitary hormones LH, FSH, ACTH, GH, TSH, and prolactin as good substrates for PCM, whereas the posterior pituitary hormones oxytocin and vasopressin were poor substrates for PCM. Kim et al.[59] found PCM activity in whole homogenates of the posterior and anterior lobes to be essentially saturated with respect to endogenous substrates. Much of the endogenous substrate(s) in the pituitary has been shown to be associated with the particulate fraction, implying a localization of MAPs in neurosecretory storage granules.[58,59] Furthermore, the MAPs of these fractions have molecular weights less than 25000, suggesting that MAPs of the pituitary gland are, in fact, polypeptide hormones and neurophysins. Edgar and Hope[60] have demonstrated that posterior pituitary neurophysin, in contrast to the peptide hormones vasopressin and oxytoxin, is an excellent substrate for PCM, and Gagnon, Axelrod, and Brownstein[61] found by gel electrophoretic analysis that over 80% of neurohypophysial MAP appears to be neurophysin. After stimulation of the hypothalamo–neurohypophysial axis via salt loading in the rat, a gradual decrease in neurohypophysial MAP was observed, consistent with glandular depletion of secretory products.

Kim, Pearson, and Paik[59] have noted increases in endogenous PCM levels of the hypothalamo–neurohypophysial complex over several days in culture which closely parallel the increase in newly synthesized neurophysin in this system, suggesting that neurophysin is indeed a substrate for PCM in the intact hypothalamo–neurohypophysial axis.

Thus, secretory peptides and carrier proteins of the pituitary are substrates for PCM. It has been suggested that protein carboxymethylation may function in the inactivation of anterior pituitary hormones both in the pituitary[54] and at their target organs.[62] The evidence cited

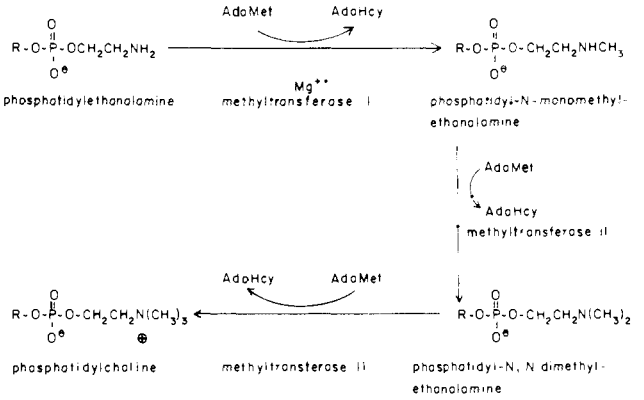

Figure 3. AdoMet-dependent biosynthesis of phosphatidylcholine. R = α,β-diacyl glycerate.

is involved in packaging and/or secretion in the neurohypophysis in some as yet undiscovered way.

Protein carboxymethylation activity has also been observed in every brain region examined, with the highest subcellular activity being associated with the synaptosomal fractions.[51] PCM has been extensively purified from calf brain;[21] however, no attempts have been made to identify brain MAPs. A crude synaptosomal fraction from rat hypothalamus has been used by Eiden et al.[63] to explore the possible role of protein carboxymethylation in neurosecretion. This crude synaptosome fraction was shown to activate methionine to AdoMet and to form protein methyl esters, indicating the presence of the AdoMet-synthesizing enzyme (ATP:L-methionine *S*-adenosyltransferase; EC 2.5.1.6), endogenous PCM, and endogenous MAPs. When the synaptosomes were exposed to conditions which produce increased secretory activity (e.g., high potassium or veratridine), a decrease in methylated MAP formation was observed. The effects of potassium on protein carboxymethylation was shown to be calcium dependent and the effects of veratridine could be blocked by tetrodotoxin, indicating a relationship between this protein methylation and exocytotic release of neurotransmitters.[63] However, inhibition of this synaptosomal PCM activity had no effect on either basal catecholamine release or that evoked by potassium stimulation.[64] These results suggest that protein carboxymethylation is not an obligate step in hypothalamic neurosection. The relationship between protein carboxymethylation and neurosecretion in neural tissue, therefore, has yet to be established.

Phospholipid Methylation. Phospholipids are a major component of biomembranes and provide the fluid matrix for movement of other membrane components (e.g., protein). Phosphatidylcholine (lecithin) is one of the major phospholipids found in mammalian membranes.[65] Phosphatidylcholine is biosynthesized by two pathways: CDP–choline incorporation into α,β-diacylglycerate or the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine using two AdoMet-dependent methyltransferases.[66–68] Since the elucidation of this Ado-

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Met-dependent pathway in 1978 by Hirata et al.,[66,67] many profound effects on membrane structure and function have been observed as a result of changing the activity of these phospholipid methyltransferases. In the following paragraphs, I have attempted to summarize the most recent advances in this area.

The AdoMet-dependent methylation of amino groups of phospholipids to form phosphatidylcholine was first reported by Bremer and Greenberg[68] in 1961. In 1978, Hirata et al. identified two methyltransferases which were involved in converting phosphatidylethanolamine to phosphatidylcholine in bovine adrenal[66] and rat erythrocytes.[67] Subsequently, these enzymes have also been identified in rat brain synaptosomes,[69] rat reticulocytes,[70] and rabbit leucocytes.[71] The first enzyme, methyltransferase I, which was shown to catalyze the methylation of phosphatidylethanolamine to phosphatidyl-*N*-methylethanolamine, has a high affinity for AdoMet and requires Mg^{2+} (Figure 3). Methyltransferase II, which was shown to catalyze the transfer of two methyl groups to phosphatidyl-*N*-methylethanolamine to form phosphatidylcholine via phosphatidyl-*N*,*N*-dimethylethanolamine, has a low affinity for AdoMet and does not require Mg^{2+} (Figure 3).[66,67] These enzymes, like phospholipids,[72,73] are asymmetrically distributed in the biological membrane. Methyltransferase I and its substrate phosphatidylethanolamine are localized on the cytoplasmic surface of the membrane, whereas methyltransferase II and its product phosphatidylcholine are found on the outer surface.[66,67] Phosphatidyl-*N*-methylethanolamine, the product of the first enzyme and the substrate for the second enzyme, appears to be neither on the inside nor on the outside of the membrane but is embedded in the membrane. The phospholipid asymmetry may, therefore, be maintained in the membrane as a result of their pathway of biosynthesis and the methyltransferase asymmetry.

The degree of membrane phospholipid methylation apparently has dramatic effects on the membrane's physical–chemical properties (e.g., fluidity and charge). Hirata and Axelrod[74] showed that incubating erythrocyte ghosts with AdoMet caused a marked decrease in membrane viscosity. These viscosity changes could be abolished when AdoHcy, a methyltransferase inhibitor, was included in the incubation mixture. The erythrocyte fluidity changes were shown to be mainly the result of the intramembrane synthesis of monomethylated phospholipids, via the action of methyltransferase I (Figure 3).

The degree of membrane phospholipid methylation has also been shown to be affected by and to affect the β-adrenergic receptor in Hela cells,[75] in rat reticulocytes,[70,76-78] and in C_6astrocytoma cells.[79] For example, with rat reticulocytes stimulation of the β-adrenergic receptors increases the methylation of both phospholipid substrates and also enhances the rate of their translocation.[70] It appears that binding of the β-adrenergic receptor agonist, but not activation of adenylate cyclase, increases membrane phospholipid methylation.[70] Increases in phospholipid methylation in reticulocytes were shown to enhance the coupling between the β-adrenergic receptor and adenylate cyclase[78] and to increase the number of β-adrenergic binding sites.[77] In both rat reticulocytes[76,77] and Hela cells[75] inhibition of phospholipid methylation resulted in a marked reduction in β-adrenergic binding sites as measured by [^{3}H]dihydroalprenolol binding. Using C_6astrocytoma cells, Strittmatter et al.[79] have shown that stimulation of a benzodiazepine receptor with antianxiety benzodiazepines and stimulation of the β-adrenergic receptor with β-agonists increase phospholipid methylation. Furthermore, simultaneous addition of benzodiazepine and β-adrenergic agonists increases methylation in an additive manner. Therefore, it would appear as if phospholipid methylation is emerging as a crucial mechanism for regulating ligand–receptor interaction and receptor–adenylate cyclase interactions. The potential implications of these findings on our understanding of the mechanism of hormone–receptor interactions are profound.

Phospholipid methylation has also been shown to affect the activity of another membrane-bound enzyme, that being Ca^{2+}-ATPase. Increases in phospholipid methylation in human erythrocytes causes an increase in Ca^{2+}-ATPase, which was attributed to the changes in membrane fluidity.[80]

Phospholipid methylation,[71] like protein carboxymethylation,[40-43] has been shown to play a crucial role in leucocyte chemotaxis. Hirata et al.[71] observed that the treatment of rabbit peritoneal leucocytes with a chemoattractant such as fMet-Leu-Phe resulted in an increased degradation of phosphatidylcholine. Only phosphatidylcholine synthesized by the AdoMet-dependent pathway was affected by these chemoattractants. The degradation of the phosphatidylcholine was accompanied by the release of arachidonic acid, suggesting that the chemoattractant activated a phospholipase A_2, which removes an unsaturated fatty acid from phospholipids. Chemotaxis of leucocytes is one manifestation of inflammatory reactions. The release of arachidonic acid from phospholipids is potentially important because this fatty acid is a precursor of prostaglandins and prostacyclines, which play a critical role in inflammation. These results suggest a close association between the metabolism of methylated phospholipids and chemotaxis is leucocytes.

Recent data have also suggested that the synthesis and degradation of phosphatidylcholine are an intrinsic part of the biochemical mechanism modulating histamine release from mast cells.[81] Phospholipid methylation has also been suggested as a signal modulator for lymphocyte mitogenesis.[82]

RNA Methylation. Posttranscriptional modification of ribonucleic acids (RNAs) by AdoMet-dependent methyltransferases is a process common to both procaryotic and eucaryotic organisms. tRNAs are the most highly

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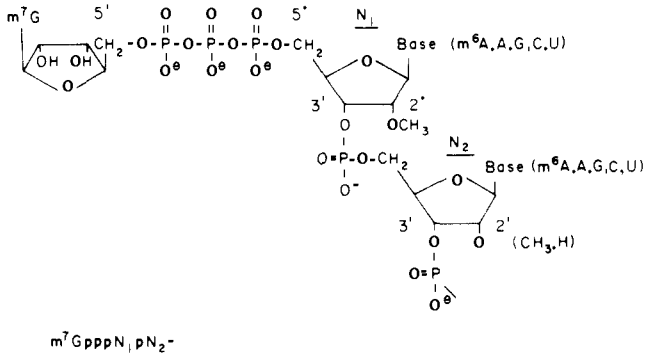

Figure 4. 5′-Methylated and capped structure of eucaryotic mRNA.

modified type of RNA, containing over 30 different methylated nucleosides.[83,84] The methylated nucleosides of tRNA appear to play an important role in protein synthesis at the level of the codon–anticodon recognition process.[85,86] rRNAs are also quite heavily methylated. Methylation of eucaryotic rRNA occurs on nucleolar precursor rRNA, and the methylated regions of the precursor rRNA are conserved during processing to the mature rRNA. The methylation appears to be necessary for processing to the mature rRNA.[87] Of the RNA methylations, the most well-defined and the best understood from a function standpoint is that of mRNA methylation. Eucaryotic mRNA, but not procaryotic mRNA, has been shown to have a methylated and blocked 5′-terminal structure which is important for the ribosomal binding of the message and its eventual translation to proteins. mRNAs from a variety of eucaryotic cells and viruses have been found to contain a 5′-methylated and capped structure of the general type shown in Figure 4.[4,88] Considering the well-defined nature of the viral mRNA methyltransferases and their attractiveness as targets for the design of potential chemotherapeutic agents, a brief review of the pertinent literature follows.

The presence of a viral mRNA methyltransferase was first reported by Furuichi[89] in 1974. Since his original observation using silkworm cytoplasmic polyhedrosis virus, other AdoMet-dependent mRNA methyltransferases were reported in reovirus,[90] vaccinia virus,[91] vesicular stomatitis virus,[92] and Newcastle disease virus.[93] In 1975, Furuichi and Miura[94] elucidated the methylated 5′-terminal structure for polyhedrosis mRNA to be $m^7G(5')ppp(5')Am-$. Subsequently, similar structures have been reported for other cytoplasmic virus mRNAs.[90-98] The structural features common to the cytoplasmic mRNA capped structures include the 7-methylguanosine (m^7G) and a penultimate purine ribonucleotide in general base and sugar methylated. Thus, the cytoplasmic viruses produce either a cap 0, m^7GppN, or cap 1, $m^7GpppNm$, structure in vitro. Methylation of nucleosides internal to the penultimate position does not occur in vitro but in vivo. This position can be methylated by host methyltransferases.

Extensive work has been done on the viral mRNA synthesizing and modifying enzymes. As an example, vaccinia virus, which is a very large double-stranded DNA poxvirus,[95-98] synthesizes and modifies (methylated and capped) mRNA using core-associated enzymes as outlined in Figure 5. These viral-associated enzymes include a DNA-dependent RNA polymerase (step 1), guanylyltransferase (step 2), AdoMet (guanine-7-)-methyltransferase (step 3), and AdoMet (nucleoside-2′-)-methyltransferase.[98] The guanine 7-methyltransferase[99-101] and (nucleoside-2′-)-methyltransferase[102] have been purified from vaccinia virus, and both enzymes require AdoMet as a methyl donor. The order of methylation involves first the guanine 7-methyltransferase, followed by nucleoside 2′-methyltransferase. The guanylyltransferase, guanine 7-methyltransferase, and (nucleoside-2′-)methyltransferases have been detected in the cytoplasm of vaccinia infected cells.[103] This and other data indicate that the guanylyltransferase and mRNA methyltransferases are probably early (prereplicative) viral gene products.

Mammalian cells also have the capacity to produce 5′-capped and methylated heterogeneous nuclear RNA (hnRNA) and mRNA.[104] The results of pulse–chase experiments on mouse L cells indicate that the majority of methylated cap structures in hnRNA are conserved in the processing to mRNA.[105] It is significant that hnRNA contains only cap 1, $m^7GpppNm$, and not cap 2, $m^7GpppNmpNm$, while mRNA contains both caps 1 and 2.[106] More detailed studies by Perry and Kelley[105] and Friderici et al.[107] indicated that cap 2 structures of mRNA result from cytoplasmic methylation of a select class of cap 1 terminated mRNA. Both hnRNA and mRNA of mammalian cells contain internal 6-methyladenine (m^6A) residues. Recent results indicate that both caps and internal m^6A are conserved in the processing of hnRNA and are transported to the cytoplasm where m^6A's are rapidly removed by a m^6A demethylase.[108] Some of the mRNA

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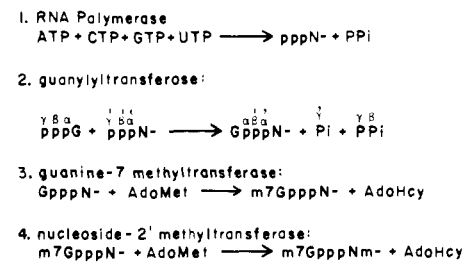

Figure 5. Vaccinia virus core-associated enzymes.

methyltransferases from mammalian cells have been isolated and studied.[109,110]

Relative to the function of the methylated capped structure of mRNA, substantial evidence suggests that methylation facilitates the initiation of protein synthesis.[111,112] This was first demonstrated with reovirus and vesicular stomatitis viral mRNAs when it was shown that these mRNAs required a 5'-terminal m^7G for efficient translation in a wheat germ cell-free protein-synthesizing system.[113] mRNA from other eucaryotic sources also require a methylated cap for maximal translation in vitro, for example, globin mRNA[114] and vaccinia mRNA.[115,116] The importance of caps in vivo was suggested from the observation that in vesicular stomatitis virus-infected cells, the polysomal mRNA was capped and methylated, while the portion of the mRNA that was not methylated remained unassociated with ribosomes.[117] Ribosomal selection of capped and methylated over unmethylated reovirus mRNA was also observed in vitro.[116]

The m^7G in the cap functions at an early stage in initiation. Reovirus mRNA with the 5' termini $m^7GpppGm$–, pppGm–, ppG–, and GpppG– were evaluated for their ability to bind to 40S initiation complexes.[118] The m^7G-capped mRNA was the only mRNA that bound efficiently to the 40S initiation complex. The ribosome binding ability of 7-ethylguanosine-capped mRNA has also been quantitated and was found to form a stable complex with the ribosomes.[112] The translational activities of ethylated or methylated capped mRNAs were computed and it was found that the activities were similar.[112] This and other results imply that a positive charge on the terminal guanosine is required for efficient ribosome binding rather than a specific requirement for a methyl group. The positively charged 7-alkylguanosine probably interacts with the triphosphate bridge, giving a rigid structure which may be related to the methylated cap's ability to facilitate ribosome binding.

The preceding discussion on mRNA methylation was not intended to be all comprehensive but rather to simply communicate the apparent importance of this type of methylation to the efficiency of translation of eucaryotic cellular and viral mRNA. Agents which inhibit this methylation, therefore, might have potential as antiviral or antineoplastic agents (see following discussion).

Inhibitors of AdoMet-Dependent Methyltransferases

A general reaction scheme depicting the role of AdoMet in methylation reactions is given in Figure 6. Each of the AdoMet-dependent methyltransferases in this category has its own requirements for a methyl acceptor.[3,4] These requirements are in general very specific, so that a particular methyltransferase will utilize only a single methyl acceptor substrate or a limited number of structurally related molecules. All of the methyltransferases in this category have a strict requirement for AdoMet as the methyl donor. AdoMet is biosynthesized from ATP and methionine through the reaction catalyzed by ATP:L-methionine S-adenosyltransferase (EC 2.5.1.6, adenosyltransferase).[119]

Besides the common requirement for AdoMet, each of these methyltransferases also exhibit a sensitivity to inhibition by AdoHcy, one of the products of the transmethylation reaction.[120] The product inhibition by AdoHcy and the enzymes involved in the metabolism of AdoHcy are part of a biological regulatory mechanism for AdoMet-dependent transmethylations.[121] The two major pathways for the enzymatic metabolism of AdoHcy[122-124] are depicted in Figure 6. The enzyme AdoHcy hydrolase (EC 3.3.1.1), which is present in yeasts, plants, birds, and mammals, catalyzes the reversible hydrolysis of AdoHcy to adenosine and L-homocysteine.[122,124-127] AdoHcy hydrolase has been shown by Palmer and Abeles[128,129] and Richards et al.[130] to be a NAD-dependent enzyme, which catalyzes both the synthesis and degradation of AdoHcy by an oxidation–reduction mechanism.

Recently, the importance of this enzyme in the metabolism of AdoHcy has been verified in vivo using rat and mouse brain, kidney, and liver homogenates[130-132] and neuroblastoma cells.[133] These studies have shown that the major metabolic pathway for AdoHcy in these mammalian tissues is the cleavage of the homocysteine–ribose bond (via AdoHcy hydrolase) with subsequent metabolism of adenosine to nucleotides or oxypurines.

The second major pathway for AdoHcy metabolism is via the action of AdoHcy nucleosidase (EC 3.2.2.9), which to date has only been detected in bacteria.[123,124] AdoHcy nucleosidase catalyzes the cleavage of the glycosyl linkage of AdoHcy to yield adenine and 5'-ribosylhomocysteine.

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Based on this basic biochemical information, one might conceive of several approaches to designing inhibitors of AdoMet-dependent methyltransferases. These approaches might include inhibitors that function directly on a particular methyltransferase (e.g., analogues of either the methyl acceptor substrate, the methylated product, AdoHcy or AdoMet) or inhibitors that function indirectly by initially inhibiting AdoMet biosynthesis (e.g., adenosyltransferase) or AdoHcy metabolism (e.g., AdoHcy hydrolase, AdoHcy nucleosidase).

The approach of using analogues of the methyl acceptor substrate or the methylated products has been extensively exploited in the design of inhibitors of "small molecule" methyltransferases.[3,4] This approach has not been used for the design of macromolecule methylase inhibitors. The reasons probably include the difficulty in synthesis of such compounds and the potential problems with their transport and metabolism. However, in reality, little is known about the active sites or substrate specificity of macromolecule methyltransferases. It is conceivable, therefore, that the enzymes bind only a small segment of the natural substrate. If that were the case, it should then be possible to synthesize inhibitors containing only those segments that are involved directly in enzymatic binding.

One approach that has been explored in an effort to inhibit AdoMet-dependent methyltransferases[134,135] is that of altering the activity of adenosyltransferase. The result of inhibiting this enzyme would be to decrease intracellular levels of AdoMet, thereby inhibiting all AdoMet-dependent enzymes. Numerous analogues of L-methionine have been prepared and evaluated as inhibitors of adenosyltransferase.[134-140] Administration of some of the most potent adenosyltransferase inhibitors to rodents in vivo resulted in the expected accumulation of L-methionine and depression in the levels of AdoMet in several tissues examined.[141] The apparent disadvantages of this approach include (1) the high structural specificity of adenosyltransferase for methionine, resulting in the poor inhibitory activity of methionine analogues, and (2) the fact that inhibition of this biosynthetic enzyme will result in a general inhibitory effect on all AdoMet-dependent methyltransferases, as well as polyamine biosynthesis.

Analogues of AdoMet have also been examined as possible inhibitors of AdoMet-dependent methyltransferases. The enzymatic preparation of such analogues has been somewhat hindered because of the high specificity of adenosyltransferase.[142] However, several analogues of AdoMet have been prepared enzymatically,[142,143] whereas many others have been prepared by chemical procedures.[144-146] In general, the analogues of AdoMet have exhibited poor substrate and/or inhibitor properties for methyltransferases.[144,145] These results indicated that the enzymes have a very high specificity for the structural features of AdoMet. This disadvantage, coupled with the unstable nature of AdoMet analogues and the problems associated with cellular transport of these compounds, has diminished interest in this approach to inhibiting methyltransferases.

Several recent observations, however, have suggested that a reexamination of AdoMet analogues as methylase inhibitors might be warranted. One particularly significant observation is the potent inhibitory activity of Sinefungin and A9145C (Figure 7) toward various methyltransferases.[147-152] Sinefungin and A9145C are antifungal antibiotics which were isolated from *streptomyces griseolus*[153] and are structurally similar to AdoMet.

In recent years, significant advances have been made in our understanding of the mechanism by which AdoMet donated its methyl group in enzyme-catalyzed reactions. Specifically, these advances include the elucidation of the absolute configuration of the sulfonium center of AdoMet,[154] the characterization of the mechanism of enzymatic methyl transfer from AdoMet,[155-159] the determination of the stereochemistry of enzymatic methyl transfer from AdoMet,[160] and the characterization of the chemical properties of AdoMet.[161] The availability of this information should now permit the rational design of transition-state type inhibitors of methyltransferases. Transition-state analogues would provide the desired specificity that is lacking in AdoMet analogues or in inhibitors of adenosyltransferase.

The AdoHcy regulatory mechanism (Figure 6) has attracted considerable attention as a potential target for the design of methyltransferase inhibitors. Two general approaches have been pursued: (1) the design of AdoHcy analogues that would selectively bind to specific methyltransferases or (2) the design of compounds that would alter AdoHcy metabolism, thereby raising the intracellular level of this natural methyltransferase inhibitor. The latter possibility suffers from the disadvantage that the end re-

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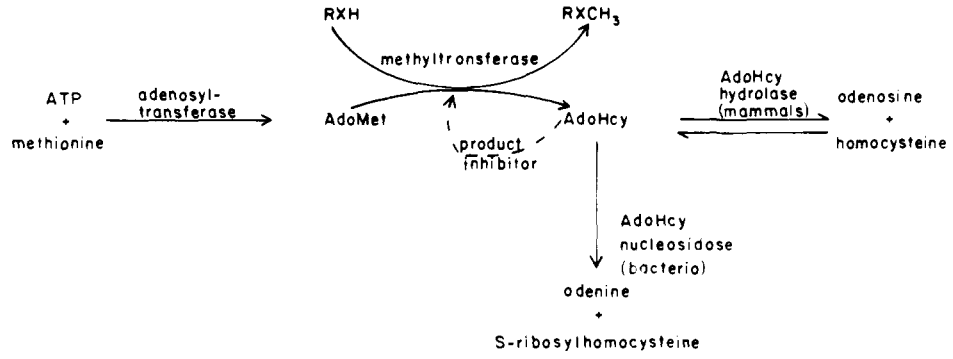
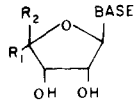


Figure 6. AdoMet-dependent methyltransferases and AdoHcy metabolism.



COMPOUND	BASE	R_1	R_2
AdoMet	adenine	H	$-CH_2\overset{+}{S}(CH_3)CH_2CH_2CH(NH_2)CO_2H$
AdoHcy	adenine	H	$-CH_2SCH_2CH_2CH(NH_2)CO_2H$
TubHcy	7-deazaadenine	H	$-CH_2SCH_2CH_2CH(NH_2)CO_2H$
Sinefungin	adenine	H	$-CH_2CH(NH_2)CH_2CH_2CH(NH_2)CO_2H$
A9145C	adenine	-	$=CHCH(NH_2)CH_2CH_2CH(NH_2)CO_2H$
3-Deazaadenosine	3-deazaadenine	H	$-CH_2OH$
SIBA	adenine	H	$-SCH_2CH(CH_3)_2$
3-Deaza SIBA	3-deazaadenine	H	$-SCH_2CH(CH_3)_2$

Figure 7. Inhibitors of AdoMet-dependent methylations.

sult would be inhibition of all AdoMet-dependent methyltransferases.

Several laboratories have reported the synthesis of base, amino acid, or sugar modified analogues of AdoHcy.[120,162-164] The general approach has been to systematically alter the structure of AdoHcy and to evaluate the derivatives as inhibitors of AdoMet-dependent methyltransferases. Hopefully, the AdoHcy binding sites are sufficiently different to permit the design of inhibitors specific for particular methyltransferases. Some interesting and encouraging results have arisen from these studies.

Analogues of AdoHcy have been evaluated in vitro and in vivo as inhibitors of various "small molecule" methyltransferases (e.g., catechol *O*-methyltransferase, phenylethanolamine *N*-methyltransferase, histamine *N*-methyltransferase, hydroxyindole *O*-methyltransferase, and indolethylamine *N*-methyltransferase)[120,144,149,162-165] and macromolecule methyltransferases (e.g., tRNA methylases, mRNA methylases, and protein methylases).[147,148,150-152,166-176] The structure–activity relationships for the binding of AdoHcy analogues to purified tRNA methylases,[166] viral mRNA (guanine-7-)- and (nucleoside-2′)-methyltransferases,[147,151,167,175] and protein carboxymethyltransferase[150,176] have been examined, and significant differences in their requirements for binding of AdoHcy have been detected. The AdoHcy analogues exhibiting the most interesting activity in vivo include Sinefungin,[147,148,151,152] 5′-deoxy-5′-(isobutylthio)adenosine (SIBA)[171-174] and *S*-tubercidinyl-L-homocysteine[167-170] (Figure 7). *S*-Tubercidinyl-L-homocysteine has been shown to inhibit RNA methylation in phytohemagglutin-stimulated rat lymphocytes and in Novikoff hepatoma cells.[168,169] Several analogues of AdoHcy have also shown inhibitory effects on vaccinia virus replication in L cells,[147,151] Rous sarcoma virus transformation of chick embryo fibroblasts,[148,171] polyoma virus plague formation in mouse embryo fibroblasts,[172] mouse sarcoma virus foci formation in mouse embryo fibroblasts,[152] mouse mammary tumor virus cloning efficiency in transformed tumor cells,[173] and *Herpes simplex* virus replication.[174] Some AdoHcy analogues have also shown antimitotic activity in mouse lymphocytes stimulated by various mitogens[177] and antiparasitic activity.[153,174] It should be noted, however, that there is limited evidence to attribute the antiviral, antimitotic, or antiparasitic activity of these compounds to direct inhibition of crucial AdoMet-dependent methyltransferases. The molecular mechanisms responsible for these interesting in vivo effects will be the subject of considerable research interest in the future.

Another approach to inhibiting AdoMet-dependent methyltransferase, which has attracted considerable attention and proved quite successful in vivo, is that of altering the metabolism of AdoHcy so as to raise its intracellular level. The metabolic pathway receiving the most attention is that catalyzed by AdoHcy hydrolase (Figure 6). AdoHcy hydrolase catalyzes the reversible hydrolysis of AdoHcy to adenosine and homocysteine via an oxidation–reduction mechanism.[128,129] The proposed mecha-

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nism[128,129] involves the oxidation of the 3′-hydroxyl group of AdoHcy by an enzyme-bound NAD. Following oxidation, L-homocysteine is eliminated to give 3′-keto-4′,5′-dehydroadenosine, which reacts with water in a Michael-type addition to form 3′-ketoadenosine, which is then reduced to adenosine. The mechanism of catalysis appears well suited for the possible design of K_{cat} inhibitors but, to our knowledge, no efforts have yet been made in this area.

The structure–activity relationship for AdoHcy binding to AdoHcy hydrolase has been extensively investigated by Chiang et al.[178] The enzyme shows very strict specificity for AdoHcy as a substrate, with 3-deazaadenosyl-L-homocysteine being the only analogue that showed significant substrate properties. Hydrolysis of this analogue yields 3-deazaadenosine, which was shown itself to be a very potent inhibitor of AdoHcy hydrolase.[178] As a result of these observations, 5′-deoxy-5′-(isobutylthio)-3-deazaadenosine (3-deaza-SIBA; Figure 7) was also synthesized and shown to inhibit AdoHcy hydrolase.[179]

Administration of 3-deazaadenosine causes an increase in AdoHcy and 3-deazaadenosyl-L-homocysteine levels in rat liver, whereas in spleen only an elevation of AdoHcy was observed.[180,181] These results confirmed similar data obtained in isolated hepatocytes.[179] Zimmerman et al.[182] and Chiang et al.[42] have shown that in leucocytes 3-deazaadenosine also causes an elevation of AdoHcy and 3-deazaadenosyl-L-homocysteine. These observations are extremely interesting because adenosine analogues such as 3-deazaadenosine could inhibit methylation by a mechanism involving inhibition of AdoHcy hydrolase, raising the intracellular concentration of AdoHcy, or by a mechanism in which it is converted to an AdoHcy analogue such as 3-deazaadenosyl-L-homocysteine which could directly bind to methyltransferases.

The inhibition of AdoHcy hydrolase in vivo by nucleosides such as 3-deazaadenosine has been shown to produce some pronounced biological effects. For example, 3-deazaadenosine and 3-deaza-SIBA have been shown to produce antiviral activity against Rous sarcoma virus in chick embryo fibroblasts and Gross murine leukemia virus in mouse embryo cells.[179,180,183] 3-Deazaadenosine has also been shown to produce antimalarial activity against *Plasmodium falciparum* in culture[184] and to inhibit macrophage phagocytosis.[185] 3-Deazaadenosine has also been shown to be a potent inhibitor of leucocyte chemotaxis.[42,43,71,82] The mechanism of this effect is unclear, since the resulting elevated levels of AdoHcy could inhibit both protein carboxymethylation and phospholipid methylation. Pike et al.[41] have shown that incubation of human monocytes with an adenosine deaminase inhibitor, adenosine, and L-homocysteine thiolacetone produces a concentration-dependent increase in intracellular AdoHcy levels, which correspondingly inhibits protein carboxymethylation and monocyte chemotaxis. 3-Deazaadenosine with and without an adenosine deaminase inhibitor and L-homocysteine has also been shown to inhibit protein carboxymethylation in hypothalamic synaptosomes.[64]

Nucleosides such as 3-deazaadenosine have also been used to study the physiological role of phospholipid methylation. For example, 3-deazaadenosine was shown to inhibit histamine release and phospholipid methylation induced by concanavalin A in rat mast cells.[81] 3-Deaza-SIBA was shown to inhibit phospholipid methylation and mitogenesis of lymphocytes.[82]

Other nucleosides such as 2′-deoxyadenosine and adenine arabinoside also inhibit AdoHcy hydrolase[186,187] and produce elevated intracellular AdoHcy levels, which have subsequent inhibitory effects on cellular methylation reactions.[187–189] These results have suggested that the toxic effects of certain nucleosides and the symptoms of human severe combined immunodeficiency disease may be related to their effects on AdoMet-dependent transmethylations.

Interestingly, inhibition of AdoHcy hydrolase by 2′-deoxyadenosine and adenine arabinoside[186] has been suggested to occur by a K_{cat} or suicide mechanism. Also of interest is the observation by Hoffman[190] that AdoHcy hydrolase is irreversibly inactivated by adenosine dialdehyde, the periodate oxidation product of adenosine. This inactivation by adenosine dialdehyde and related compounds appears to be specific for the active site of AdoHcy, and they exhibit kinetic characteristics of affinity-labeling reagents.[191]

Therefore, it appears that various compounds can affect AdoHcy hydrolase activity in vitro and in vivo. The effects of these agents in vivo are often quite pronounced, suggesting an important role for AdoHcy hydrolase in the regulation of AdoMet-dependent methyltransferases. To date, a minimal amount of effort has been made to identify inhibitors of AdoHcy hydrolase but it would appear from these preliminary data that this enzyme might be an ideal target for the design of potential chemotherapeutic agents.

Conclusions

Methylation catalyzed by AdoMet-dependent methyltransferases is rapidly being recognized as a general mechanism for regulating the activity of macromolecules in both procaryotes and eucaryotes. AdoMet-dependent methylations have been shown to be involved in posttranslational modification of proteins, in posttranscriptional modification of nucleic acids, and in the biosynthesis of phospholipids. The modifications of a macromolecule by methylation has been shown to play an integral role in processes such as protein synthesis, bacterial and leucocyte chemotaxis, peptide hormone degradation, enzyme catalytic activity, ligand–receptor and receptor–adenylate cyclase interactions, and neurosecretory processes.

To date, a fairly limited number of attempts have been made to inhibit the enzymes responsible for these methylations. The preliminary results of these studies, however, have been very encouraging. Agents which inhibit macromolecule methylations in vitro have been shown to exhibit antiviral, antimitotic, and antiparasitic activities in vivo.

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Based on these preliminary observations, it would appear that macromolecule methyltransferases offer excellent targets for the design of some new and novel chemotherapeutic agents. The interest in these enzymes by medicinal chemists should rapidly develop in the next several years as the biologists, biochemists, and pharmacologists continue to unravel the role of these macromolecule methylations in regulating important cellular processes.

Articles

Synthesis of Potential Inhibitors of Hypoxanthine–Guanine Phosphoribosyltransferase for Testing as Antiprotozoal Agents. 1. 7-Substituted 6-Oxopurines

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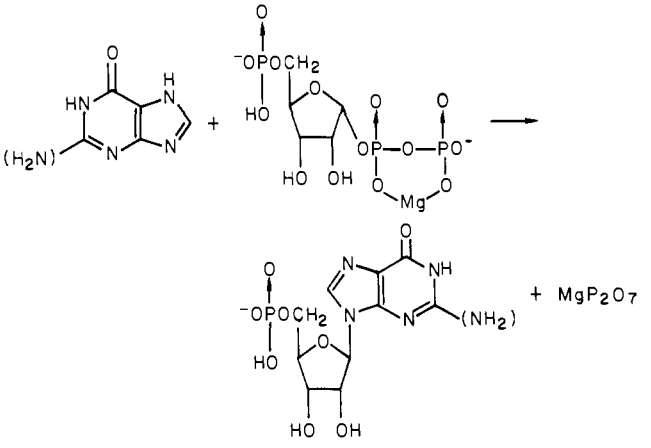
Biological evidence indicates that the enzyme hypoxanthine–guanine phosphoribosyltransferase (EC 2.4.2.8) is vital for cell proliferation in malarial parasites but nonessential for mammalian cells. 7-Substituted guanines and hypoxanthines in which the 7 substituent bears functional or hydrophobic groups were prepared with the aim of finding a suitably constituted compound whose resemblance to the normal substrate allows it to compete for the reversible purine binding site of HGPRTase while allowing a substituent group of the inhibitor molecule to form a covalent bond or strong hydrophobic bond with appropriate sites on the enzyme. Multistep syntheses that began with hydroxyalkylations and alkylations of guanosine led to four key guanines substituted at the 7 position by the following chains: 2-aminoethyl, 3-amino-2-hydroxypropyl, 3-aminobenzyl, and 4-aminobenzyl. Similarly, 7-(4-aminobenzyl)hypoxanthine was prepared. Reactions at the side-chain amino groups with bromoacetic anhydride (or, alternatively, 4-nitrophenyl bromoacetate) and 3- and 4-(fluorosulfonyl)benzoyl chlorides afforded derivatives bearing functional groups capable of forming covalent bonds with enzymes through displacement reactions. 4-Chlorobenzyl derivatives were similarly prepared as potential inhibitors that might act through hydrophobic bonding. Three 7-substituted guanines whose side chains bear other functions (7-guanine-3-propanesulfonic acid, guanine-7-acetaldehyde, and the ethyl ester of 7-guanine-4-crotonic acid) were prepared as potential inhibitors and for possible use as intermediates. None of these compounds extended the life span of *P. berghei* infected mice or showed significant in vitro inhibition of HGPRTase from H.Ep.-2 cells.

Work on purine metabolism in malarial parasites[1-12] indicates that these parasites are unable to biosynthesize purine nucleotides de novo and must depend on preformed purines of the host for the synthesis of nucleic acids necessary for multiplication, providing a firm basis for the concept that sufficiently potent inhibitors of hypoxanthine–guanine phosphoribosyltransferase (HGPRTase) should possess in vivo antimalarial activity. Adenosine is rapidly deaminated to inosine and then deribosylated to hypoxanthine on the surface or outside the parasite, while conversion of hypoxanthine to inosinic acid and, subsequently, to adenosine triphosphate occurs most probably inside the parasite.[12] Thus, hypoxanthine, a normal exit metabolite in mammalian purine metabolism (via oxidation to uric acid), is apparently the building block of the

Scheme I. Function of Hypoxanthine–Guanine Phosphoribosyltransferase



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