The enzyme thymidylate synthase (TS) catalyzes the methylenetetrahydrofolate-dependent conversion of deoxyuridine-5'-monophosphate (dUMP) to thymidine-5'-monophosphate, the key substrate in cellular DNA biosynthesis. Early trials to selectively combat cancer cells were based on interference with DNA biosynthesis. Therefore TS inhibition has been for over 50 years used in anti-cancer chemotherapy. Fluorouracil is the first and still very often used drug, transformedin a cell to a deoxynucleotide form, fluorodeoxyuridine-5'-monophosphate (FdUMP). FdUMP, a strong TS inhibitor, recognized by the enzyme as a substrate, participates in the enzyme-catalyzed reaction up to a certain point, when it is stopped due to the presence of fluorine in the inhibitor molecule. At this step, the enzyme, inhibitor and methylenetetrahydrofolate constitute a ternary complex that is covalently bound. Crystallographic studies results, concerning this complex, became the milestone on the path leading to learning the mechanism of TS-catalyzed reaction.

N^4-hydroxy-dCMP (N^4-OH-dCMP) is also a strong TS inhibitor, participating in the enzyme-catalyzed, methylenetetrahydrofolate-dependent reaction. The reaction leads to enzyme inactivation whose exact inhibition mechanismhas not been solved yet. We have been studying this mechanism for a long time, and some time ago succeeded to discover certain facts indicating this mechanism to be different from that of inhibition by FdUMP. Those differences suggested that learning the mechanism may help to understand so far unknown elements of TS-catalyzed reaction mechanism and to indicate possibilities to design new enzyme inhibitors, thus potential chemotherapeutics.

The project is aimed at verifying a hypothesis concerning mechanism of TS inhibition by N^4-OH-dCMP, formed based on the results of our crystallographic studies that shed a completely new and unsuspected light on that mechanism. Earlier the inhibitor has been assumed to form, similar to FdUMP, a covalently bound complex with the enzyme and methylenetetrahydrofolate, causing inactivation for unknown reasons. The latter picture was questioned by the results of the above mentioned crystallographic studies. In particular, by the 3D structure, solved with a very high resolution of 1.17 Å, of TS complex that crystallized in the presence of N^4-OH-dCMP and methylenetetrahydrofolate. The latter structure suggested the enzyme to catalyze in the presence of N^4-OH-dCMP and methylenetetrahydrofolate of an abortive reaction, leading to enzyme inactivation by irreversible binding of the inhibitor alone (unlike with FdUMP, where binding of inactivating inhibitor molecule leads to the formation of a covalently bound enzyme-FdUMP-methylenetetrahydrofolate ternary complex), with accompanying non-covalently bound dihydrofolate. It should be mentioned that TS-catalyzed reaction may be considered composed of two coupled simpler reactions: (i) methylene group transfer from methylenetetrahydrofolate to the fifth carbon atom of dUMP pyrimidine ring and (ii) reduction of this group by the remaining tetrahydrofolate molecule, non-covalently bound at the active center, with the latter undergoing oxidation to dihydrofolate. The above mentioned structure indicates "uncoupling" of the two reactions in the presence in the active center of N^4-OH-dCMP molecule. Also, the methylene group is apparently not transferred on this molecule (or the product of such a transfer is unstable, therefore leaving no trace), and the fifth carbon atom of the pyrimidine ring undergoes reduction that may be seen thanks to the above mentioned high resolution. Thus N^4-OH-dCMP interferes with the catalyzed reaction in a completely different way than FdUMP, thus constituting a potentially very useful tool to study the reaction mechanism, particularly that of the methylenetetrahydrofolate oxidation stage. The latter is of interest, as the mechanism of just this reaction stage is not well known.

Thus the proposed research approach is aimed not only at better understanding of the mechanism of the title inhibitor, with the idea of using this knowledge by designing new inhibitors, but also at applying N^4-OH-dCMP as a tool in studies of TS-catalyzed reaction mechanism. In order to reach those objectives, N^4-OH-dCMP and methylenetetrahydrofolate molecules will be applied with chosen atoms substituted by stable or radioactive isotopes (atoms of the same chemical element, differing in neutron number). Application of those "labeled" compounds in the enzyme-catalyzed reaction should allow to follow fates of chosen chemical groups and answer different questions concerning this reaction. Besides, the role of amino-acid residues, suspected (based on the crystal structure) of participation in the title inhibitor binding in the enzyme active center, will be tested with the use of 5 mutated TS variants, each with one of the suspected amino-acid altered (by genetic engineering). Each mutant protein will be assayed for enzyme activity, and with those variants preserving this activity, inhibition by N^4-OH-dCMP, as well as a potential to catalyze the methylenetetrahydrofolate-dependent transformation by the inhibitor, leading to permanent inactivation. I addition, the experimental studies will be supplemented by calculations, involving molecular modeling with the use of quantum mechanics, and molecular mechanics and dynamics, aimed at testing possible reaction paths, leading to formation of the above mentioned crystal structure.