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Development of inhibitors of biomolecules of pharmacological interest

Ph.D. thesis of

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S.S.D. CHIM/03

1 INTRODUCTION

At present the therapeutic treatment of cancer is strongly limited in its effectiveness due to the low selectivity of the drugs used for the cancer modified cells[1]. It is based on the administration of cytotoxic drugs which are highly harmful also for the healthy cells of the individual mostly because of their interference with the normal physiology functions. This lack of selectivity of anticancer drugs is the cause of their high toxicity *in vivo*.

Moreover the unwanted secondary effects are not compensated by a long-lasting remission, especially in cases of advanced solid tumors, which still represent an incurable disease with survival chances tending, in the long term, to zero.

The current trend in research on anticancer drugs is to exploit particular traits unique to cancer cells. Despite the fact that cancer displays a great heterogeneity in clinical behavior, most human tumors share a limited set of acquired capabilities that define the malignant state[2]. These include self–sufficiency in growth signal, insensitivity to antigrowth-signals, avoidance of programmed cell death, unlimited replicative potential, sustained angiogenesis, tissue invasion and metastasis. Among these hallmarks, the acquisition of unlimited replicative potential is a key step to ensure expansive tumor growth.

The activation of a telomerase maintenance mechanism seems indispensable for immortalizing human cells. Telomeres and telomerase, the protein that allows their maintenance, have therefore been proposed as preferential targets for anticancer drug development.

1.1 Telomeres

In the 1960s, Hayflick and collaborators[3] demonstrated that the proliferative capacity of normal human somatic cells is finite, and that after a limited number of cell divisions, cells stop dividing and enter a state termed senescence. During the past 40 years or more, considerable research interest has focused on identifying the mechanism that regulates the replicative capacity of normal somatic cells.

One molecular pathway thought to regulate somatic cell proliferation is that mediated by telomeres, specialized DNA-protein structures present at the end of all linear chromosomes[4]. The telomere is a simple sequence of nucleotides together with a group of specific proteins. In humans, the sequence TTAGGG is repeated dozens or even thousands of times at each

chromosome end. The telomere is involved in several essential biological functions[5]. It prevents chromosomes from recombination, end-to-end fusion and, recognized as damaged DNA, it provides a means for complete replication of chromosomes, contributes to the functional organization of chromosomes within the nucleus, participates in the regulation of gene expression and serves as a molecular clock that controls the replicative capacity of human cells and their entry into senescence[6]. In particular, the replication of chromosome ends pose a special problem for the cells. In fact the DNA is progressively lost from the ends of chromosome each time cell divides, because the conventional DNA polymerase is not fully able to replicate the 3'-end of the lagging strands of the linear molecule. This "end replication problem" leaves a gap between the final priming events and the end of chromosomes[7,8]. Without an appropriate mechanism to offset telomere shortening, normal human cells proliferate for a certain number of divisions (the Hayflick limits), followed by an irreversible growth arrest phenomenon known as cellular senescence[9,10]. Rare survivor cells are able to maintain telomere length. In normal mammalian cells, one well-characterized mechanism of telomere maintenance and elongation is mediated by the enzyme telomerase[11].

1.2 Telomerase

Telomerase is a RNA-dependent DNA-polymerase that can add the hexameric repeats, TTAGGG, to chromosome ends, extending and maintaining the length of the telomeres and thereby extending the number of divisions the cell may undergo[12].

It is a unique reverse transcriptase composed of both protein subunits (among which the catalytic subunit hTERT in humans with reverse transcriptase activity)[13] and a RNA component (hTR in human) [14] that serves as the template for the addition of the repeated sequence to 3'-chromosome ends[15]. hTR is highly expressed in all tissue regardless of telomerase activity[16], with cancer cells generally having fivefold-higher expression than normal cells. In contrast, the expression of the human catalytic component hTERT is estimated at less than 1 to 5 copies per cell[17]. hTERT is generally repressed in normal cells and upregulated in immortal cells, suggesting that hTERT is the rate-limiting component of the telomerase enzyme and its activity[18].

The advent of a highly sensitive telomere repeat amplification protocol (TRAP assay) allowed the screening of a variety of human tumors[19]. The TRAP assay allowed the evaluation of telomerase activity of normal human tissues and of a whole spectrum of human tumors[20]. In normal human cells, telomerase activity appears to be strictly regulated during development.

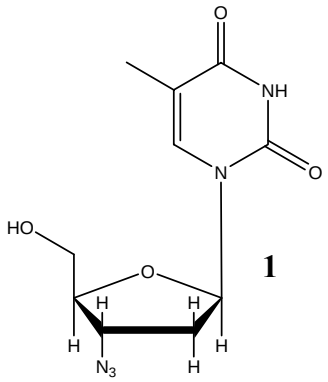
Its activity is extinguished during embryonic differentiation and so most somatic cells are devoid of telomerase activity after birth, but remain active in some tissue, such as male germ cells, activated lymphocytes, and certain types of stem cell population[21]. A low level of telomerase activity continues to express in some specific proliferating epithelial cells found in the breast, endometrial tissues, the basal layer of the skin including hair follicles[22-25]. This telomerase activity is reactivated in 85-90% of diverse human tumors[20] while adjacent healthy tissue is typically telomerase-negative.

Therefore, the evidence that most cancer cells activate telomerase whereas normal cells are usually devoid of telomerase activity has naturally led to extensive investigations in order to detect this protein and its activity for a potential use in cancer diagnosis and prognosis and to eventually monitor the tumor response to therapy. Finally, these data have greatly inspired the development of various strategies to target telomere and telomerase for cancer therapy.

1.3 Telomerase as target of cancer therapy.

The target of anti-telomerase drugs can principally be the catalytic subunit hTERT and the RNA subunit hTR, or the regulation of the expression of telomerase and its associated proteins[26].

Targeting the hTERT catalytic subunit, various nucleoside or non-nucleoside reverse transcriptase inhibitors are under investigation, and these represent interesting candidates for clinical drug development. Nucleoside analogues such as 3'-azido-3'deoxythymidine **1** (AZT) are small molecules with reverse transcriptase inhibitors effect.



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These types of molecules block the incorporation of dNTPs into the neosynthesized DNA during the reverse transcription activity. In early studies, AZT was able to partially reduce the telomerase activity, but the cells showed only some weak proliferative impairment. This compound with other nucleoside analogues showed a stronger inhibitory effect[27], but there is

an indirect effect such as the incorporation of the nucleoside analogs into mitochondrial DNA causing the depletion of mtDNA and mitochondrial toxicity.

Non-nucleoside inhibitors are compounds that inhibit the telomerase by binding the active site of telomerase, but they do not have effect on other DNA and RNA polymerases. These small molecules cause progressive telomere shortening in pharmacological screening programs. One of the most promising molecules is probably the BIBR 1532, which is a very specific drug and non-competitive inhibitor of telomerase enzyme, suggesting that the binding site is different from DNA primer and nucleotide binding site[28]. It leads to progressive telomere shortening, followed by a senescence-like growth arrest, and further induces a significant decrease of the tumorigenic potential *in vivo*.

Targeting the RNA hTR is a strategy that aims at blocking the access of telomerase to its template RNA resulting in inhibition of telomerase activity. Another promising approach is to employ mutant hTRs that introduce mutations into the telomeres indirectly impairing the structure of telomeres.

Targeting directly the element that seems to force cells into crisis, i.e. the length of telomeres, has largely enriched the development of anticancer candidates. Emerging evidence suggests it is the shortest telomeres rather than average telomere length that cause chromosome end fusions and apoptosis in telomerase inhibited cells[29], even if it is still not fully known what mechanism and molecular actors are implicated in the telomere dysfunction related cell crisis.

However, the testing of inhibitor compounds has shown that the time required for the shortening of the telomere to the critical length is too long to contrast the progression of tumors, especially in cases of advanced solid tumors[30].

A different approach that we envisaged is to exploit the activity of telomerase instead of inhibiting it, using a specific molecule, a prodrug that can be activated only in tumor tissues.

1.4 Prodrug strategies.

In recent years attempts have been made to administrate drugs as prodrugs[31-34]. From a therapeutic point of view, a prodrug is an inactive compound, which can be transformed *in vivo* into an active drug, i.e. into a therapeutically active compound, thanks to a chemical or an enzymatic transformation of its structure. Normally, in a prodrug, the active agent is linked to a carrier (also known as a promoiety), and the activation occurs by hydrolysis, oxidation or reduction. However, there are prodrugs that do not contain a promoiety and are equally activated by oxidation, reduction or other chemical reaction[33].

Normally prodrugs can be designed to overcome pharmaceutical, pharmacokinetic, or pharmacodynamic barriers such as insufficient chemical stability, poor solubility, insufficient oral absorption, inadequate blood-brain barrier permeability, irritation or pain, toxicity and also unacceptable taste or odor. A developing field of high importance is that of rationally designed prodrugs for tissue or cell targeting.

Among the objectives of prodrug design the improvement bioavailability is one of the most important. There are a variety of factors limiting bioavailability: aqueous solubility, passive intestinal absorption and targeted active absorption.

Inadequate aqueous solubility is an important factor limiting parenteral, percutaneous and oral bioavailability. Charged promoieties (e.g. phosphates, hemisuccinates, aminoacyl conjugate) and neutral promoieties (e.g. polyethylen glicols, PEG) can be used in order to overcome the problem. Two examples of this kind of prodrugs, PEG-taclitaxel and fosphenytoin, are showen in Figure 1. Improved aqueous solubility for better administration has been demonstrated for the taxol derivate PEG-taclitaxel[35]. Fosphenytoin is a hydrophilic phosphate prodrug of the anticonvulsant phenyton and is hydrolyzed rapidly by phosphatases[36].

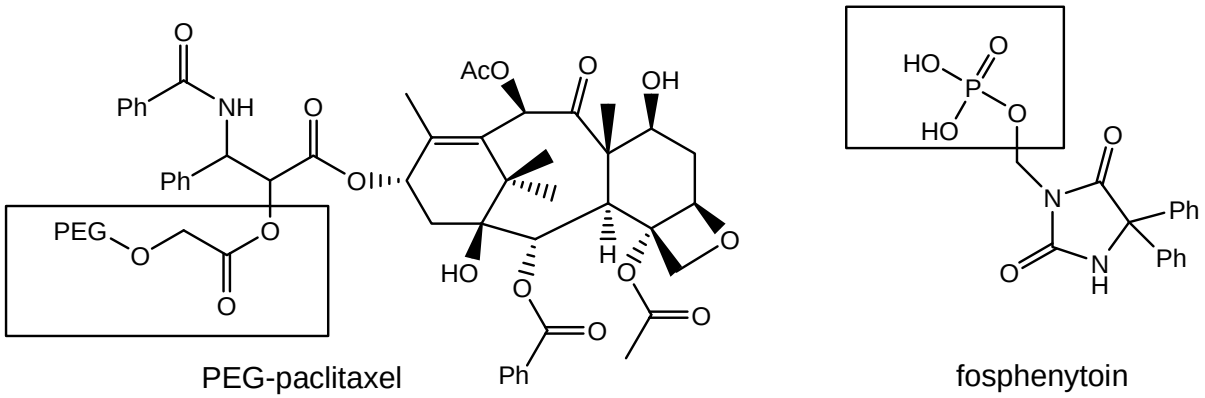


Figure 1. Examples of promoietis for improved aqueous solubility

To increase passive intestinal absorption a prodrug strategy is often adopted . Various esters of carboxylic acids, a frequently encountered group of carried-linked prodrugs, are cleaved by hydrolysis, enzymatic and/or chemical, to liberate the active compound[37]. There are many examples such as the ACE inhibitors benazepril and fosinopril, the statin lovastatin, and some antibiotics such as pivampicillin (Figure 2)[32].

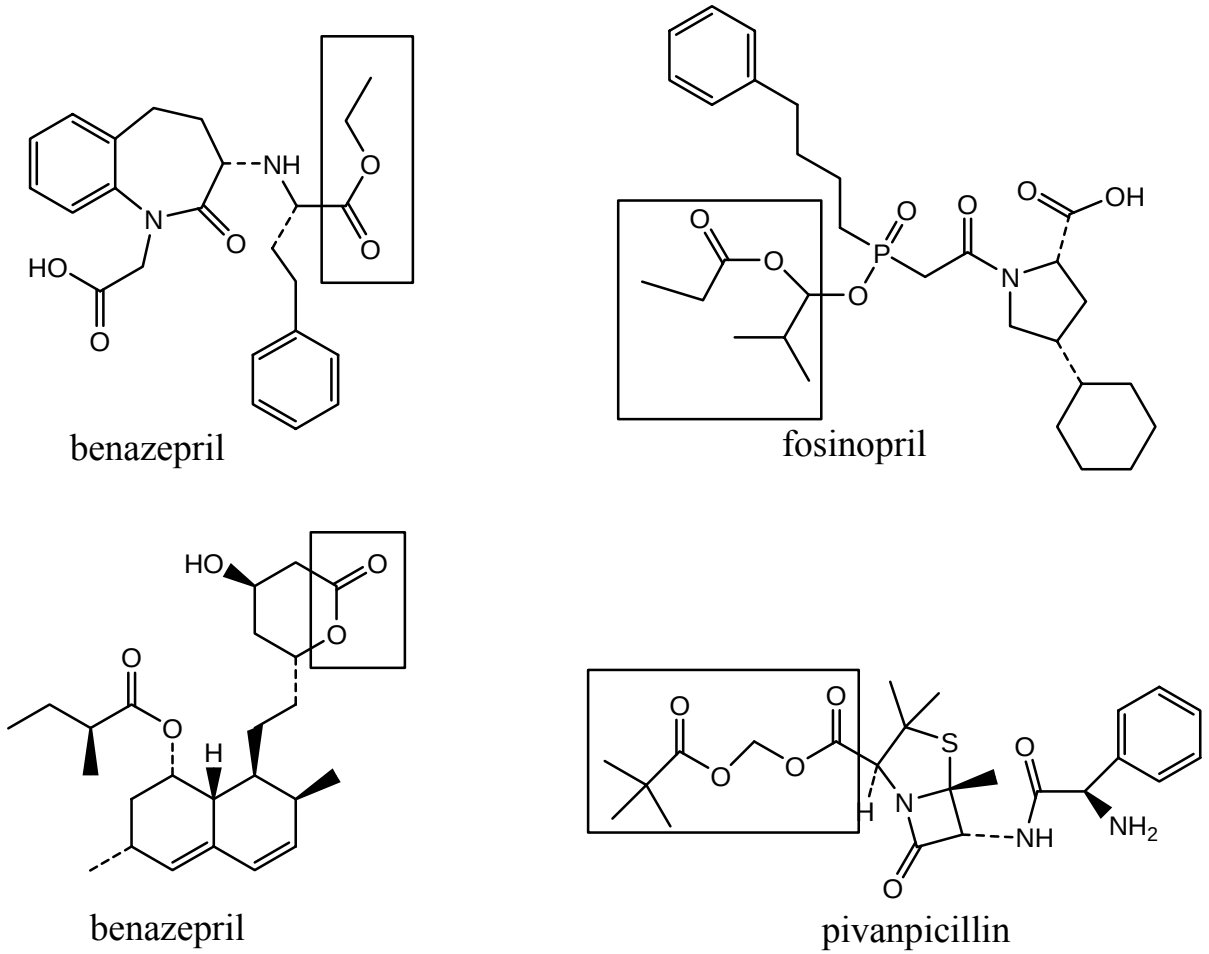


Figure 2. Examples of ester prodrugs showing improved passive intestinal absorption.

Some prodrugs use carrier-mediated transport to increase intestinal absorption. For example valacyclovir and valganciclovir (Figure 3), the valine esters of acyclovir (**ACV**) and ganciclovir (**GCV**), show an bioavailability 5 times higher than that of acyclovir. This is due to transport by the intestinal dipeptide transporter PEPT1[34].

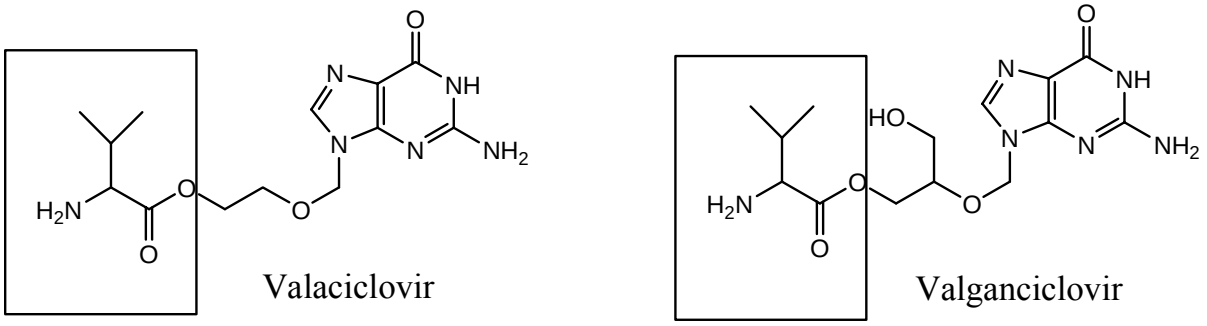


Figure 3. Examples of prodrugs utilizing oligopeptide transporters.

Finally an additional prodrug strategy relies on the organ – or tissue-selective delivery of a drug[33]. Rationally designed tissue targeting is probably the most exciting objective of a

prodrug strategy. This can be achieved by passive enrichment in the target tissue, targeting specific transporters, targeting surface antigenes and targeting tissue- or cell specific enzyme.

Examples of passive enrichment in target tissue are cytostatic and cytotoxic agents conjugated to PEG with MW > 50000 (e.g. PEG-paclitaxel, Figure 1). They are reported to exhibit an improved therapeutic efficacy due to their longer half-life and selective accumulation in tumors cells. This passive tumor targeting is called the "enhanced permeability and retention" (EPR) effect, and its mechanism is not fully understood. The low drug load achievable by such macromolecular conjugates requires more potently cytotoxic agents than traditional anticancer drugs.

The number of examples of prodrugs targeting tissue-specific transporters is relatively limited. Levodopa constitutes an example of enrichment resulting from active import of the prodrug into the brain followed by tissue entrapment of the active metabolite dopamine(Figure 4).[38] It is a substrate for the neutral amino acid transporters present at the blood-brain barrier. After brain entry, levodopa is decarboxylated to dopamine, which can act locally, being no longer a substrate for the neutral amino acid transporter.

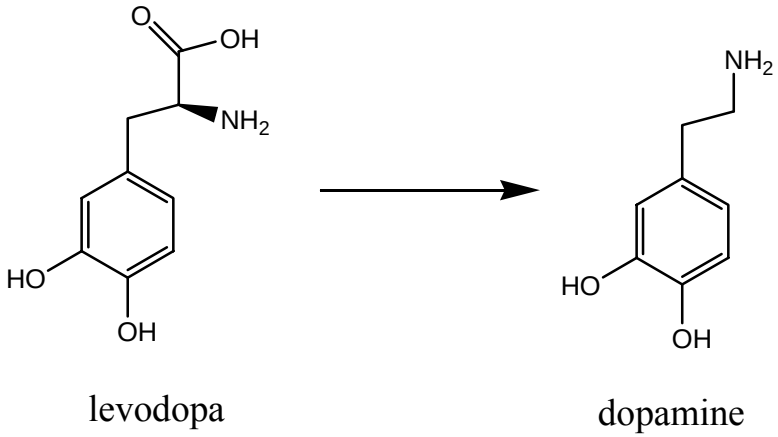


Figure 4. Example of prodrugs targeting the brain.

There are many examples of prodrug activated from tissue- or cell-specific enzyme. Specific targeting of virus-infected cell is elegantly realized by the antiviral prodrug acyclovir and all other selective antimetabolites used as antiviral agents. The activity of such antimetabolites implies their intracellular phosphorylation by kinases to form a nucleotide analogue that inhibits DNA synthesis. The selective antimetabolites are phosphorylated only by kinases coded by the viral genome and expressed in infected cells. As a result, acyclovir and all other selective antimetabolites are active only in infected cells[39].

The selective delivery of cytotoxic drugs to tumor cells, without concomitant damage to normal tissues, is a major challenge in cancer chemotherapy. Prodrugs for active tumor

targeting are therefore of high interest. Tumor specificity can be achieved by targeting trasporter system or enzyme having a higher activity in tumor cells.

Prodrugs of cytotoxins have been used in cancer therapy for some time (e.g. araC esters[40], cyclophosphamide[41]), but they undergo a non-specific activation and are primarily used to modify drug uptake or pharmacokinetics by controlling physicochemical properties.

Capecitabine, an orally available triple prodrug of 5-fluorouracil (5-FU), offers a good example of a prodrug activated by tumor specific enzyme[42]. It is well absorbed orally and undergoes three activation steps resulting in high concentration of the active drug in tumor. It is first hydrolyzed by liver carboxylesterase, the resulting metabolite being a carbamic acid which spontaneously decarboxylates to 5'-deoxy-5-fluorocytidine (Figure 5). The enzyme cytidine deaminase, which is present in liver and tumor, then transforms 5'-deoxy-5-fluorocytidine in 5'-deoxy-5-fluorouridine. Transformation into 5-FU is catalyzed by thymidine phosphorylase and occurs selectively in tumor cells.

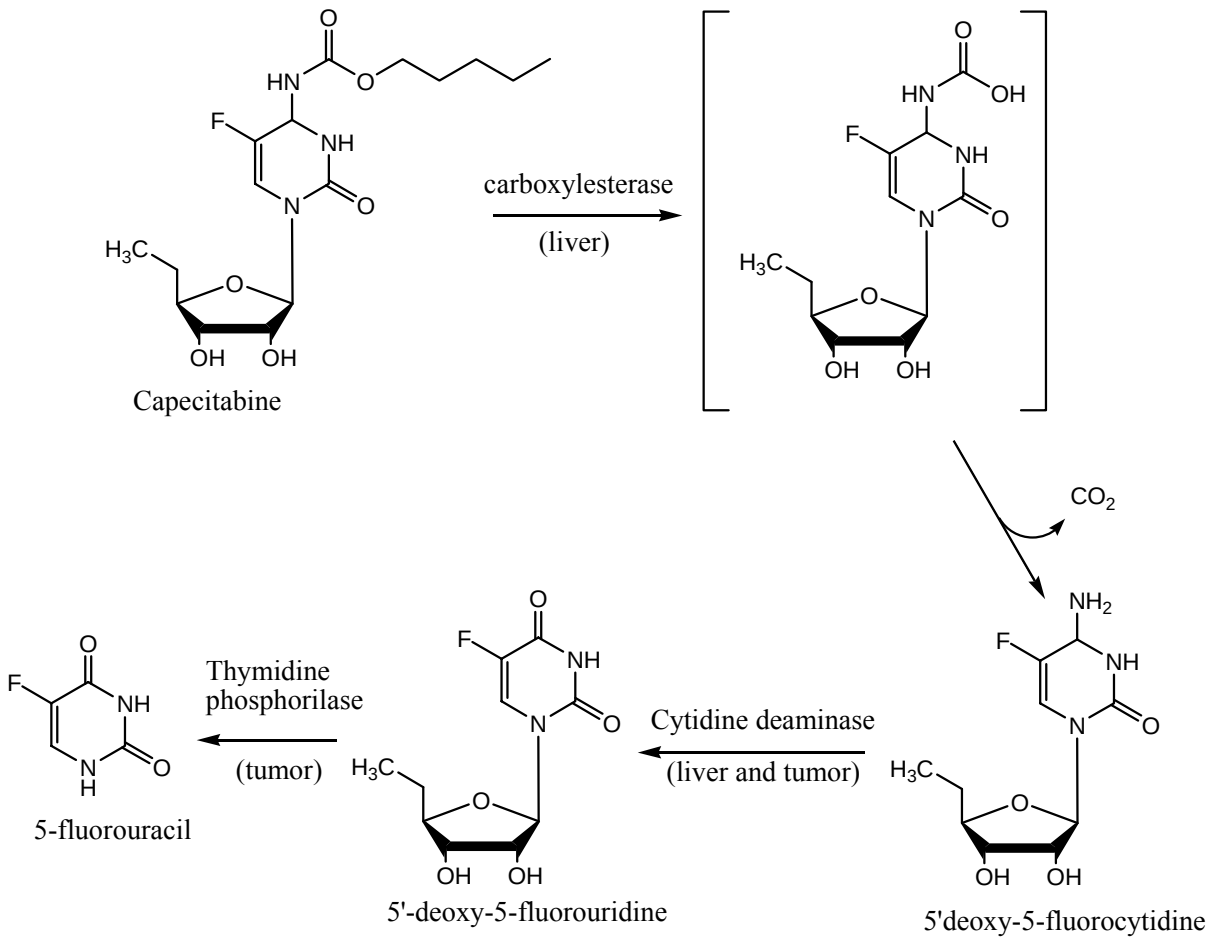
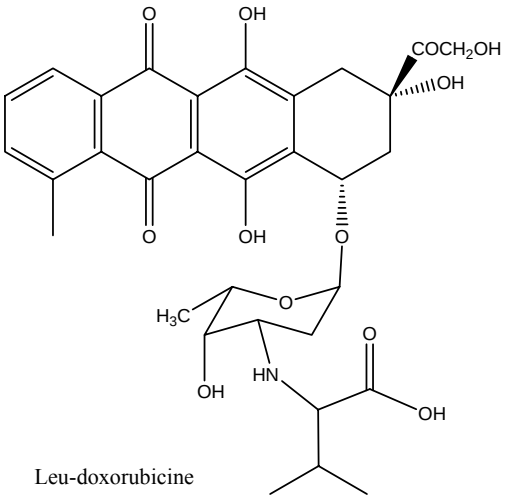


Figure 5. The stepwise activation of capecitabine to the antitumor drug 5-fluorouracil.

Another example of prodrug activated from specific enzyme is Leu-doxorubicin (Figure 6). It was first studied in human ovarian cancer cell xenografts in mice showing conversion to

doxorubicin in tumor cells by cathepsin-like enzyme, resulting in superior antitumor activity than with doxorubicin itself. The role of the conjugated aminoacid in this prodrug is not to physically locate the prodrug on tumor cells, but to serve as a substrate for designated enzymes that are produced and secreted preferentially by tumor cells. The prodrug gave lower levels of the cardiotoxic doxorubicin in heart tissue.



Leu-doxorubicine

Figure 6. Strutture of leu-doxorubicine.

Regardless of the mechanism of prodrug selective activation, it is useful to consider an anticancer prodrug as comprised of three distinct domains: trigger and effector units joined by a linker[43,44] (Figure 7). The role of the trigger unit is to undergo efficient metabolism by tumor-specific mechanism. The role of the linker is to deactivate the prodrug up to the trigger metabolism. The role of the effector is to kill cells rapidly.

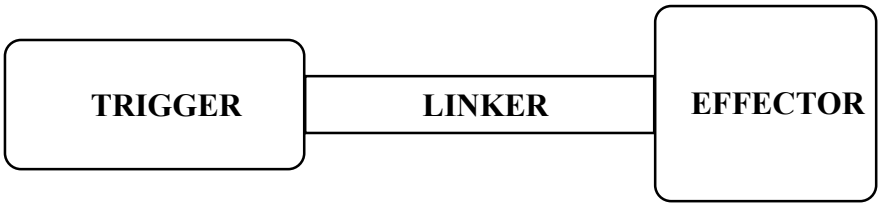


Figure 7. Schematic structure of a prodrug

In the case of prodrug activated by a specific enzyme, the trigger must be recognized from its catalytic portion. For this reason, it must have a structure similar to a physiological substrate of enzyme. Moreover, the effector must not interfere with the catalytic portion of the enzyme. In the last example, doxorubicin prodrug (Figure 6), the trigger is the aminoacid leucine, the linker is the peptide bond and the effector is doxorubicin.

Another important characteristic of a prodrug is its *in vivo* stability, since it must be able to reach the specific site, the tumor cells, without degradation in the blood or in the intracellular space. In addition, it must be able to cross the cellular membrane. For this reason it must be small and lipophilic or able to use an active transport present in the cellular membrane.
Using this approach, several clinically successful prodrugs have been developed; however, few prodrugs developed show tumor selectivity. This can be rationalized by the fact that, unlike bacteria and viruses, cancer cells do not contain molecular targets that are completely foreign to the host[33]. Therefore it seems necessary to exploit phenotypic differences between normal and tumor cells, especially the presence of enzymes that may activate prodrugs. Enzymes which can be used for local activation of prodrugs include tyrosinase, thymidylate synthase, thymidine phosphorilase for example, but also telomerase.

1.5 Prodrugs activated by telomerase.

As outlined above, the structure of prodrugs activated by telomerase must have some specific characteristic:

- it must obviously be a telomerase substrate;
- it must be stable to enzymatic attack in the blood and in the intercellular media;
- it must be selective to telomerase respect to DNA polymerases, since otherwise it loses selectivity to cancer cells;
- it must be able to release a cytotoxic compound into the cells.

The physiological telomerase's substrates are the 2'deoxynucelotide-5'-triphosphates dGTP, dATP and TTP, because telomerase adds thousands of hexameric repeats TTAGGG at the end of the telomere. The catalytic site of telomerase recognizes the nucleotide triphosphate, adds the nucleotide monophosphate into the telomere, freeing a molecule of pyrophosphate. The structure of the prodrug should therefore be similar to a dNTP, but with a cytotoxic group in the γ position (Figure 8): the TS group is the telomerase's substrate, while the CT moiety is the cytotoxic compound.

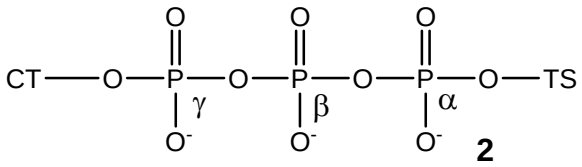


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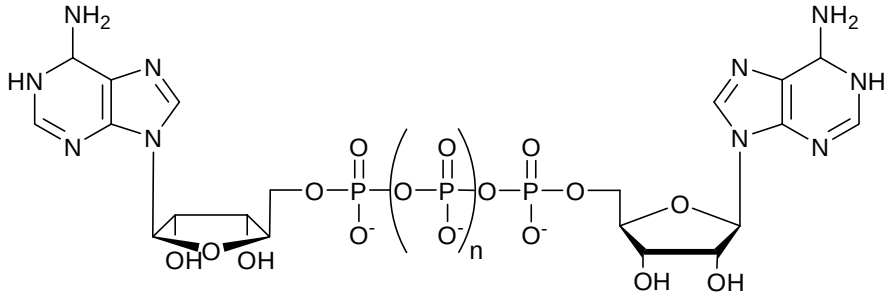
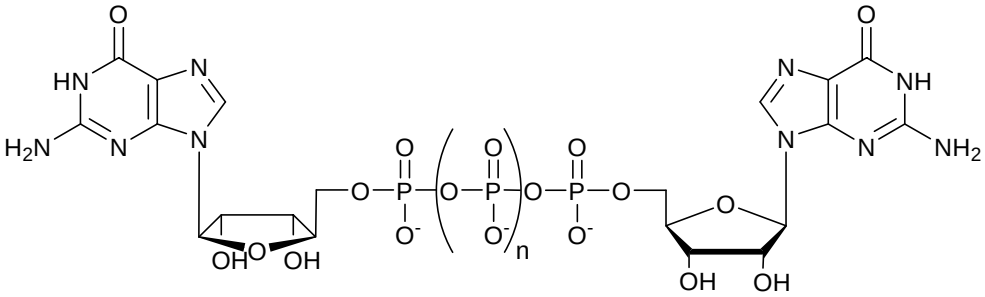
Figure 8 Schematic structure of telomerase-activated prodrugs.

Both TS and CT should be nucleosides or nucleoside analogues. The TS groups can be chosen among the physiological substrates of telomerase: deoxyguanosine, deoxyadenosine and thymidine (Figure 11). As to the candidates for CT moiety, it is possible to choose between the cytotoxic nucleoside analogues used as anticancer (e.g. arabinocytosine **AraC**, etc.) or antiviral (e.g. acyclovir **ACV**, ganciclovir **GCV**, etc.) drugs (Figure 12).

A prodrug with the structure **2** is similar to a dinucleotide oligophosphate. The dinucleotide oligophosphates (DNOPs) are ubiquitous compounds formed by two nucleotides bridged by two to seven phosphates. The most widely investigated are diadenosine polyphosphates (**Ap_nA**) and diguanosine polyphosphates (**Gp_nG**) (Figure 9). They are natural compounds and some of them, in particularly diadenosine oligophosphates, are involved in extracellular and intracellular signaling.[45] Extracellular diadenosine oligophosphates are involved in blood pressure regulation, stimulation of DNA synthesis in cells and in many aspects of messenger RNA metabolism[46].



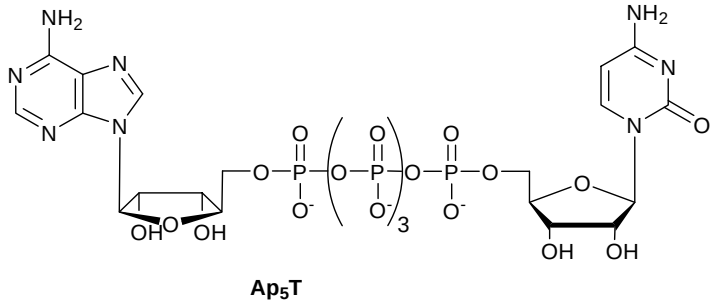
Ap_nA (n=0-5)



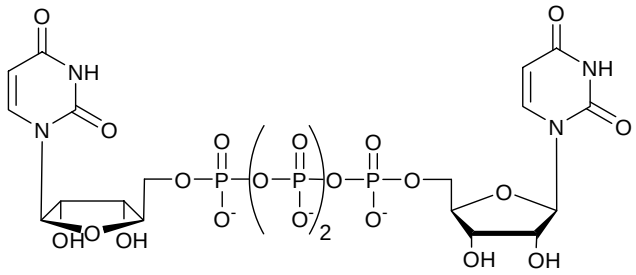
Gp_nG (n=1-2)

Figure 9. Structures of adenosine oligophosphates and guanosine oligophosphates.

They are stable *in vivo*, unlike NTPs and dNTPs that are rapidly dephosphorylated in intercellular media and during their diffusion into cells. Thus, molecules with a structure similar to the DNOPs are very likely to be stable in the blood and in intercellular media[47]. For this reason many DNOPs are studied as potential drugs and they are used as probes to the active sites of enzymes in which nucleosides and/or nucleotides are involved in phosphate transfer reactions[48-52]. Some examples of non-natural DNOPs are shown in figure 10.



Ap$_5$T



Up$_4$U

Figure 10. Example of non-natural DNOPs.

The DNOP **Ap$_5$T** is a good inhibitor of thymidine kinase and thymidilate kinase, two enzymes that are elevated in malignancy and are essential to DNA synthesis[50,53].

Diuridine tetraphosphate **Up$_4$U** has shown to have beneficial properties in the treatment of variuos diseases. It is a potent and selective agonist of P2Y receptor. It has demonstrated to facilitate the clearance of mucous secretion from the lungs in subject needing therapy for various diseases (e.g. cystic fibrosis, chronic bronchitis, asthma, bronchiectasis, post operative mucous retention and other diseases that involve mucus secretion).[54,55]

It is not clear if DNOPs are able to be uptaken from cells for their high charge, but nucleotide triphosphates with substituent groups in γ position increase the retardation factor (Rf) on thin layer chromatography (TLC), showing that the substitution increases the lypophilicity of the molecules[15,56].

The presence of a group in the γ position probably increases the selectivity towards telomerase at the expenses of DNA polymerase. In fact it is known that the presence of a group bound to the γ-phosphate increases the selectivity towards reverse transcriptase of HIV,

which has a very similar activity to telomerase, becoming at the same time a bad substrate for DNA-polymerase α, β.

In a molecule with the structure **2** the TS groups may be recognized from telomerase and the telomerase should add the TS groups into the telomere, freeing the cytotoxic fragment as a diphosphate. This fragment can play a cytotoxic function in this form (e.g. arabinocytosine-diphosphate, **AraCDP**, is a inhibitors ribonucleotide reductase, RNR), or can be transformed from kinases into a triphosphate and incorporated into DNA by DNA polymerase (e.g. if CT is acyclovir or dideoxycytidine).

In order to verify these hypotheses, a library of different molecules with the general structure **2** has been synthesized using different TS and CT groups. As TS groups the physiological substrate of telomerase (**dA**, **dG** and **T**) and inhibitors of telomerase (**AZT**) were chosen; as CT groups acyclovir (**ACV**), dideoxycytidine (**ddC**), ganciclovir (**GCV**) arabinocytidine or (**AraC**) and 3'-O-nitro-2'-deoxycytidine (**Nitro-C**) were chosen. **ACV**, **GCV** and **ddC** are the chain terminator used in antiviral therapy[39], while **AraC** is an antimetabolite agent[57]. **Nitro-C** is a new antimetabolite agent that is a chain terminator but also able to release the NO radical specie inside the cells. This radical can damage the DNA chain, then killing the host cell. **Nitro-C**, as 5'-monophosphate, could act as an irreversible inhibitor of thymidilate synthase, whereas the triphosphate could serve as an unnatural substrate for, or inhibitor of, DNA polymerase to elicit a cytotoxic anticancer/antiviral effect[58].

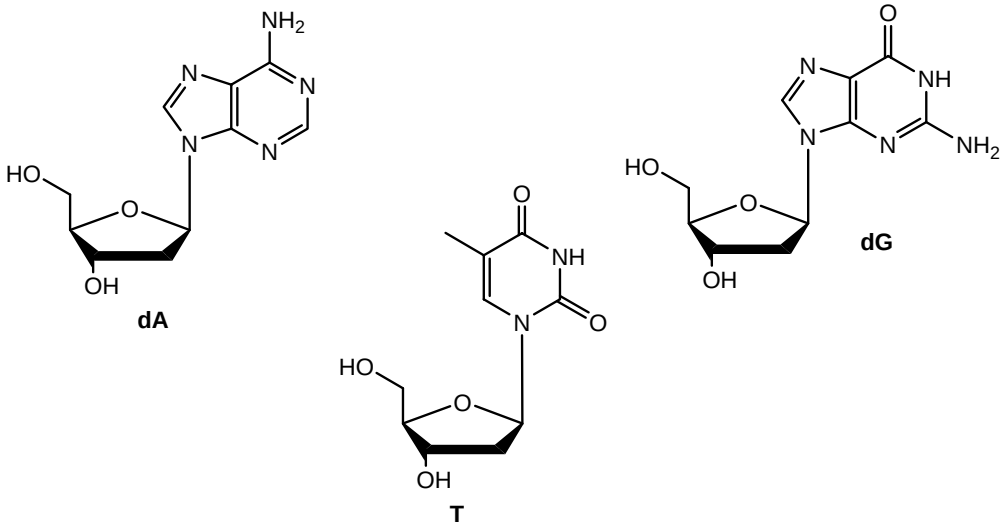


Figure 11. Telomerase substrates (TS).

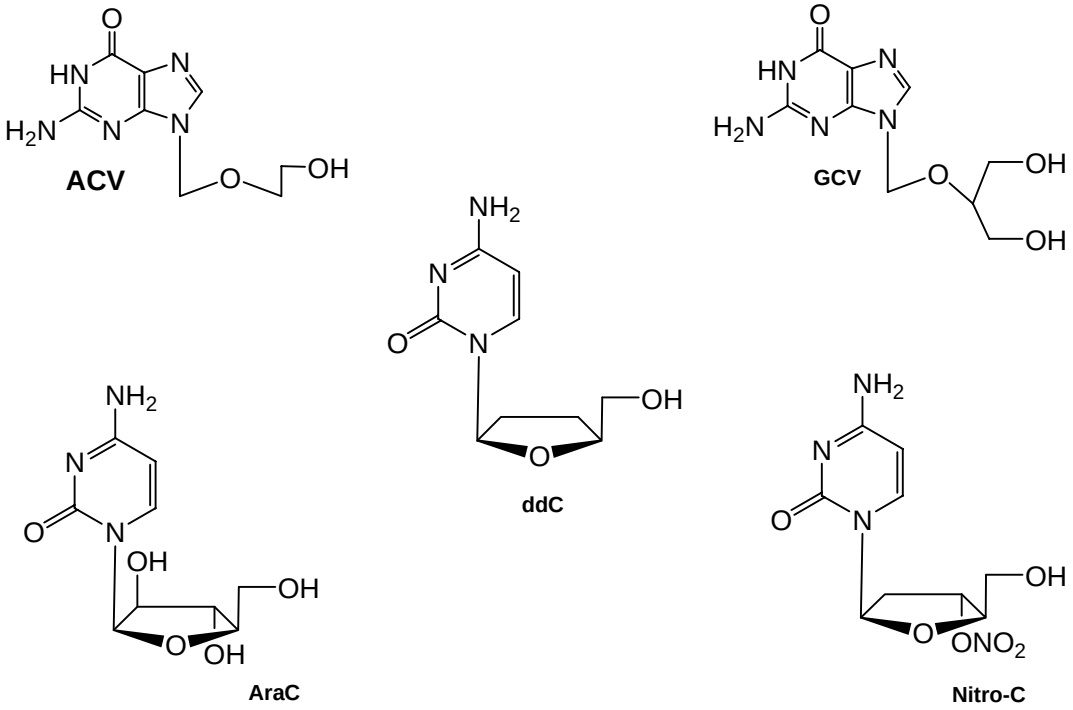
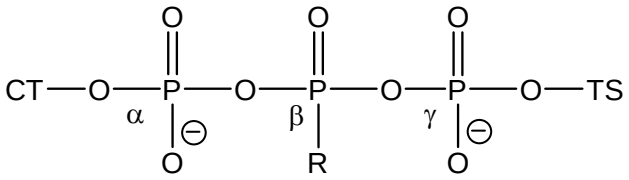


Figure 12. Cytotoxic compounds.

An attempt has also been made to prepare analogues to dinucleosides triphosphate **3**, where the β-phosphate is replaced by a phosphonate or alkyl- aryl-phosphonate in order to remove a negative charge in the phosphate bridge, to increase the molecular hydrophobicity, and to test how this set of molecules can cross the cell membrane.



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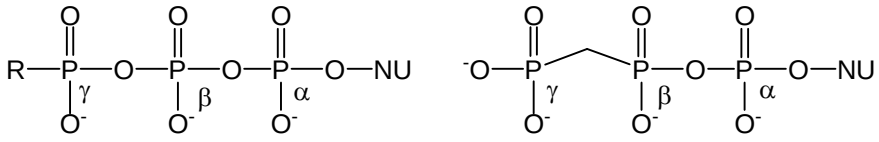
R = CH_3, Ph, OCH_3, OPh

Figure 13. Schematic structure of β-phosphonates.

The replacement of a phosphate group with a phosphonate group (R = CH_3 or Ph) or alkyl-aryl-phosphate group (R = OCH_3 or OPh) in α, β or γ position in dNTPs or NTPs has already been used to increase molecular stability to the dephosphorilation or to increase molecular selectivity in case of a particular enzyme (HIV reverse transcriptase versus DNA polymerase)[56]. However, the structure of these molecules provides either a γ-positioned phosphonate[59] or the presence of a β,γ –positioned methylendiphosphonate unit[56] (Figure 14).



R = CH_3, Ph

Figure 14. Example of phosphonate.

In our case the phosphonate units must replace the phosphate in β-position, because this is the less sensible position for the action of telomerase and DNA polymerase enzyme. In fact the phosphate in γ-position is the one to be incorporated, together with the base, into the telomere, while the phosphate in α-position is the one that should be recognized by DNA polymerase and incorporated in DNA chain, thus carrying out its cytotoxic activity. The presence of a substituent in one of these positions would make the phosphate hardly usable by these two enzymes.

The molecules prepared were tested on cellular cultures of different cancer cell lines and a healthy cell line. The most efficient compounds were then tested on mice.

2 SYNTHESIS OF NEW DINUCLEOTIDE TRIPHOSPHATES

2.1 General considerations

Dinucleotide triphosphates, as nucleotide triphosphates, are difficult to make, isolate and characterize[60] due to several factors. Firstly, many of the methods for their preparation involve combinations of charged ionic reagents with more lipophilic substrates (e.g. orthophosphates and protected nucleosides). Consequently, it is difficult to find appropriate reaction media and the purification procedures must involve isolation of a charged water-soluble product from a mixture of hydrophilic and hydrophobic impurities. Secondly, these compounds, and especially their intermediates (e.g. nucleotide diphosphate), are not particularly robust. It is difficult to make quantitative generalizations about their decomposition rates, but it is certain that the hydrolysis of these compounds is accelerated under both basic and acidic conditions.

Their isolation protocols involve gravity chromatography of the crude product on DEAE-cellulose [(diethylamino)ethylcellulose] using a gradient of increasing volatile protic ammonium salt in water (e.g. triethylammonium bicarbonate) as eluant.

^{31}P-NMR spectra of dinucleotide triphosphates feature two resonances at around -11 ppm (the α and γ phosphorus atoms) and a resonance at about -22 ppm (the β phosphorus atom). Chemical shift values for these kinds of molecules are highly pH and counter ion dependent. The ease with which these signals are observed is inversely related to the exchange processes that occur on the NMR time scale. These exchange processes, usually involving the triphosphate counter ions, can be slowed down by buffering the medium. The coupling constant between the two geminal phosphorus atoms is about 19Hz.

Proton NMR spectra of dinucleoside triphosphates are also influenced by exchange phenomena. Moreover, inadequate freeze-drying can result in trialkylammonium salt impurities that can obscure and/or overshadow relevant peaks in the spectra. The spectral characteristic of these compounds are also influenced by pH.

2.2 Synthetic pathway

The most widely used synthesis of dinucleoside triphosphates involves the disconnection shown in Figure 15. Only the nucleoside monophosphate **5**, the orthophosphate **9** and the cytotoxic compound **11** are commercially available. All the other compounds need to be prepared.

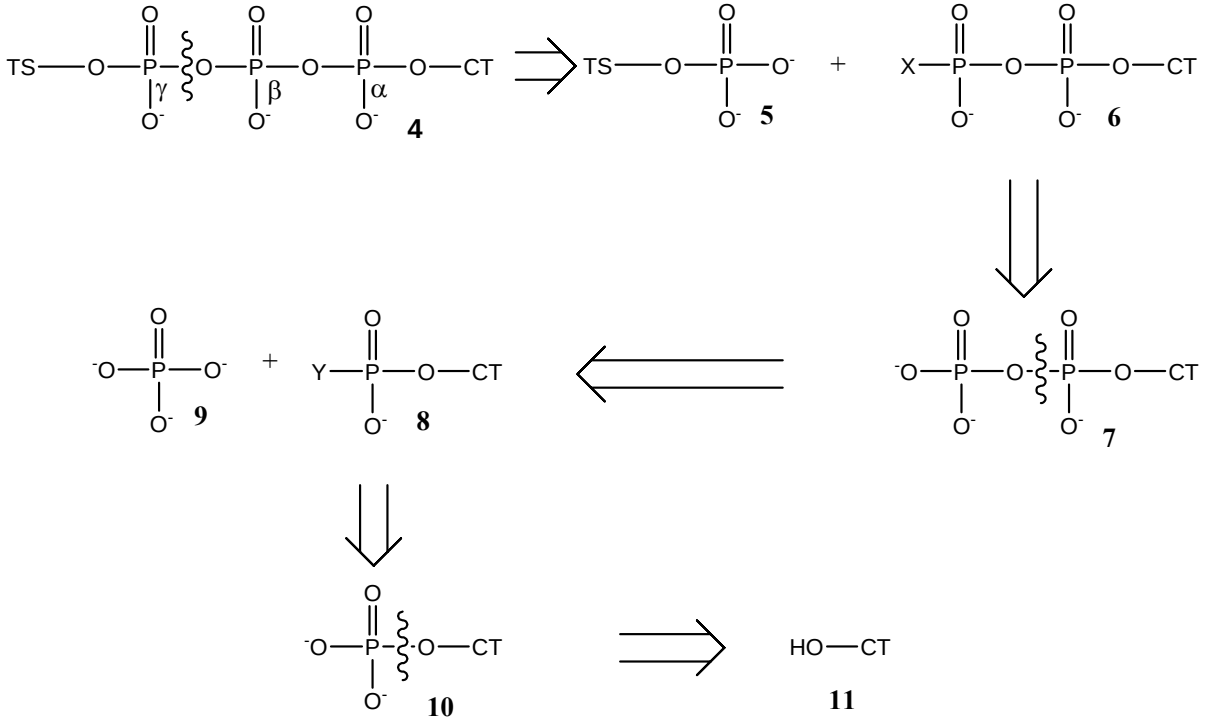


Figure 15. Retrosynthetic pathway of dinucleotide triphosphates.

The dinucleotide triphosphates preparation is carried out by coupling the two phosphates **5** and **7**. This coupling is made by activating one of the two phosphates (in our case **7**) and by letting it react with the other nucleotide.

There are two ways in which nucleotide phosphates are transformed into activated compound **6**. The most common methods use 1,1'-carbonyldiimidazole (CDI) or morpholine or piperidine to transform the nucleotide phosphate into a nucleotide-5'-phosphoramidate[61] (Figure 16). This intermediate is not isolated but is coupled with a second nucleotide at room temperature. The phosphoramidate derivates have frequently been utilized as useful intermediates in the coupling of phosphates groups[60].

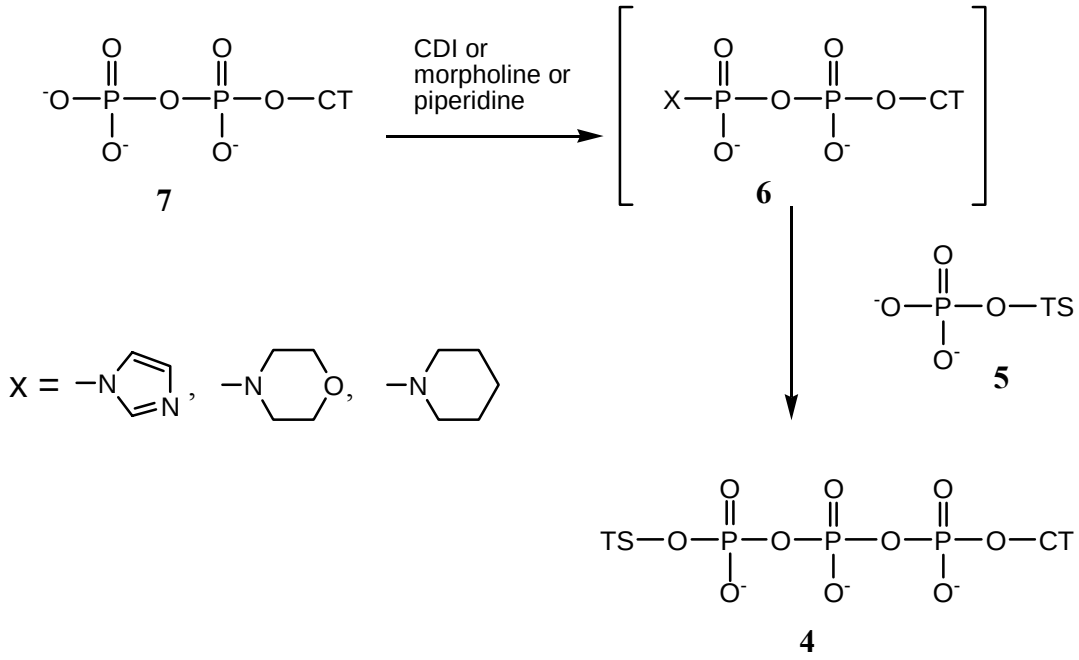


Figure 16. Activation of a nucleotide phosphate in coupling reactions with phosphorammidate method.

Another method consists in the activating of the phosphate by reaction with diphenyl chlorophosphate, thus forming an intermediate **6** whose terminal phosphate can be easily replaced by the monophosphate **5** (figure 17).

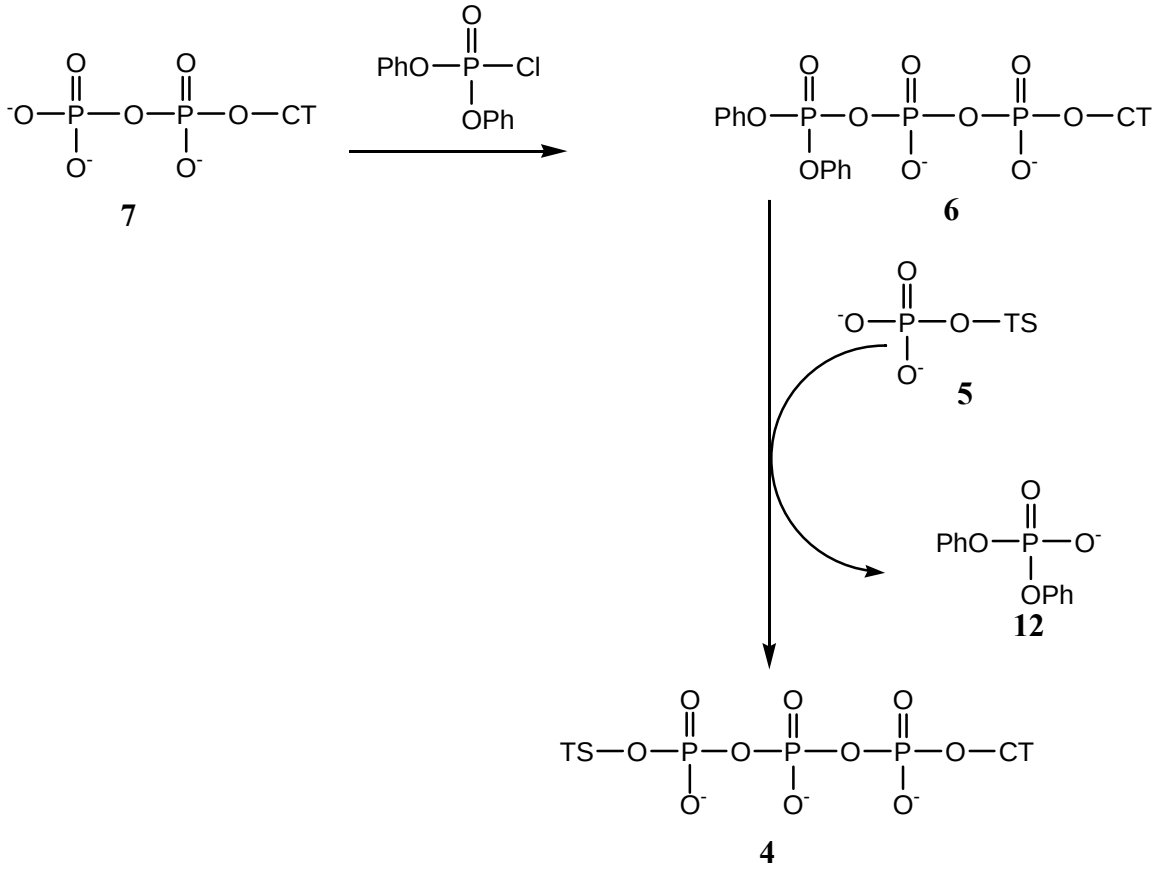


Figure 17. Activation of a nucleotide phosphate in coupling reactions with diphenyl chlorophosphate.

The yield is always low with both methods and never exceeds 50%. The most frequent method in litterature has therefore been used, employing CDI as reagent in order to activate diphosphate **7** to phosphoramidate **6**, since it generally yields more[61].

Using the same activation methods it is possible to obtain the diphosphate **7** from monophosphate **10** and orthophosphate **9**.

Monophosphate nucleotides **10** are not often available, especially in case of modified nucleotides and, when their synthesis is required. According to a common procedure, phosphorus oxychloride ($POCl_3$) is used with a polar solvent, such as trimethylphosphate o triethylphosphate, and is then hydrolyzed to form a monophosphate nucleotide. This procedure was first used by Yoshikawa and co-workers who also discovered that the phosphorylation rate of nucleosides was accelerated by using trimethyl- or triethylphosphate as solvent[62]. This method is practically the only way used to prepare nucleoside monophosphate, because this monophosphorylation is selective for the 5'-hydroxyl group of the sugar moiety in unprotected nucleosides (Figure 18).

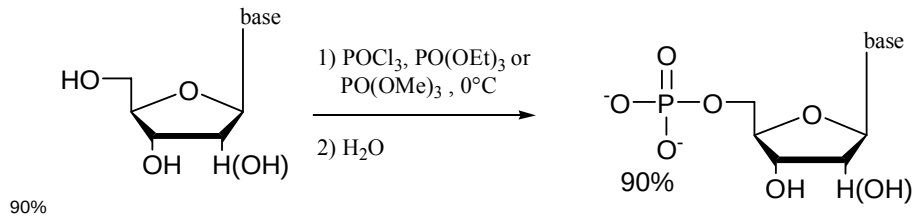


Figure 18. Monosphosphates synthesis.

The major difficulties of the above mentioned reactions lie in the preparation of reagents and in the purification of the reaction's results. Phosphates are too polar compounds to be solubilized in usual organic solvents (e.g. dimethylformamide), whereas this would be required in order for the reaction to take place. The most common way to overcome the problem is to transform the phosphate into its tributylammonium salt: the ionic pair phosphato-tributylammonium is now soluble in DMF and therefore enables a reaction.

This transformation is made possible by first converting the sodium phosphate salt into the pyridinium salt by chromatography on cationic exchange polystyrenic resin in pyridinium form (eluant water:methanol 1:1). The solution, reduced to small volume, is then treated with 1 mol equiv of tributylamine/mol of phosphorus. At the end of this procedure, it is necessary to completely remove the water from the tributylammonium salt, before starting a new reaction. Water can in fact react with phosphoramidate to give the deactivated reagent (Figure 19).

Coevaporation with anhydrous pyridine and anhydrous dimethylformamide is usually necessary to remove all water. In order to increase the anhydricity of the reaction condition we have also used molecular sieves in the reaction's flask both for diphosphates and triphosphate synthesis.

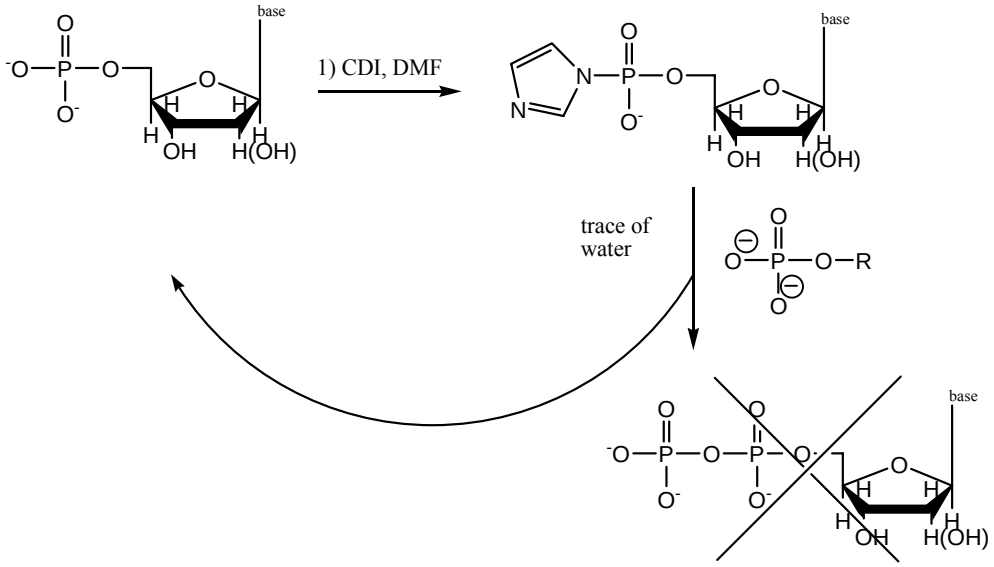


Figure 19. Reaction of phosphoroamidate in presence of water.

2.3 3'-O-nitro-2'-deoxycytidine

This non conventional nucleoside analogue is not commercially available. It has therefore been prepared using a known procedure[58]. The nucleoside analogue was prepared in five steps starting from 2'-deoxyuridine **12** (Figure 20). The 2,3'-anhydro-5'-O-benzoyl-2'-deoxyuridine **13** was prepared by a one-pot tandem Mitsunobu reaction, using triphenylphosphine, diisopropyl azodicarboxylate and benzoic acid in 82% yield. The ring was opened with ammonium nitrate in DMF to form the O-nitrate esters **14** in 25% yield which was then reacted with 1,2,4-triazole and 4-chlorophenyl dichlorophosphate in pyridine to afford the corresponding 4-(1,2,4,-triazolo) derivate **15** in 72% yield. Subsequent treatment of **15** with aqueous ammonia in dioxane yielded the 5'-O-benzoyl-3'-O-nitro-2'-deoxycytidine **16** (53%), which on deprotection with NaOMe in methanol afforded the target 3'-O-nitro-2'-deoxycytidine **17** in 63% yield.

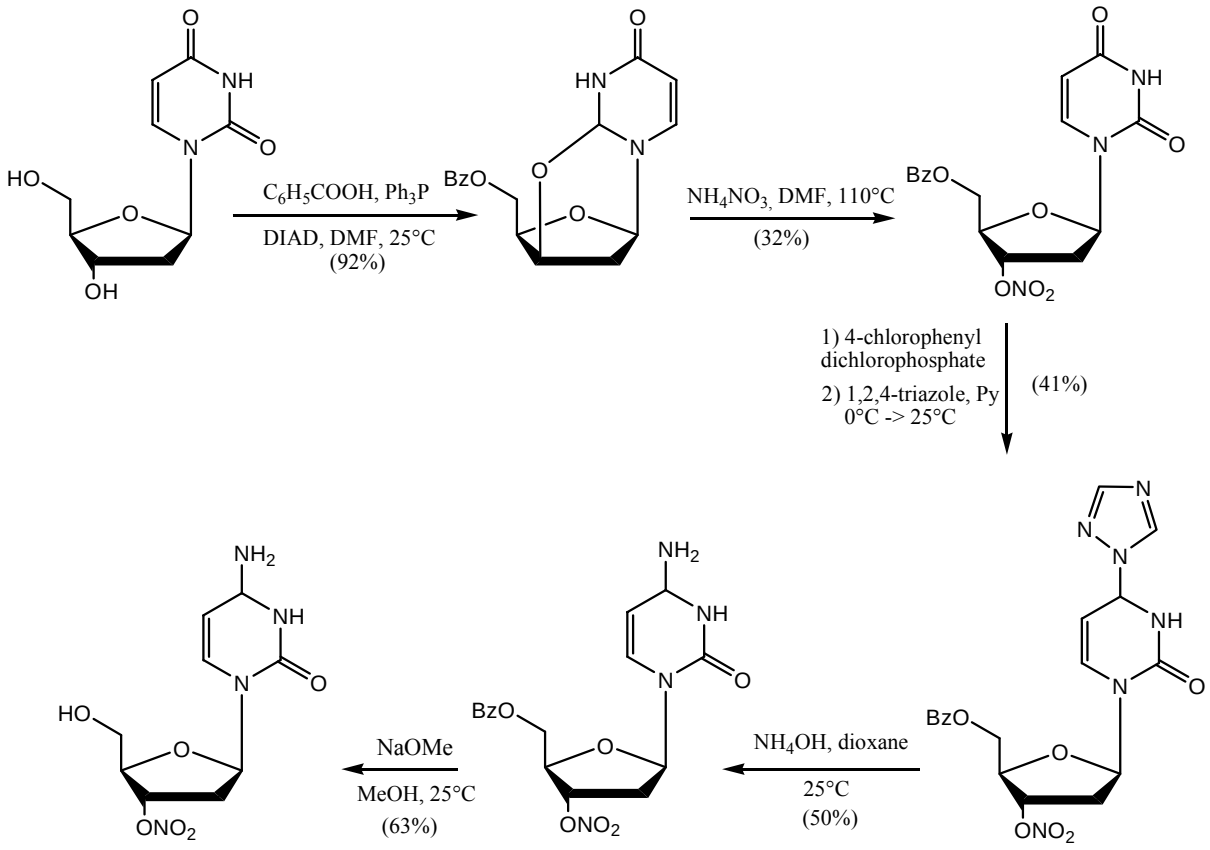


Figure 20. Synthesis of 3'-O-nitro-2'-deoxycytosine.

2.4 Monophosphates synthesis

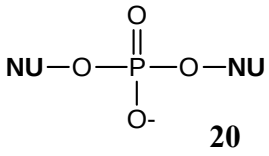
The first synthetic targets were the monophosphate compounds. Different compounds, both nucleosidic and non-nucleosidic, have been used for the implementation of the monophosphates synthesis and their purification.

Aiming at studying the monophosphatation of acyclovir, 2-benzyloxy-ethanol **18** and 2-((1H-benzo[d]imidazol-1-yl)methoxy)ethanol **19** have been used as starting substrates, in order to establish reaction conditions and in particular purification methods. The synthesis of **19** was performed as reported from Yavorskii and co-workes[63]. The synthesis of monophosphates with phosphorus oxychloride and the above mentioned substrates has been made at 0°C in diethyl ether or THF, which turns out to be better for the solubilization of reagents, and the work-up has been made using a solution of $NaHCO_3$.

A first purification of the monophosphate **18**, made by DEAE-cellulose chromatography, allowed the recovery of some fractions containing the desired monophosphate contaminated by ortophosphoric salt, which was eliminated as barium salt precipitation from water/ethanol solution adding $BaCl_2$.

The monophosphatation reaction of nucleosides analogues (**ACV**, **AZT**, **ddC**, **araC** and **GCV**) have been performed using $PO(OEt)_3$ as solvent. The work-up has been made using $NaHCO_3$ (acquous, 5%) and than adjusting to pH 7 with NaOH 1M. The purification method with barium salt cannot be used for nucleosides. These compounds precipitate as barium salt together with orthophosphate. Therefore, in order to obtain a proper purification in these cases, the operation must be repeated twice through DEAE-cellulose.

This monophorilation method presents a disadvantage that is not enough mentioned in the literature: the formation of a phosphoric diester **20** resulting from the double reaction of nucleoside with $POCl_3$.



20

Figure 21. Phosphoric diester

Even though the reaction is carried out at 0°C by adding a nucleotide to an excess of $POCl_3$ in triethylphosphate, the substition reaction to the phosphorus oxychloride has a speed similar to that of the monosubstituted intermediate. In order to solve this problem the reaction was performed at -10°C, with a lower concentration of reagents. Thus, the formation of the by-product was minimized.

Using this standard procedure a library of nucleoside monophosphate not commercially available has been prepared (Table 1).

The preparation of ganciclovir monophosphate (**GCVMP**) required the protection of one of the hydroxyl groups before performing the reaction with $POCl_3$ in order to prevent the diphosphorilation. The protection was performed with acetate group. The mono-O-acetate ester of ganciclovir **22** was prepared by reaction of ganciclovir with an excess of trimethyl orthoacetate followed by acidic hydrolysis of the cyclic orthoester **21** intermediate[64] (Figure 22).

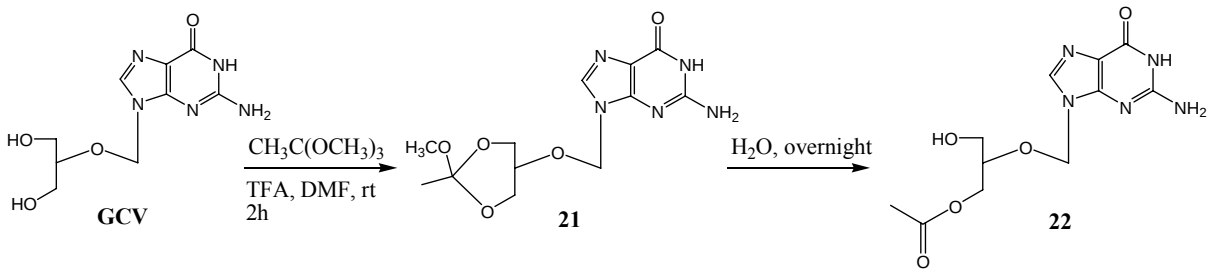


Figure 22. Selective monoprotection of ganciclovir.

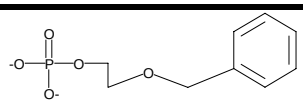
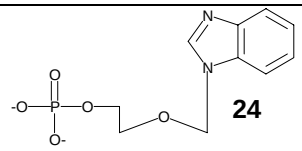
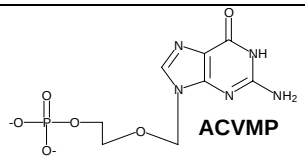
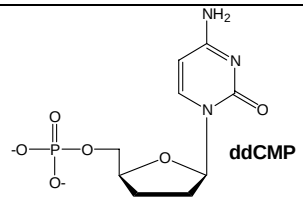
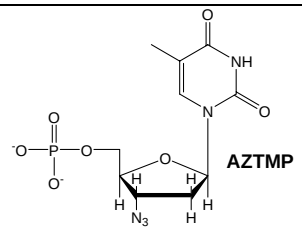
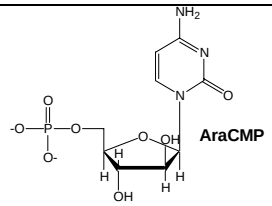
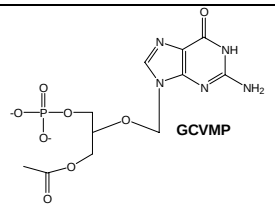
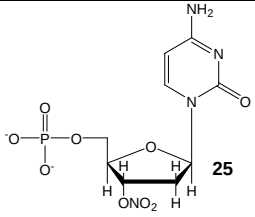
Entry	Starting material	Product	yield
1	**18**	 **23**	70%
2	**19**	 **24**	60%
3	**Acv**	 **ACVMP**	59%
4	**ddC**	 **ddCMP**	59%
5	**AZT**	 **AZTMP**	40%
6	**AraC**	 **AraCMP**	50%
7	**GCV**	 **GCVMP**	60%
8	**17**	 **25**	21%

Table 1. Library of monophosphate.

2.5 Nucleotide diphosphate's synthesis.

The most common ways to prepare a nucleotide diphosphate are principally two: either the phosphorylation of its monophosphate with tributylammonium orthophosphate in the presence of carbonyldiimidazole (CDI)[65] or the direct diphosphorylation of nucleosides with tributylammonium pyrophosphate in the presence of dicyclohexilcarbodiimide[66]. The direct diphosphorylation approach was tried for the preparation of **ACVDP**, but no product was recovered from the reaction mixture. Hence for the preparation of every diphosphate a two steps procedure adapted from Hoard and Ott[65] was used. The monophosphate was first transformed into the tributyl ammonium salt as described above. The tributylammonium orthophosphate necessary for the reaction was generated from reaction between anhydrous orthophosphoric acid and tributylammine. Than the two compounds were coupled in presence of CDI (Figure 23).

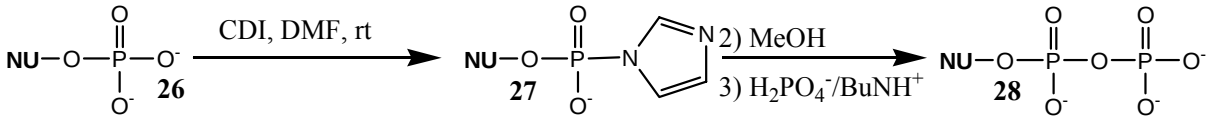


Figure 23. Nucleotide diphosphate's synthesis.

Using this procedure we have the formation of the imidazolidate **27** from a nucleotide monophosphate **26** and excess 1,1'-carbonyldiimidazole (CDI). The reaction is complete in about 2 hours at room temperature. Unreacted CDI is decomposed with methanol, before tributylammonium orthophosphate is added, to eliminate formation and subsequent necessity for separation of inorganic polyphosphate. Using an excess of orthophosphate the precipitation of imidazolium orthophosphates has taken place. The phosphorylation is essentially complete within 16 hours, and the product is purified by anion-exchange chromatography on DEAE-cellulose.

Results obtained with various monophosphates are given in Table 2. The transformation of the nitro-cytosine monophosphate **25** and ganciclovir monophosphate **GCVMP** into diphosphate did not take place. These results are not very clear. In the case of **GCVMP** we found that during the work-up the acetate group was last and the free hydroxyl group could probably interfere during the coupling reaction.

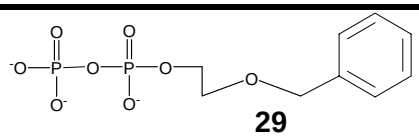
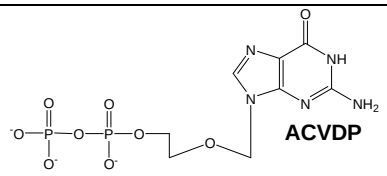
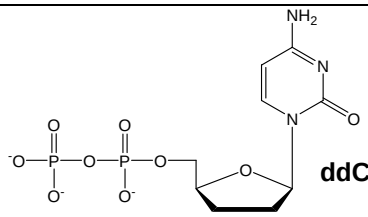
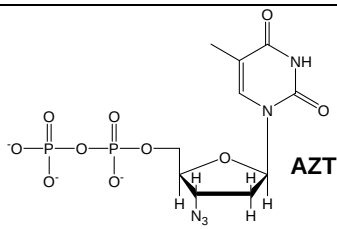
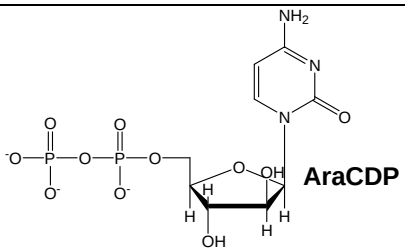
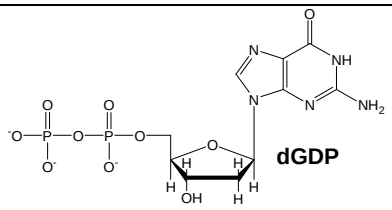
Entry	Starting material	Product	yield
1	**23**	 **29**	76%
2	**ACVMP**	 **ACVDP**	70%
3	**ddCMP**	 **ddCDP**	70%
4	**AZTMP**	 **AZTDP**	40%
5	**AraCMP**	 **AraCDP**	62.5%
7	**dGMP**	 **dGDP**	40%

Table 2. Library of diphosphates.

2.6 Dinucleotide triphosphate synthesis.

The preparation of dinucleotides was performed with a procedure similar to that used for the preparation of nucleotide diphosphates. The phosphoroimidazolidates resulting from activation of nucleoside diphosphates with CDI were coupled with an excess of nucleoside

monophosphate commercially available. Both the nucleotide diphosphate and nucleotide monophosphate were first transformed in the tributylammonium salts using standard procedure.

Also in this case the reaction was performed in 16 hours and the yields ranged between 30% and 60%.

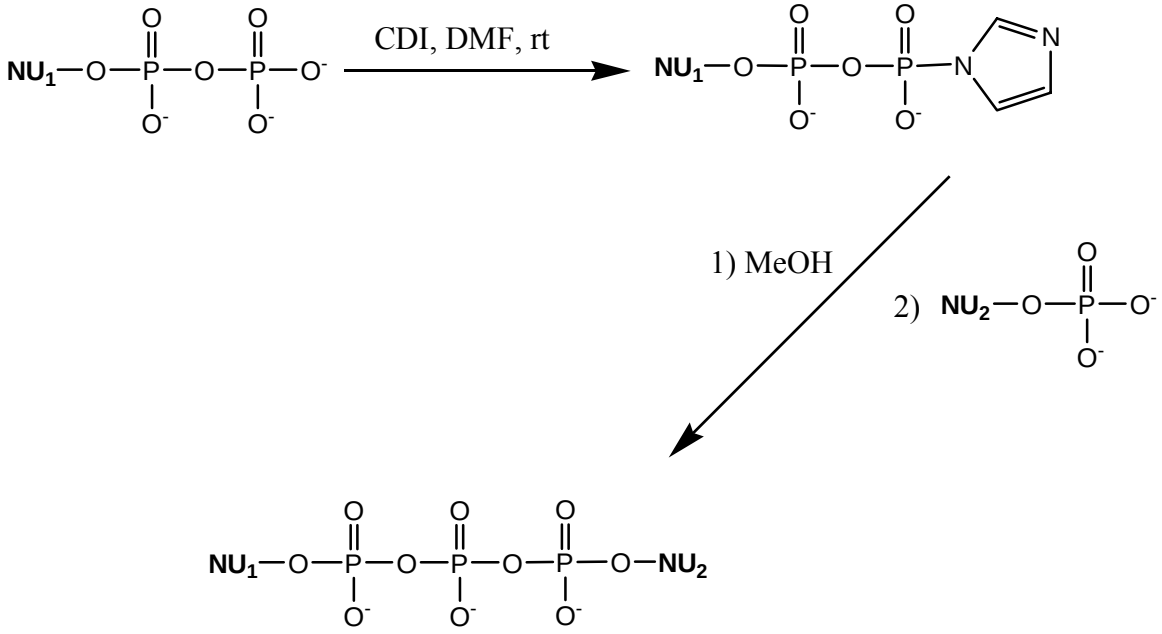


Figure 24. Synthesis of DNOPs.

The library of eight different dinucleotide triphosphates reported in Table 3 was prepared using these procedure. As TS group 2'-deoxycytidine was also used. This nucleoside is the only deoxynucleotide not used from telomerase. The synthesis of a dinucleotide triphosphate with this moiety instead of a telomerase substrate was necessary to verify the prodrug's activity mechanism.

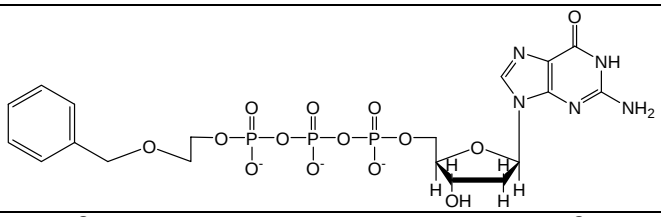
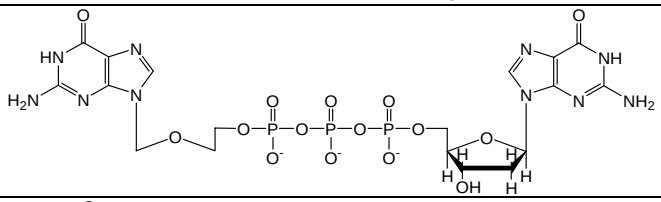
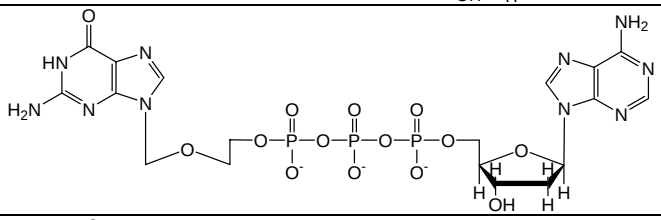
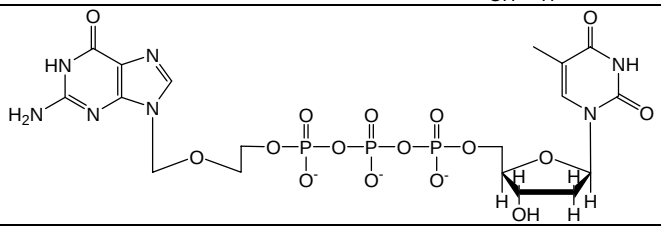
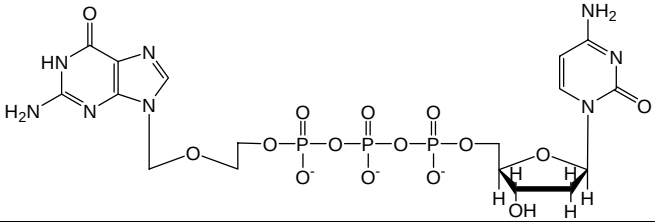
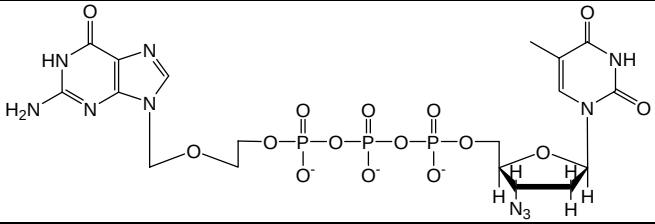
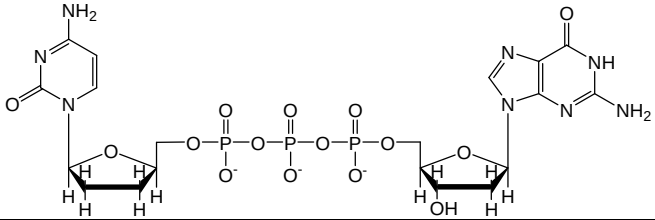
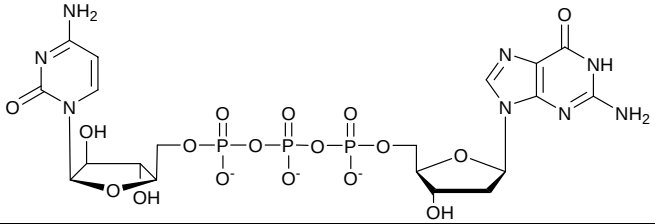
entry	CT- DP	TS-MP	CT-TP-TS (yield)	Structure
1	**29**	dG-MP	Ph-TP-dG (28%)	
2	ACV-DP	dG-MP	ACV-TP-dG (59%)	
3	ACV-DP	dA-MP	ACV-TP-dA (39%)	
4	ACV-DP	T-MP	ACV-TP-T (46%)	
5	ACV-DP	dC-MP	ACV-TP-dC (39%)	
6	ACV-DP	AZT-MP	ACV-TP-AZT (33%)	
7	ddC-DP	dG-MP	ddC-TP-dG (28%)	
8	AraC-DP	dG-MP	AraC-TP-dG (18%)	

Table 3. Library of triphosphates.

2.7 Synthesis of β-Phosphonates

The synthesis of dinucleotide triphosphate analogues with a phosphonate in β position has proved to be difficult, because of the lower reactivity of the phosphonate in comparison with normal phosphate in the coupling processes.

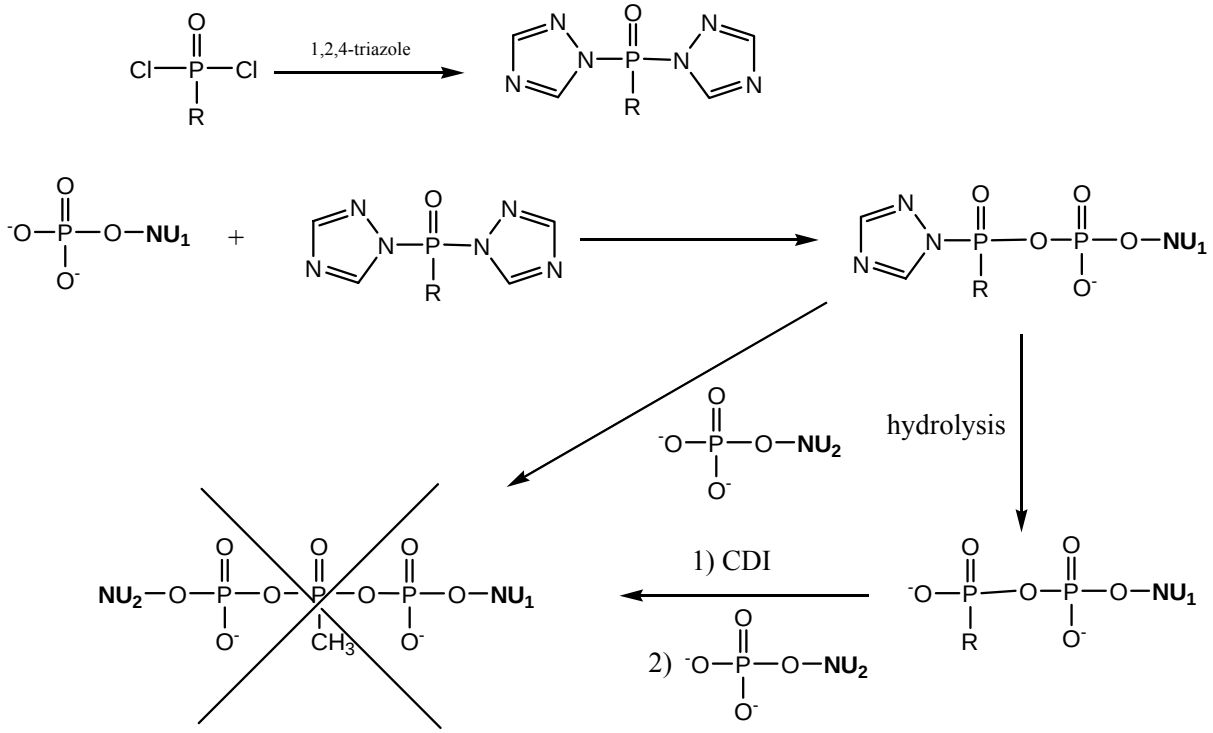


Figure 25. Phosphonate's synthesis.

At first we used a modified procedure by Alexandrova[56] (Figure 25), according to which methylphosphonic dichloride was activated with triazole .The first tributylammonium salt of monophosphate nucleotide was added to the solution of methylphosphonic bis(1,2,4-triazolide), followed after 1 hour by the second nucleotide. Unfortunately the reaction was not successful and stopped at the first substitution.

In a second attempt the β-phosphonate nucleoside was again activated with CDI and the second nucleoside added, but again no coupled product was obtained.

Believing that the intermediate β-phosphonate has a low reactivity towards a substitution reaction with phosphate, we then activated the monophosphate nucleoside with CDI and used the phosphonate as nucleophile. Unfortunately the result was not successful again. The above mentioned attempts showed a low reactivity of a phosphonate, both as electrophile and as nucleophile. Also changing methylphosphonic dichloride with phenylphosphonic dichloride did not lead to any positive result.

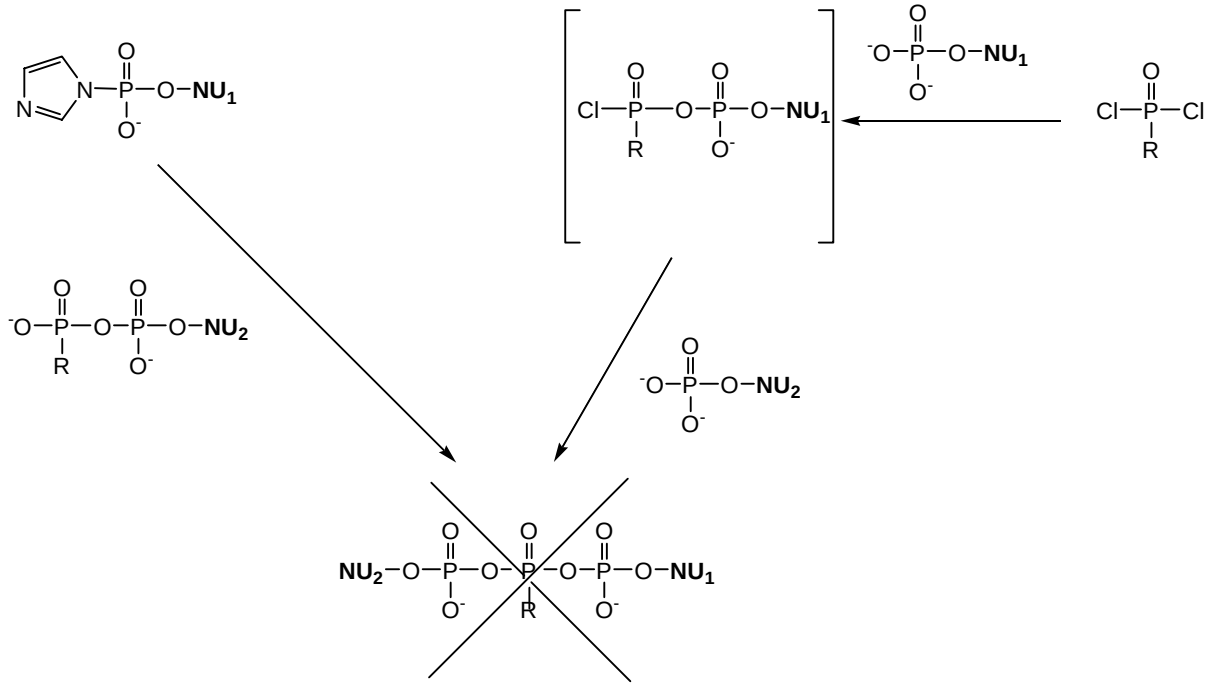


Figure 26. Different strategy for phosphate's synthesis.

3 EVALUATION OF PRODRUGS ACTIVITY *IN VITRO*

3.1 General considerations

Every compound prepared was then evaluated for growth inhibitory activity against different cancer cell lines using different methods: viability and proliferation methods.

Cell viability, related to cell activity, was evaluated measuring the ATP present in the cell by a spectrophotometic analysis. High level of ATP means high cell viability.

Cell proliferation was evaluated measuring the bromo-deoxyuridine incorporated in DNA. High quantity of BrdU incorporated during the DNA synthesis means high proliferation activity.

The tests were performed using two different protocols. As shown in Table 4, according to protocol A, the addition of prodrug was done two days after cells seeding and the activity measurement after five days. In protocol B the addition and measurement were performed after one day and two days respectively.

Day	0	1	2	3	4	5
Protocol A	S		A			M
Protocol B	S	A	M			

Table 4. Protocol used in cell test. S = Cells seeding; A = Prodrug addiction; M = Measurement.

3.2 Studies on cytotoxic activity of the prodrugs ACV-TP-*TS*

The first prodrug used to measure the cytotoxic activity toward cancer cells was the **ACV-TP-dG**. A preliminary test of this prodrug candidate (using protocol A on colo-rectal carcinoma cell line SW620) showed a lower growth of treated cancer cells (Figure 27) in comparison to the control colture (Figure 28).

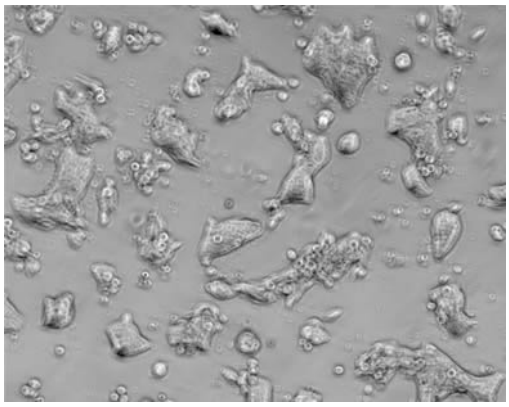


Figure 27. SW620 colo-rectal carcinoma cells tested with ACV-TP-dG

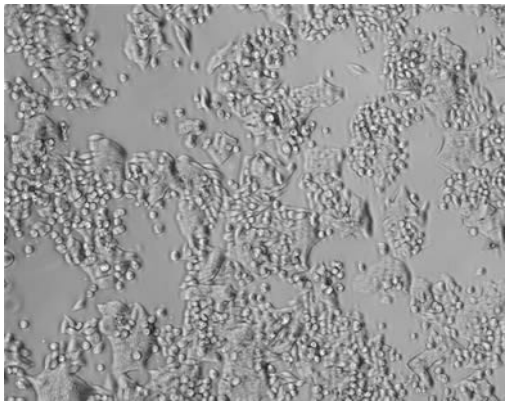


Figure 28. SW620 colo-rectal carcinoma cells .

The same **ACV-TP-dG** was then tested using protocol A once again and measuring the viability at different concentration of prodrug on different cancer colo-rectal carcinoma cell lines. These kind of cells were chosen because colo-rectal carcinoma cell lines have generally high telomerase activity.

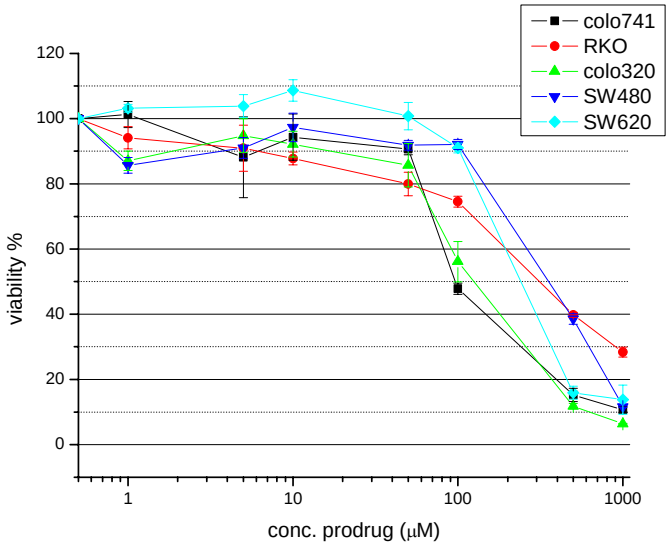


Figure 29. Cell viability on 5 colo-rectal carcinoma cell lines of **ACV-TP-dG** (protocol A).

The test showed a moderate cytotoxicity towards this kind of cells: the IC50s were between 100 and 350 μM (Figure 29). These were the first interesting results on activity of the prodrug towards cancer cells.

In order to verify if telomerase is involved in the activity of the prodrug, studies of base's effect were made. It is known[12] that the telomerase uses series of TTAGGG to build telomeres. This means that our prodrug should be hydrolyzed by telomerase releasing three cytotoxic compounds for every **ACV-TP-dG**, two for every **ACV-TP-T** and only one for **ACV-TP-dA**. The viability of the prodrugs **ACV-TP-dA** and **ACV-TP-T** (Figure 30) was measured using the same protocol. It is interesting to note that the activities of **ACV-TP-dG** and **ACV-TP-T** are high in comparison with **ACV-TP-dA**. As expected, the prodrug toxicity follows the order indicated above with **ACV-TP-dG** and **ACV-TP-dA** being the most and the least toxic compounds respectively.

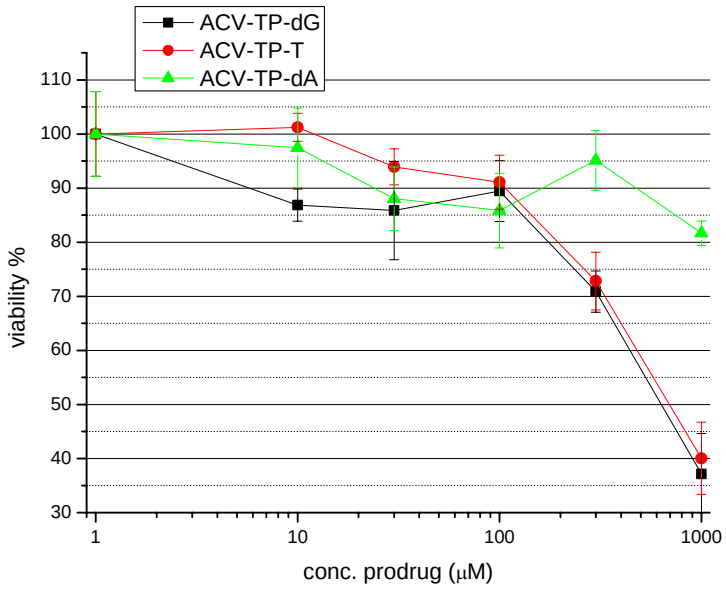


Figure 30. Cell viability on SW620 colo-rectal carcinoma cell lines with different TS groups (protocol A).

Similar results were obtained using the protocol B and measuring both cell viability and proliferation (Figure 31). Moreover, if the TS group is not recognized by the telomerase, as in the case of **ACV-TP-dC**, the cytotoxicity is even lower. These results could be a prove of the involvement of telomerase on the prodrug's activation.

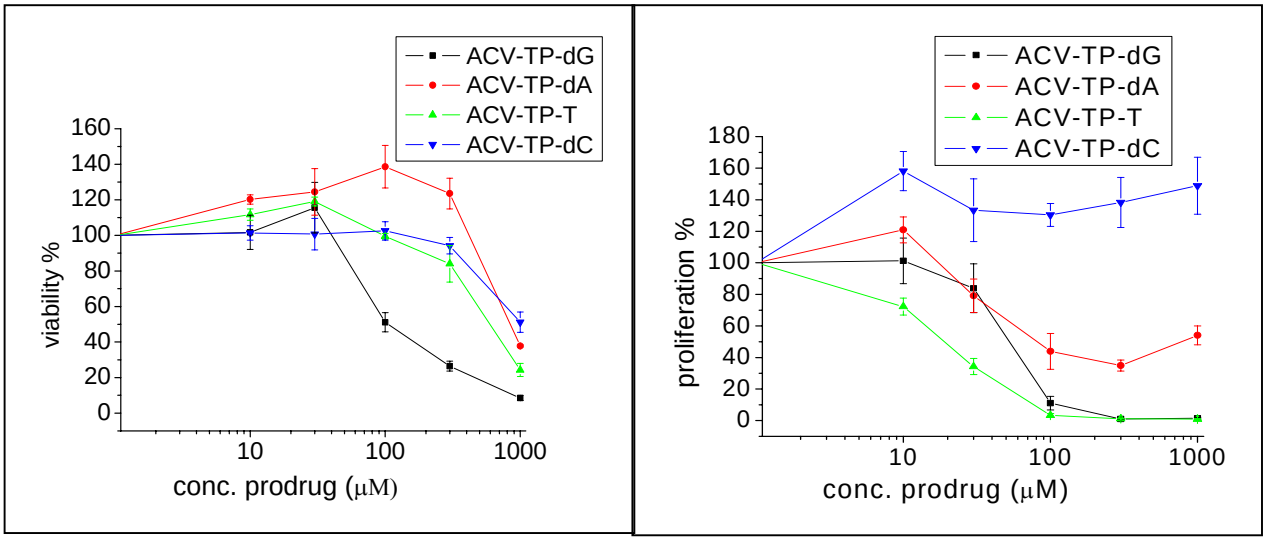


Figure 31. Cell viability and proliferation of the four **ACV** prodrugs on colo320 colo-rectal carcinoma cell line (protocol B).

The tests show that the most active compound is **ACV-TP-dG.** Moreover, the activity measured in protocol B was higher than that in protocol A. These results pushed us to determine the activity of the prodrug within 52 hours. The cytotoxicity of **ACV-TP-dG** was compared with that of the 5-fluorouracil, an antimetabolite drug used in anticancer therapy. The test showed that the prodrug immediately stops the proliferation, but after a few hours the cells start growing again. On the other side 5-fluorouracil only showed activity after more than 24 hours (Figure 32). These different activities can be due to the different activity mechanisms of the two molecules. The 5-fluorouracile acts by an uptake into target cells, followed by an intracellular conversion to 5-fluorodeoxyuridine-5'monophosphate which covalently binds to thymidylate synthase, thereby blocking the synthesis of **dTTP** and hence the replication of DNA. For this reason the activity of 5-fluorouracil only showed after several hours.

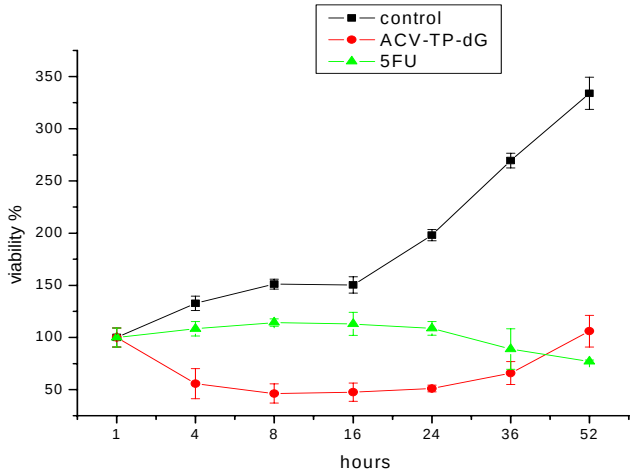


Figure 32. Cell viability with respect to control during 52 hours of **ACV-TP-dG** (100 μM) and 5-fluorouracil (5FU, 100 μM) on SW620 colo-rectal carcinoma cell line.

According to the activity mechanism supposed, the prodrug **ACV-TP-dG** should initially be hydrolyzed freeing the **ACVDP**, which is being transformed from a kinase into acyclovir triphosphate (**ACVTP**), and therefore incorporated in the DNA chain, thereby blocking its synthesis. The resistance shown towards the prodrug after 48 hours, could be due to DNA repair mechanisms normally active in the cells.

It was therefore necessary to use protocol B in order to evaluate the highest cytotoxicity of the prodrug on different cell lines.

Applying this protocol, the **ACV-TP-dG** was used on other cell lines to verify its activity on different kinds of tumors: SW620, SW480, colo320 and colo741 between colo-rectal carcinoma cell lines, HT1080 as fibroblastoma cell line and HeLa A1 for cervical cancer cell line. **ACV-TP-dG** was also tested on 18Co normal colon epithelial cell line. All these tests showed that the cytoxicity of the prodrug is higher for cancer cells than for healthy ones, where the telomerase activity is not present (Figure 33). This is an important result, both because an anticancer drug must have low toxicity towards healthy cells and because it is another evidence that the telomerase is involved in the activity of prodrugs.

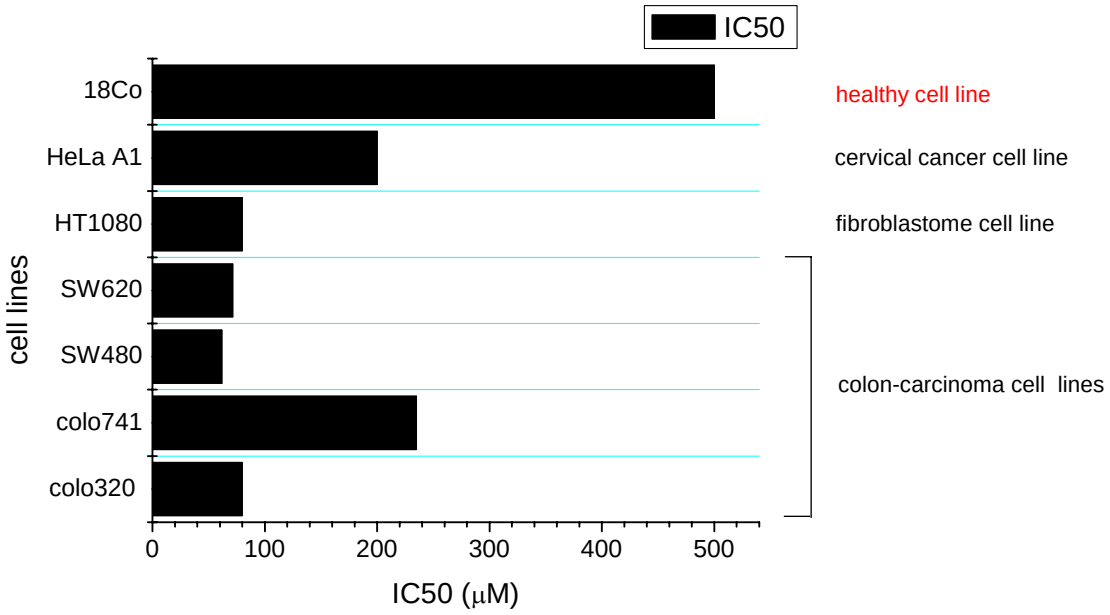


Figure 33. IC50 on various cell lines of **ACV-TP-dG** (protocol B).

It is important to note that, in order to be active, the prodrug needs to retain its original structure which allows it to cross the cellular membrane. Indeed, when we treated SW620 cells with the fragments **ACV-MP** or **ACV-DP**, no activity was detected, thus indicating that the hydrolyzed fragments are not able to enter the cell. The addition of lipids is effective in helping the membrane crossing in the case of **ACV-MP** and **ACV-DP**, while the **ACV-TP-dG** compound shows the same activity both in the presence and in the absence of lipids. This clearly indicates that the cytotoxicity of our prodrugs is due to its structural features that allow them to enter the cell and to release the active drug once inside, due to the telomerase activity (Figure 34).

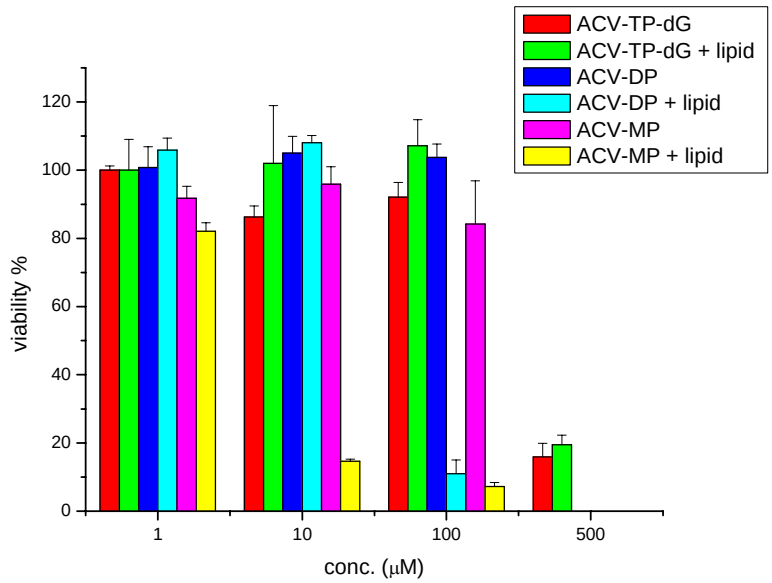


Figure 34. Effect of liposome on cell viability of SW620 colo-rectal cancer line with different cytotoxic compounds (protocol A).

3.3 Studies on different CT portion

The change of the **CT** portion (**ACV**) maintaining the same **TS** (**dG**) fragment has also been tested. The toxicity of a series of compounds, **ddC-TP-dG** and **AraC-TP-dG** in addition to **ACV-TP-dG**, was analyzed obtaining similar results in terms of cell vitality (Figure 35), but interestingly better in terms of cell proliferation for **AraC-TP-dG** (Figure 36). This can be attributed to the fact that the **CT** of the new prodrug, **AraC**, is endowed with a different cytotoxic activity mechanism in comparison with **ACV** and **ddC**. In fact **ACV** and **ddC** are chain terminators while **AraC** is an antimetabolite agent that is able both to inhibit the ribonucleotide-reductase causing a lack of deoxynucleotide into the cell and to terminate the synthesis of DNA. Thus the cell is not able to synthesize new DNA, and the proliferation is then completely inhibited.

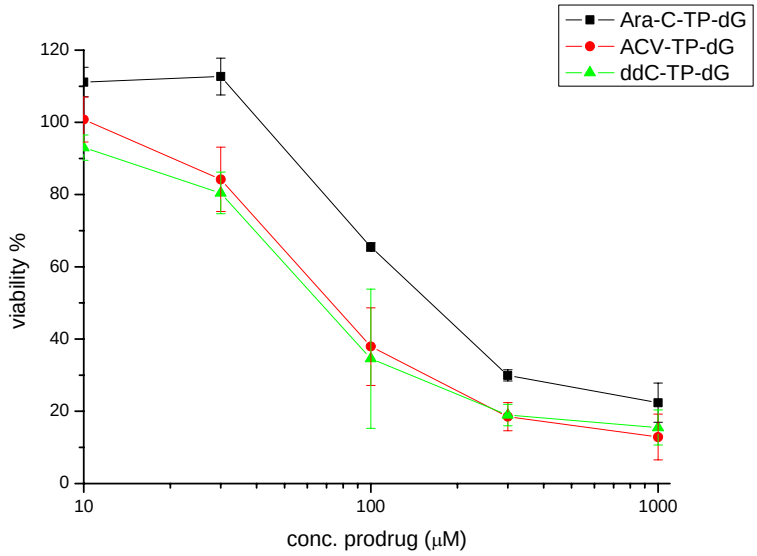


Figure 35. Cell viability of colo 320 cells treated with prodrugs showing different CT moiety (protocol B)

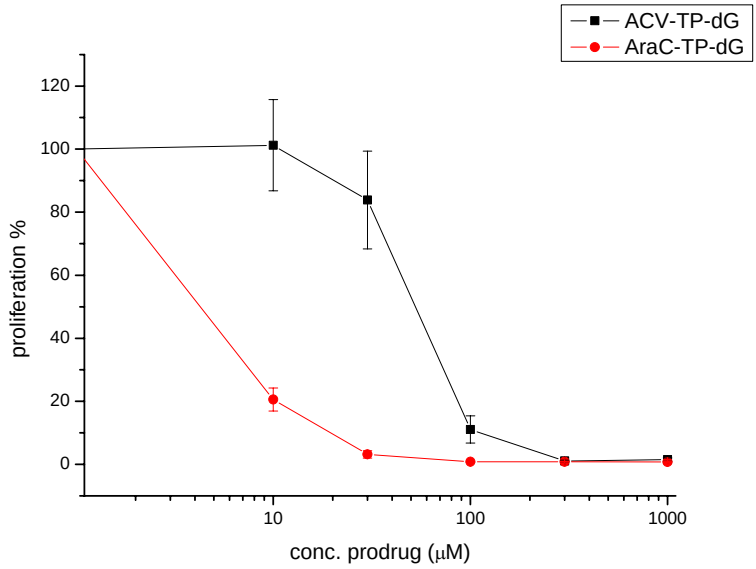


Figure 36. Cell proliferation of colo 320 cells treated with prodrugs showing different CT moiety (protocol B).

On the whole, the results obtained with prodrug **ddC-TP-dG** were quite similar to those obtained with **ACV-TP-dG**: this shows that, if the activity mechanism of the cytotoxic compound freed in the cell is the same, the cytotoxicity values are similar.

Further tests made with **AraC-TP-dG** also showed, by and large, a noteworthy difference in the IC50 values, measured according to viability or proliferation (Figure 37). In particular, it seems clear that this molecule blocks cell proliferation more effectively if compared with the

other prodrugs synthesized and tested, rather than reducing its viability and hence causing its apoptosis.

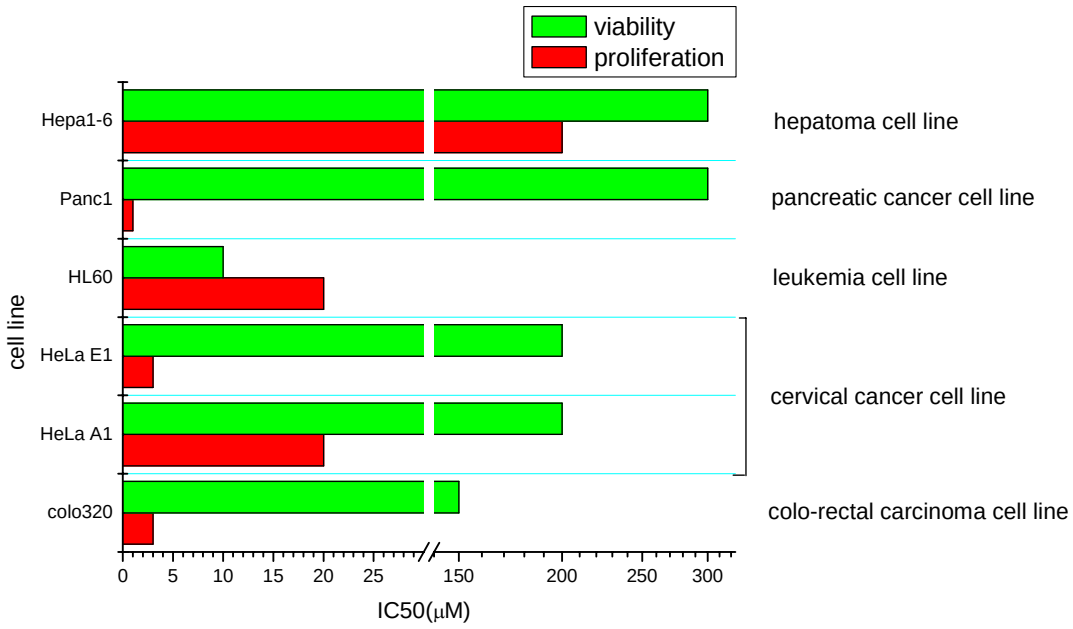


Figure 37. Values of IC50 measured with cell viability or proliferation in different cancer cell treated with **AraC-TP-dG**.

Of particular relevance were the results obtained with pancreatic cancer cell lines (Panc1, IC50 = 1 μM), a kind of cell line which is particularly resistant to treatments with usual anticancer drugs. The results obtained with leukemia cell line were the only ones that had a good IC50 value with both methods, viability and proliferation.

The IC50 values obtained by proliferation methods on the two cell lines HeLa showed an activity of the **AraC-TP-dG** molecule about 6-7 times higher in HeLa E1 cell line rather than in HeLa A1. The two lines differ from each other only because in HeLa E1 the telomerase activity is about 25 times higher than in HeLa A1[67]. Hence, the fact that the prodrug is more active towards the first cell line can further confirm the activity mechanism of the prodrug.

This interesting result with **AraC-TP-dG** prompted us to use this prodrug in tests on leukemia cell line in comparison with arabinocytosine (**AraC**). **AraC** is an antimetabolite agent used in antileukemia therapy. It is, at the moment, the most efficient drug used for this kind of disease, but has many side effects.

The tests on HL60 leukemia cell line showed similar cytotoxicity for the two compounds. The results of the viability tests and proliferation test are shown in Figure 38. This result could be

of some importance if, together with a high antiproliferation activity, a lower toxicity *in vivo* with respect to AraC were detected.

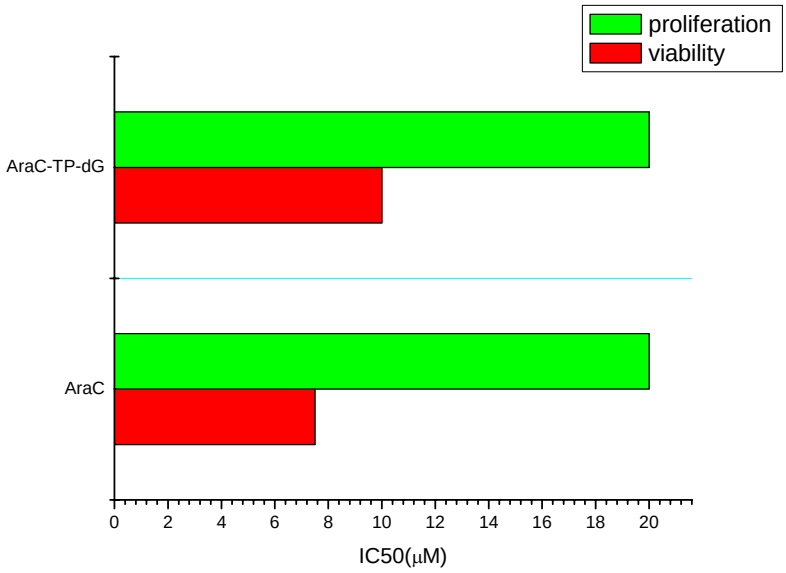


Figure 38. Comparison between activity of AraC-TP-dG and AraC in HL60 leukemia cell line using viability and proliferation assays.

3.4 Short summary

According to the tests made so far, telomerase seems involved in the activity mechanism of the prodrug. This can be firstly proven by the fact that the **ACV-TP-dC**, when the TS group is 2'-deoxycytidine (i.e. the nucleoside which is not recognized by telomerase), shows very low toxicity (Figure 31). As a matter of fact if the compound were hydrolyzed by a DNA-polymerase or by any phosphatase instead of by telomerase, the toxicity of the molecule would not depend on the type of TS group chosen, since these enzymes can indifferently use any nucleoside (dG, dA, dC or T) as substrates.

Another positive sign is the lower toxicity of these molecules on healthy cells, even though the usual anticancer drugs are normally more active towards cancer cells, because of their cellular activity, which is higher than in healthy cells.

Even the different toxicity found in HeLa E1 and HeLa A1 cells (in HeLa E1 the telomerase activity is almost 25 times higher than in HeLa A1), treated with AraC-TP-dG prodrug, is an evidence that strengthen our hypothesis.

The functioning of the AraC-TP-dG prodrug is also interesting, since its anti-proliferative activity turns out to be very high towards all cell lines tested, with IC value lower than 5μM and therefore close to the value of the common anticancer agents (usually more toxic towards healthy cell).

4 MICE TESTS

To complete the biological test, the prodrugs **ACV-TP-dG** and **AraC-TP-dG** were tested *in vivo* on mice in order to evaluate their toxicity and activity. These two molecules were chosen because of their more interesting IC50 values, as shown by the *in vitro* results. The prodrug **ACV-TP-dG**, in particular, turned out to be the most active towards tumors of the colo-rectal carcinoma cell line, while the results obtained with **AraC-TP-dG** on leukemia cell line were very interesting if compared with the **AraC** activity.

4.1 Activity *in vivo* of ACV-TP-dG

Preliminary tests on healthy mice showed a low acute and chronic toxicity: the LD50 in mice is between 4g/Kg and 8g/Kg, while mice chronically treated with the prodrug (two months, 3 times per week) at a dose of 0,16 g/Kg survived and showed a phenotype of hair loss. Even if the hair loss is a characteristic of many anticancer drugs, it can be related to telomerase activity because it is active in hair follicles.

The prodrug was tested on different mice with different kinds of tumor: two colo-rectal carcinoma human cell lines (colo320 and HT29), a synergic colo-rectal carcinoma cell line (CT26) and a melanoma synergic cell line (B16F10).

The mice were treated at 10 mg/mouse every other day for a total of 10 times.

In all mice the cytostatic effect on tumors measured was modest in comparison with control group as shown below (Figure 39-41).

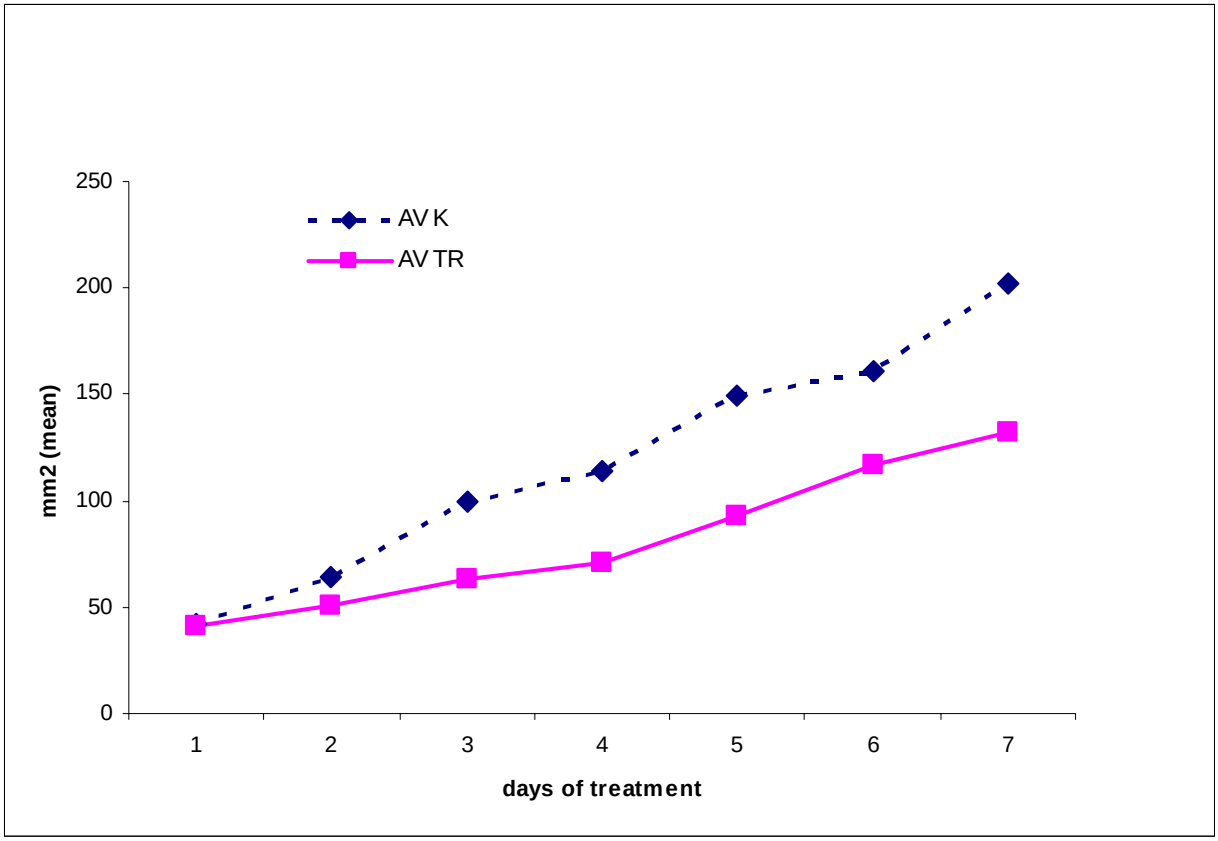


Figure 39. Comparison between control (AV K) and treated with **ACV-TP-dG** (AV Tr) starting from the same level of development nu/nu COLO320 human colo-rectal carcinoma (mean values/group)

As reported in Figure 39, the tumor growth (COLO320, human colo-rectal carcinoma cell line) is only moderately slowed down by the prodrug administration (which starts from the same dimension of the tumor). The worst result was obtained using Balb/c CT26 colo-rectal carcinoma (Figure 40), and in one case an increase in tumor dimension was obtained with respect to control (Figure 41), as though the product was a stimulator for the tumor growth.
The prodrug has therefore shown a modest activity only for the cell line where, according to *in vitro* tests, it turned out to be more active (colo320, Figure 39).

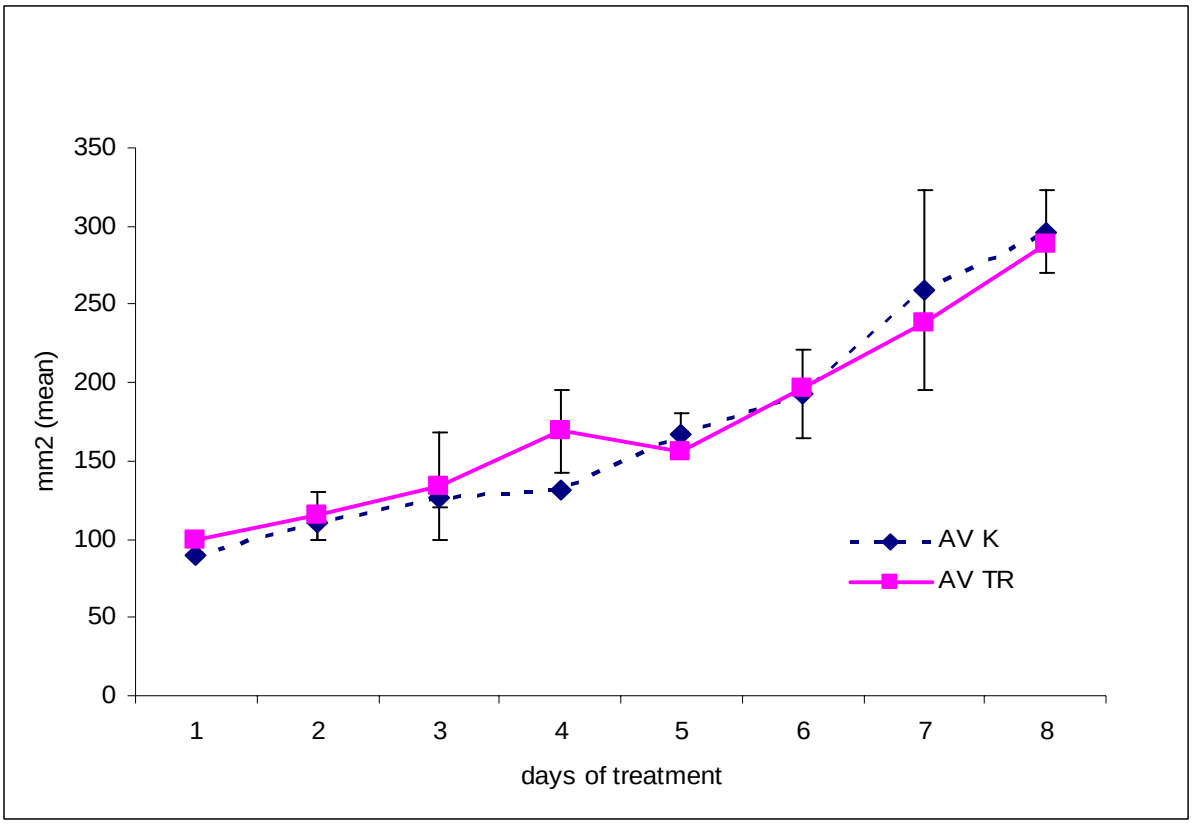


Figure 40. Comparison between control (AV K) and treated with **ACV-TP-dG** (AV TR)starting from the same level of development Balb/c CT26 colo-rectal carcinoma (mean values/group)

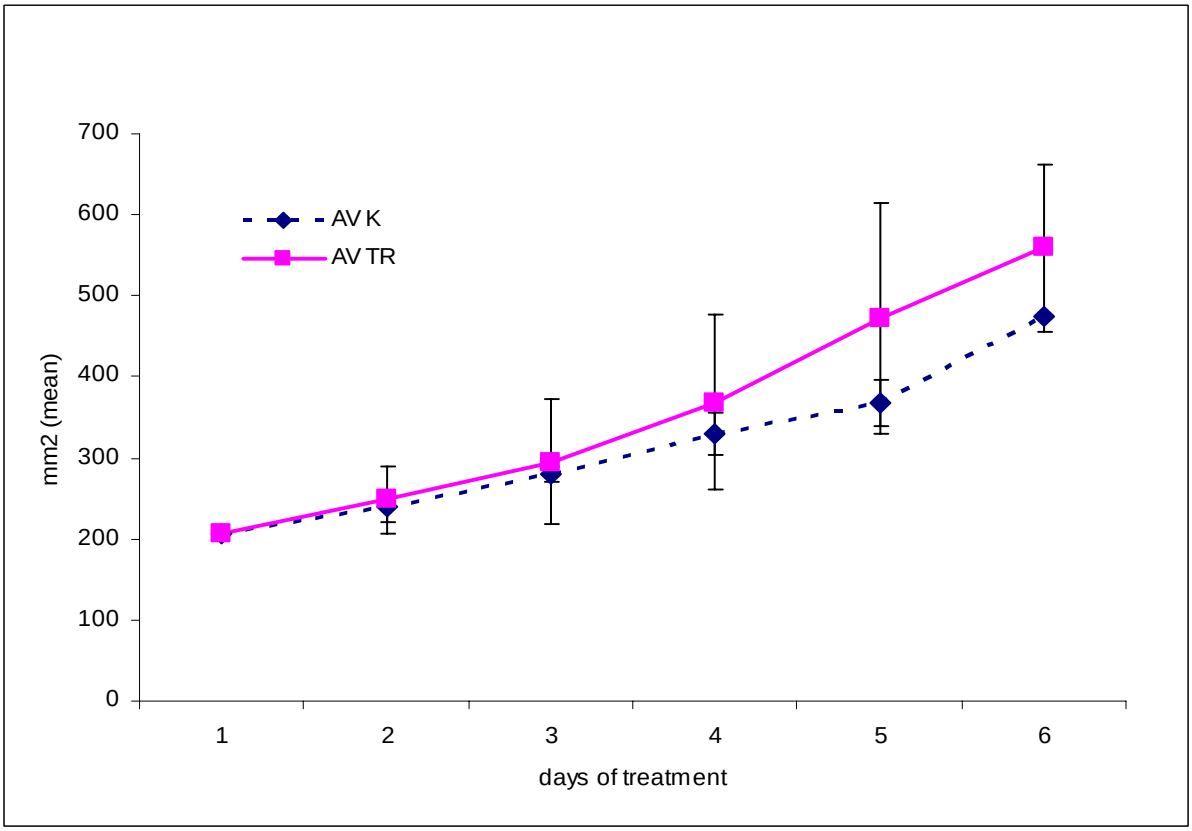


Figure 41. Comparison between control (AV K) and treated with **ACV-TP-dG** starting from the same level of development C57BL/6 B16F10 melanoma (mean values/group)

4.2 Activity *in vivo* of AraC-TP-dG

To evaluate the activity of the prodrug **AraC-TP-dG** *in vivo*, the toxicity in healthy mice and the ability of molecule to suppress the growth of HL60 human cell in nude animals were examined and compared to **AraC**.

To this purpose NOD/SCID mice were chosen. NOD/SCID mice are appropriate for this study because of their genetic immunodeficiency and their capability of tolerating the human leukemia cell inoculated.

For toxicity studies, these mice were treated with two different doses of **AraC** and **AraC-TP-dG**, as reported in Table 5, continuatively repeated for four days. Both concentrations used for the two drugs tested were equimolar. At the end of the treatment (after 3 weeks) all mice had survived.

Groups	Mice for groups	Dose and administration
Control	2	Physiological/i.p./4days
AraC	2	200mg/Kg/i.p./4days
AraC	2	800mg/Kg/i.p./4days
AraC-TP-dG	2	400mg/Kg/i.p./4days
AraC-TP-dG	2	1600mg/Kg/i.p./4days

Table 5. Summary scheme of the treatment made in NOD/SCID mice for the evaluation of pharmacological toxicity of AraC and AraC-TP-dG.

On the whole, the results of the study on toxicity, comparing AraC-TP-dG with AraC, showed:

- similar low toxicity in liver
- similar toxicity in bone marrow
- similar toxicity in kidney
- lower toxicity of AraC-TP-dG on spleen.

Thus, the tested prodrug has an *in vitro* effectiveness similar to that of **AraC** and a similar or lower toxicity especially at spleen level.

Once the prodrug toxicity was evaluated, its pharmacological effectiveness *in vivo* towards leukemia was analyzed. To this purpose, NOD/SCID mice were inoculated with HL60 human leukemia cells and, after 7 days, were treated with intraperitoneal administration of 1g/Kg of **AraC-TP-dG** or of a physiological solution in the control group, continuatively for 4 days.

The first important date for the evaluation of the disease progression was represented by FACS analysis, that was made by estimating the percentage of human leukemia cells with respect to the murine ones in the bone marrow and in peripheral blood of each mouse.
The effect appears particularly evident in the bone marrow where the percentage of human leukemia cells was reduced by over an half in comparison to the control mice (Table 6). These results were similar for the mice treated with **AraC**.

	Bone marrow	Peripheral blood
HL60 + physiological solution	5.3%	12%
HL60 + **AraC-TP-dG**	2.2%	6.2%
HL60 + **AraC**	3.25%	3.2%

Table 6. Percentage of human cells with respect to the murine ones in the bone marrow and in peripheral blood.

With respect to control group the one treated with **AraC-TP-dG** shows a substantial reduction of bone marrow angiogenesis in those groups treated with the prodrug. The leukemia cells are able to increase the degree of bone marrow angiogenesis and hence an alteration of bone marrow functionality. The reduction of the degree of bone marrow angiogenesis implies a better functional bone marrow activity and a reduced capacity of leukemia cells to penetrate in tissue or to produce metastasis.
Moreover, at spleen level, the prodrug treatment shows a clear reduction of leukemia cells. This reduction means that **AraC-TP-dG** is able to reduce the invasion of leukemia cells the outer bone marrow organs.
These dates confirm therefore that the activity of **AraC-TP-dG** can be compared to the activity of **AraC**, even though the former shows a lower toxicity, especially at spleen level.
The prodrug **AraC-TP-dG** was tested also in nu/nu mice inoculated with human colorectal carcinoma cell lines colo320. The mice were treated with intravenous administration (200mg/Kg every 3 day for 10 times) of **AraC-TP-dG** or of a physiological solution in the control group. The administration was started at the appearance of the tumor almost 16 mm^2.
During the treatment no side effects and no significant changes in body weight were observed.
The **AraC-TP-dG** demonstrated a certain efficacy in reducing the growth of colo320 human colorectal carcinoma in nu/nu mice with this protocol (Figure 42).

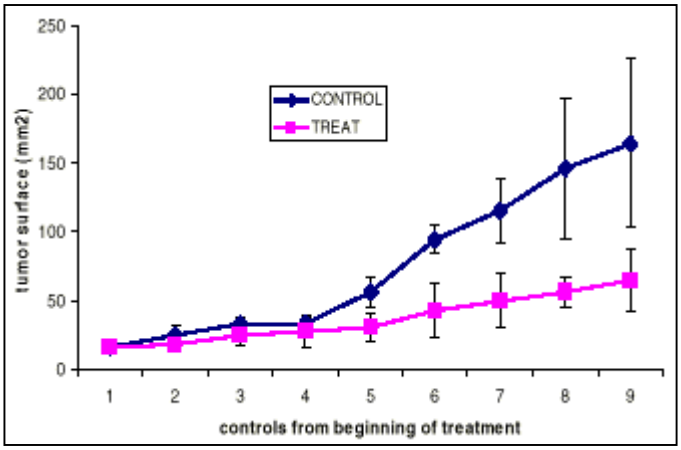


Figure 42. Comparison between CONTROL and treated (TREAT) with **AraC-TP-dG** (200mg/Kg/i.v./day every 3 day).

However, the treatment did not inhibit the metastatic spread. An autoptic control demonstrated liver metastases both in untreated and longer treated mice.

Another group of nu/nu mice inoculated with human colorectal carcinoma cell lines colo320 was treated with **AraC-TP-dG** daily with 500mg/Kg intracardiac. This high dosage produced occasional episodes of convulsions immediately after the administration. No reduction of tumor growth was shown with this protocol. On the contrary, a progressive toxicity was indicated by the reduction of body weight, by the reduced development of the animals (smaller than the controls), and by a quicker death than controls with hepatomegaly due to intense metastatic spread.

4.3 Short summary

The results obtained *in vivo* with **AraC-TP-dG** are encouraging, since they show a reduction of the disease progression in the mice inoculated with leukemia cells HL60. Unlike our expectations, however, **AraC-TP-dG** is just as toxic as AraC.

The **AraC-TP-dG** demonstrated a certain efficacy in reducing the growth of colo320 human colorectal carcinoma in nu/nu mice when the dose was low (4 mg), intermittent (every 3rd day) and prolonged.

The **ACV-TP-dG** shows on the contrary low acute toxicity but also low anticancer activity toward mice inoculated with colorectal carcinoma cells colo320.

5 MATERIALS AND METHODS.

5.1 Synthesis

5.1.1 General Method

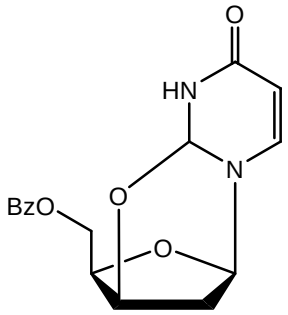
^{1}H-NMR, ^{13}C-NMR and ^{31}P-NMR were recorder on a Varian Gemini 200 (200 MHz, 50 MHz and 80 MHz respectively). Chemical shifts are quoted in ppm using residual solvent as internal standard except ^{31}P chemical shifts are given relative to 85% H_3PO_4 (external standard, δ_p = 0.00 ppm).

All purchased chemicals were used as received without further purification except $POCl_3$ that was refluxed for 1 hour and than distilled.

The reactions involving nucleotides were monitored by thin-layer chromatography (TLC) using PEI-cellulose F (Merck) and chromatography was carried out on DEAE-cellulose fast flow (Sigma) . The other reaction were monitored by TLC using silica gel silice (Merck 60, 70-230 mesh) and flash chromatography was carried out on flash silica gel (Merck 60A, 230-400 mesh).

All air- and moisture-sensitive manipulations were carried out under a dry nitrogen atmosphere using standard Schlenk techniques.

5.1.2 Synthesis of 2,3'-anhydro-5'-O-benzoyl-2'-deoxyuridine **13**



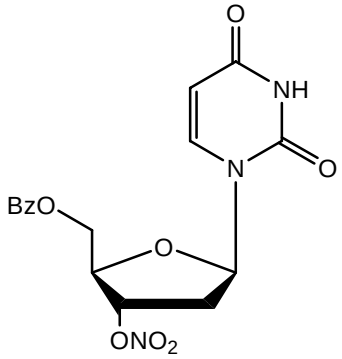
A solution containing 2'-deoxyurindine **12** (1.5g, 6.57 mmol) and triphenylphosphine (2.59g, 9.86 mmol) in DMF (9 mL) was prepared, a solution comprised of diisopropyl azodicarboxylate (1.99g, 9.86 mmol) and benzoic acid (1.2g, 9.86 mmol) in DMF (4,5 mL) was added dropwise with stirring at room temperature and the reaction was allowed to

proceed for 20 min. An addiction aliquot of triphenylphosphine (2.59g, 9.86mmol) and diisopropyl azodicarboxylate (1.99g, 9.86 mmol) was added. The reaction was allowed to proceed for 1h, diethyl ether (80 mL) was added, and the mixture was stirred for 10 min at room temperature. The resulting suspension was cooled to ice-bath temperature, and the white crystalline precipitate was collected by filtration and washed with cold diethyl ether to give 1.9g of the product **13** (yield 92%).

^{1}H-NMR (DMSO-d_6, 200MHz) δ (ppm): 2.52-2.58 (m, 2H), 4.40-4.64 (m, 2H), 5.49 (br s, 1H), 5.81 (d, J = 7.4 Hz, 1H), 6.00 (d, J = 3.8 Hz, 1H), 7.50-7.73 (m, 4H), 7.96 (d, J = 7.0 Hz, 2H).

^{13}C-NMR (DMSO-d_6, 50MHz) δ (ppm): 32.67, 62.47, 77.27, 81.87, 86.92, 107.88, 128.55, 128.93, 129.06, 133.34, 140.57, 153.50, 165.14, 170.04.

5.1.3 Synthesis of 3'-O-nitro-5'-O-benzoyl-2'-deoxyuridine **14**

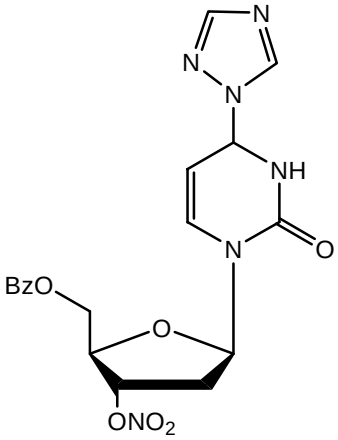


A mixture containing 2,3'-anhydro-5'-O-benzoyl-2'-deoxyuridine **13** (1g, 3.18 mmol) and NH_4OH (3.82 g, 47.75 mmol) in dry DMF (15 mL) was stirred at 110-120°C for 12 h under argon. The solvent was removed in vacuo, and the residue was purified via silica gel flash column chromatography using cyclohexane – Ethylacetate (1:2) as eluent to give the corresponding product **14** (390 mg, yield 32.5%).

^{1}H-NMR (DMSO-d_6, 200MHz) δ (ppm): 2.56-2.74 (m, 2H), 4.48-4.64 (m, 3H), 5.59 (d, J = 8HZ, 1H), 5.76 (m, 1H), 6.13 (t, J = 6.6 Hz, 1H), 7.50-7.55 (m, 2H), 7.65-7.73 (m, 2H), 7.99 (d, J = 7.0 Hz, 2H), 11.42 (br s, 1H).

^{13}C-NMR (DMSO-d_6, 50MHz) δ (ppm): 34.23, 64.03, 79.17, 83.29, 84.78, 102.06, 128.72, 129.06, 129.15, 133.47, 140.44, 150.15, 162.77, 165.32.

5.1.4 Synthesis of 4-(1,2,4-triazolo)-4-deoxy-3'-O-nitro-5'-O-benzoyl-2'-deoxyuridine **15**

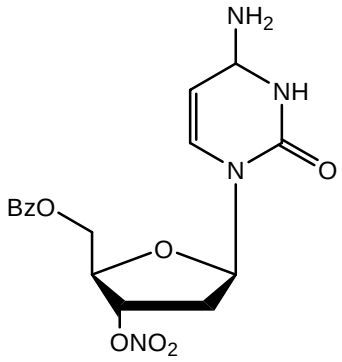


4-Chlorophenyl dichlorophophate (174.42 mg, 0.71 mmol) and 1,2,4-triazole (98.08mg, 1.42 mmol) was added to a solution of 3'-O-nitro-5'-O-benzoyl-2'-deoxyuridine **14** (268mg, 0.71 mmol) in pyridine (2.7 mL) at 0°C with stirring. The reaction was allowed to proceed at 25°C for 4 days with stirring, and the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 (30 mL), this solution was washed with water (2 x 10 mL) and $NaHCO_3$ (1 x 10 mL). The organic fractions was dried on Na_2SO_4, and the solvent was removed in vacuo. Purification of the residue by flash silica gel column chromatography using cyclohexane:ethylacetate (1:5) gave 126 mg of the product **15** (yield 41%).

^{1}H-NMR ($CDCl_3$, 200MHz) δ (ppm): 2.30-2.45 (m, 1H), 3.20 (ddd, *J* = 15.2, 5.8, 1.4 Hz, 1H), 4.63 (dd, *J* = 11.6, 3.2Hz, 1H), 4.71 (dd, *J* = 5.2, 3.0 1H), 4.80 (dd, *J* = 11.6, 3.0 Hz, 1H), 5.63 (d, *J* = 6.6 Hz, 1H), 6.20 (dd, *J* = 7.8, 6.0 Hz, 1H), 6.93 (d, *J* = 7.2 Hz, 2H), 7.34-7.43 (m, 2H), 7.49-7.58 (m, 1H), 7.88-7.92 (m, 2H), 8.10 (s, 1H), 8.22 (d, *J* = 7.2 Hz, 1H), 9.20 (s, 1H).

^{13}C-NMR ($CDCl_3$, 50MHz) δ (ppm): 38.01, 63.58, 82.46, 82.78, 88.47, 94.74, 128.68, 128.77, 129.34, 133.78, 143.18, 145.19, 153.88, 159.42, 165.72.

5.1.5 Synthesis of 3'-O-nitro-5'-O-benzoyl-2'-deoxycytidine **16**

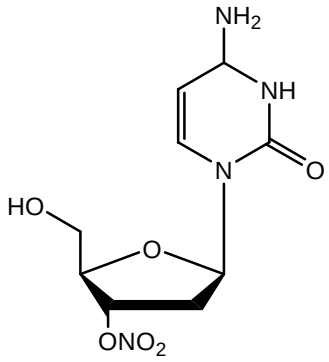


A solution of 4-(1,2,4-triazolo)-4-deoxy-3'-O-nitro-5'-O-benzoyl-2'-deoxyuridine **15** (126 mg, 0.29 mmol) in NH_4OH/dioxane (1:3, 6.5 mL) was stirred at room temperature for 5 h prior to removal of the solvent in vacuo. The residue was purified via flash silica gel column chromatography using MeOH:CH_2Cl_2 (1:9) as eluent to give 54.8mg of the product **16** (yield 50%).

^{1}H-NMR ($CDCl_3$, 200MHz) δ (ppm): 2.26-2.40 (m, 1H), 2.88 (dd, *J* = 15, 5.8 Hz, 1H), 4.56-4.66 (m, 3H), 5.58 (d, *J* = 6.6 Hz, 1H), 5.67 (d, *J* = 7.4 Hz, 1H), 6.15 (dd, *J* = 8.0, 5.8, 1H) 7.41-7.63 (m, 4H), 7.98 (d, *J* = 8.4Hz, 2H), 8.23 (br s, 2H).

^{13}C-NMR ($CDCl_3$, 50MHz) δ (ppm): 35.6, 64.20, 79.23, 83.77, 85.60, 94.30, 128.60, 129.10, 133.34, 140.68, 154.60, 165.29, 165.48, 165.80.

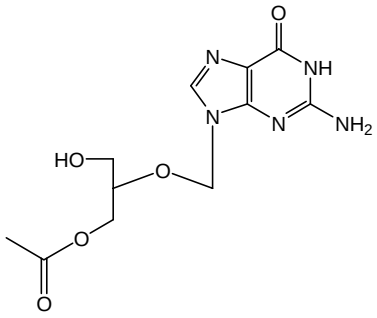
5.1.6 Synthesis of 3'-O-nitro-2'-deoxycytidine **17**



A solution 0.5 M of NaOMe in methanol (5.3 mL, 2.65 mmol) was added to 3'-O-nitro-5'-O-benzoyl-2'-deoxycytidine **16** (1g, 2.65 mmol), and the mixture was stirred at room temperature for 15 min. The solvent was removed in vacuo. Purification of the residue by flash silica gel column chromatography using $CHCl_3$:methanol (6:1) as eluent afforded 460mg of the product **17** (yield 63%).

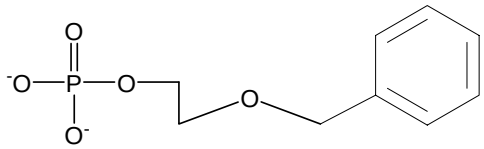
^{1}H-NMR (DMSO-d_6, 200MHz) δ (ppm): 2.23-2.38 (m, 1H), 2.40-2.49 (m, 1H), 3.65-3.67 (m, 2H), 4.20 (br s, 1H), 5.31 (br s, 1H), 5.59 (d, J = 6.2Hz, 1H), 5.77 (d, J = 7.4Hz, 1H), 6.15 (dd, J = 8.8, 5.8Hz, 1H), 7.22 and 7.26 (two br s, 2H), 7.80 (d, J = 7.8 Hz, 2H).
^{13}C-NMR (DMSO-d_6, 50MHz) δ (ppm): 35.87, 61.10, 82.78, 84.80, 85.00, 94.36, 141.70, 152.34, 163.67.
ESI-MS: calculated $C_9H_{13}N_4O_6$ (M – H) 273.09, observed 273.1

5.1.7 Synthesis of mono-O-acetate ester of ganciclovir **22**



To a mixture of 1 g of ganciclovir (3.92 mmol), 900 μM of trimethyl orthoacetate (7 mmol) and 100 DMF was added 320 μM of trifluoroacetic acid (4.16 mmol) under stirring, and the mixture was stirred at room temperature overnight. The water (8 mL) was added and the solution was stirred for 6h at room temperature. The solvent was removed under vacuum. Purification of the residue by flash silica gel column chromatography using CH_2Cl_2:methanol (6:1) as eluent afforded 1.06 g of the product **22** (yield 91%).
^{1}H-NMR (DMSO-d_6, 200MHz) δ (ppm): 1.83 (s, 3H), 3.37-3.41(m, 2H), 3.77-3.91 (m, 2H), 4.02-4.08 (m, 1H), 5.42 (s, 2H), 6.68 (br s, 2H), 7.83 (s, 1H), 10.91 (br s, 1H).
^{13}C-NMR (DMSO-d_6, 50MHz) δ (ppm): 23.42, 60.30, 63.32, 71.41, 76.85, 116.35, 137.88, 151.45, 154.01, 157.16, 173.53.

5.1.8 Synthesis of 2-benzyloxyethanol monophosphate **23**



To a solution of 300 mg of $POCl_3$ (2.5 mmol) in 2 mL of diethyl ether at 0°C were added 276 mg (2 mmol) of 2-benzyloxyethanol **18**. The mixture was allowed to warm to room temperature and was maintained under stirring for 12 hours. The solvent was removed under

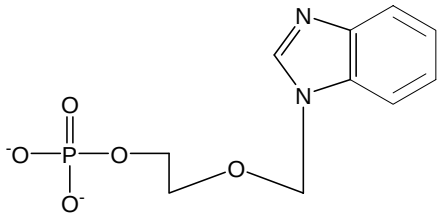
vacuum and then 20 mL of 0°C cold water were added to the solution. The pH was adjust to 9 with NaOH 2M solution. Then 10 mL of ethanol and 208 mg of $BaCl_2$ (1 mmol) was added to the solution. The white precipitate was removed by filtration and the precipitate was washed with water/ethanol 1:1. The solution was evaporated to dryness obtaining 495 mg of **23** as barium salt (70%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 3.71-3.76 (m, 2H), 3.87-3.94 (m, 2H), 4.61(s, 2H), 7.43-7.47(m, 5H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): 3.95 (s).

ESI-MS: calculated $C_9H_{12}O_5P$ (M – H) 231.05, observed 231.50.

5.1.9 Synthesis of 2-((1H-benzo[d]imidazol-1-yl)methoxy)ethanol monophosphate **24**

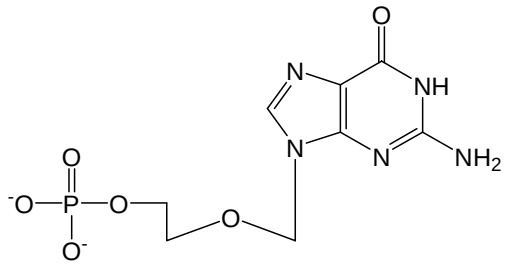


To a solution of 200 mg of $POCl_3$ (1.3 mmol) in 1 mL of THF at 0°C were added 200 mg (1 mmol) of 2-((1H-benzo[d]imidazol-1-yl)methoxy)ethanol **19**. The mixture was allowed to warm to room temperature and was maintained under stirring for 12 hours. The solvent was removed under vacuum and 10 mL of water was added. The pH was adjust to 9 with NaOH 2M and the solution was subjected to a reduction of volume (5 ml) and loaded on to a DEAE-cellulose column. The column was eluted with linear gradient of triethylammonim bicarbonate (0.05-0.3M). Appropriate fractions were evaporated under vacuum. The residual bicarbonate was eliminated by two sequential evaporation steps from 5 ml methanol. Evaporation to dryness yielded 284 mg of **24** triethylammonium salt (0.6 mmol, yield = 60%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.23-1.30 (t, *J* = 7.32, 18H), 3.13-3.24 (q, *J*=7.32, 12H), 3.84-3.87 (m, 2H), 6.00 (s, 2H), 7.66-7.73 (m, 2H), 7.85-7.89 (m, 1H), 7.95-7.99 (m, 1H), 9.42 (s, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): -0.27 (s).

5.1.10 Synthesis of acyclovir monophosphate **ACVMP**



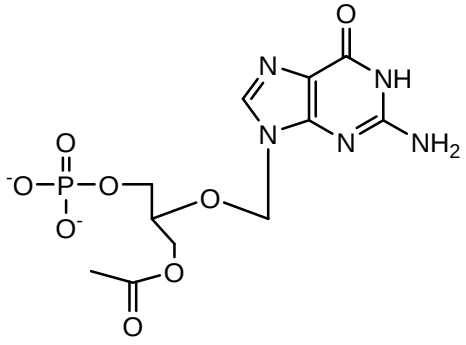
A mixture of 1g acyclovir (4.3 mmol) and 6 ml triethylphosphate was gradually added to a mixture of 4 ml triethylphosphate and 860 µl phosphorus oxychloride (8.6 mmol) at -10°C. The mixture was maintained at 0°C for 12 h under stirring. Then 100 ml of ice-cold 5% $NaHCO_3$ in water were added to the solution. After stirring at 0°C for 1 h and at room temperature for 8 h, the pH was adjusted to 7.0 with NaOH 1M. After further 12 h under stirring, the mixture was extract with 3 x 50ml of diethyl ether and the water solution was evaporated to dryness, dissolved in the minimum volume of water and loaded onto a DEAE-cellulose column. The column was eluted with a linear gradient (0.05-0.5 M) of triethylammonium bicarbonate, pH 7.5). Appropriate fractions were evaporated under vacuum. Methanol was added and evaporated again to remove triethylammonium bicarbonate obtaining ACVMP as triethylammonium salt (1.28 g, 2.53 mmol, yield = 59%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.17-1.21 (t, *J* = 7.3, 18H), 3.06-3.17 (q, *J* = 7.3, 12H), 3.69-3.77 (m, 2H), 3.85-3.94 (m, 2H), 5.51(s, 1H), 7.93 (s, 1H).

^{31}P-NMR (D^2O, pH 7.5, 80MHz) δ (ppm): 3.79 (s).

ESI-MS: calculated $C_8H_{11}N_5O_6P$ (M – H) 304.05, observed 304.30.

5.1.11 Synthesis of mono-O-acetate ester of ganciclovir monophosphate **GCVMP**

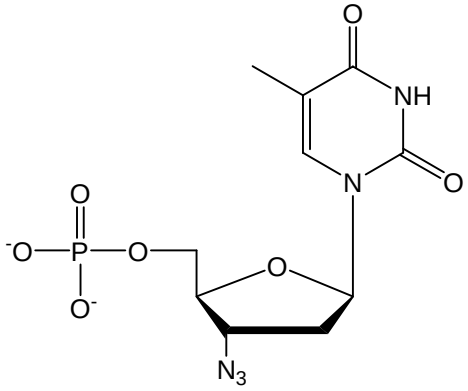


The procedure described above for the synthesis of acyclovir monophosphate **ACVMP** was followed for the preparation of mono-O-acetate ester of ganciclovir monophosphate **GCVMP,** using mono-O-acetate ester of ganciclovir **22** (1 g, 3.57 mmol) instead of acyclovir, to give the product monophosphate as white solid (1.24 g, 2.14 mmol, yield = 60%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.17-1.21 (t, *J* = 7.3 Hz, 18H), 3.06-3.17 (q, *J* = 7.3 Hz, 12H), 3.48-3.81 (m, 5H), 5.54(s, 2H), 7.81 (s, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): 4.10 (s).

5.1.12 Synthesis of 3'-azidothymidine-5'-monophosphate **AZTMP**

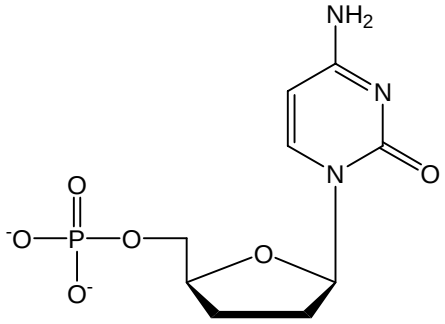


The procedure described above for the synthesis of acyclovir monophosphate was followed for the preparation of 3'-azidothymidine-5'-monophosphate, using 3'azidothymidine (**AZT**, 100 mg, 0.37 mmol) instead of acyclovir, to give 3'-azidothymidine-5'-monophosphate as white solid (90 mg, 0.15 mmol, yield = 40%).

^{1}H-NMR (D_2O, pH 7.5, 200 MHz) δ (ppm): 1.23-1.30 (t, *J* = 7.3 Hz, 18H), 1.92 (s, 3H), 2.44-2.52 (m, 2H), 3.13-3.24 (q, *J*=7.3 Hz, 12H), 3.96-4.00 (m, 2H), 4.12-4.20 (m, 1H), 4.46-4.54 (m, 1H), 6.22-6.29 (t, *J*=6.6 Hz, 1H), 7.81 (s, 1H).

31P-NMR (D2O, pH 7.5, 80MHz) δ (ppm): 2.57 (s).

5.1.13 Synthesis of 2',3'-dideoxycytidine-5'-monophosphate **ddCMP**



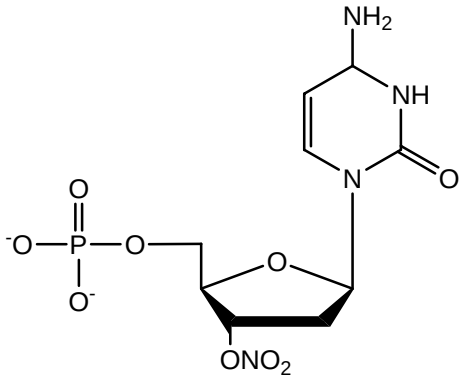
The procedure described above for the synthesis of acyclovir monophosphate was followed for the preparation of 2',3'-dideoxycytidine-5'-monophosphate, using 2'3'-dideoxycytidine

(**ddC**, 100 mg, 0.47 mmol) instead of acyclovir, to give 2'3'-dideoxycytidine-5'-monophosphate as white solid (145 mg, 0.28 mmol, yield = 59%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.23-1.30 (t, *J* = 7.3 Hz, 18H), 1.74-2.11 (m, 2H), 2.24-2.40 (m, 2H), 3.13-3.24 (q, *J*=7.3 Hz, 12H), 3.85-3.90 (m, 1H), 4.02-4.11 (m, 1H), 4.18-4.30 (m, 1H), 5.88-5.92 (m, 1H), 6.04-6.08 (d, *J*=7.3 Hz, 1H), 8.08-8.12 (d, *J*=7.3 Hz, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): 1.30 (s).

5.1.14 Synthesis of 3'-O-nitro-2'-deoxycytidine-5'-monophosphate **25**.

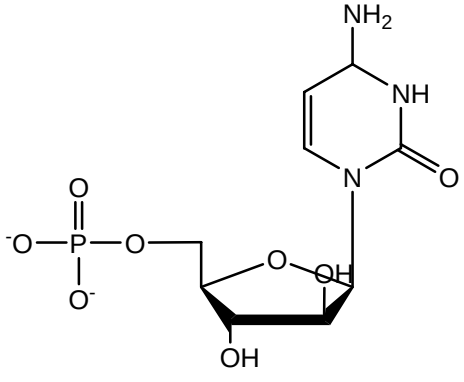


The procedure described above for the synthesis of **GCVMP** was followed for the preparation of 3'-O-nitro-2'-deoxycytidine-5'-monophosphate **25,** using 3'-O-nitro-2'-deoxycytidine **17** (450 mg, 1.63 mmol) instead of **22**, to give the product monophosphate as white solid (190 mg, 0.34 mmol, yield = 21%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.17-1.21 (t, *J* = 7.3, 18H), 2.25-2.50 (m (2H), 3.06-3.17 (q, *J* = 7.3, 12H), 3.48-3.81 (m, 5H), 5.54(s, 2H), 7.81 (s, 1H). 3.65-4.20 (m, 4H), 5.65 (d, *J* = 6.2Hz, 1H), 6.15 (m, 1H), 7.80 (d, *J* = 6.2 Hz, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): 4.55 (s).

5.1.15 Synthesis of arabinocytosine-5'-monophosphate **AraCMP**

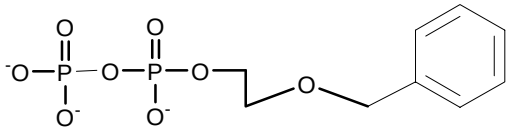


The procedure described above for the synthesis of acyclovir monophosphate **ACVMP** was followed for the preparation of arabinocytosine-5'-monophosphate **AraCMP,** using arabinocyotisine **AraC** (1 mg, 4.11 mmol) instead of **22**, to give the product monophosphate as white solid (1.08 g, 2.05 mmol, yield = 50%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.17-1.21 (t, *J* = 7.3, 18H), 3.06-3.17 (q, *J* = 7.3, 12H), 3.99-4.07 (m, 2H), 4.19-4.23 (m, 1H), 4.36 (t, *J* = 5.13Hz, 2H), 6.05 (d, *J* = 7.3, 1H), 6.17 (d, *J* = 5.13, 1H), 7.94 (d, *J* = 7.3 Hz, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): 3.80 (s).

5.1.16 Synthesis of 2-benzyloxyethanol diphosphate **29**



The tributylammonium orthophosphate, necessary for this transformation, was prepared as follows. Anhydrous orthophosphoric acid (5 g, 51 mmol) and 10 ml of CH_2Cl_2 were put in a Schlenk tube, under anhydrous condition. Tributylamine (12.25 ml, 51 mmol) was then added dropwise into the solution in 30 minutes. The mixture was left under stirring for 1 h. CH_2Cl_2 was evaporated and the reaction residue re-evaporated with 3 x 10ml anhydrous pyridine and 2 x 10ml of anhydrous DMF. The final product was dissolved in anhydrous DMF to a concentration of 1M, and stored over molecular sieves (4Å) at 4°C.

The barium salt of **23** (450mg, 1.6 mmol) was converted into its pyridinium salt using Dowex 50W-X8 (pyridinium form resin). The column was eluted with 50% aqueous methanol. The eluted was evaporated under reduced pressure to dryness, and then 10 ml methanol and 760 µl tributylamine (3.2 mmol) were added. After 30 minutes stirring, the solution was concentrated under vacuum. The residue was dried by repeated addition and evaporation of anhydrous pyridine (3 x 10ml), anhydrous toluene (1 x 10ml) and anhydrous DMF (2 x 10 ml).

The resulting tributylammonium salt of **23** was dissolved in 15 ml anhydrous DMF.

1,1'-Carbonyldiimidazole (1 g, 8 mmol) was dissolved in 3.5 ml DMF. This solution was added to the solution of **23**. The mixture was stirred at room temperature on molecular sieve. After 12 hours, 466 µl anhydrous methanol (8 mmol), and after further 30 minutes, 8 ml 1M tributylammonium orthophosphate in DMF (8 mmol) were added dropwise under stirring. After 12 h at room temperature, the precipitate was removed by centrifugation. The supernatant solution was added with 75 ml water and the resulting solution was extract with 3

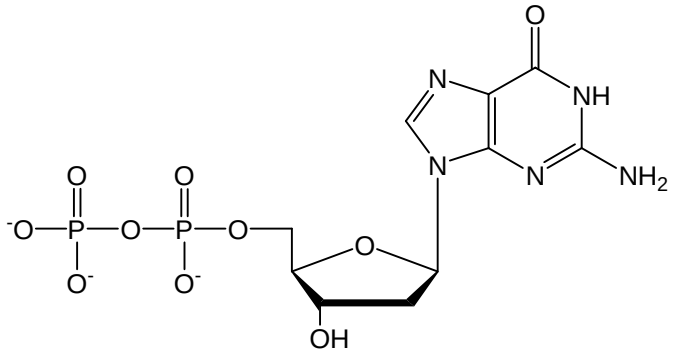
x 50ml CHCl3, subjected to a reduction of volume (5 ml) and loaded on to a DEAE-cellulose column. The column was eluted with linear gradient of triethylammonim bicarbonate (0.05-1M). Appropriate fractions were evaporated under vacuum. The residual bicarbonate was eliminated by two sequential evaporation steps from 20 ml methanol. Evaporation to dryness yielded 747 mg of **29** triethylammonium salt (1.21 mmol, yield = 76%).

^{1}H-NMR (D_2O, pH 8.5, 200MHz) δ (ppm): 3.63 (t, *J*=4.4 Hz, 2H), 3.91-4.03 (m, 2H), 4.47 (s, 2H), 7.30 (s, 5H).

^{31}P-NMR (D_2O, pH 8.5, 80MHz) δ (ppm): -6.16 (d, *J*=22.0 Hz), -10.40 (d, J=22.0 Hz).

ESI-MS: calculated $C_9H_{13}O_8P_2$ (M – H) 311.02, observed 311.50.

5.1.17 Synthesis of 2'-deoxyguanosine-5'-diphosphate **dGDP**



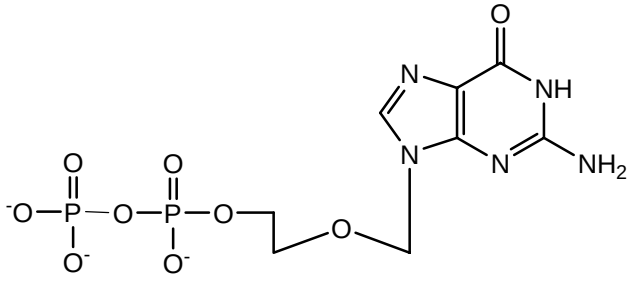
The procedure described above for **29** was followed for the preparation of 2'-deoxyguaosine-5'-diphosphate (**dGDP**) using 2'-deoxyguanosine-5'-monophosphate triethylammonium salt (867 mg, 2.5 mmol) instead of **23**, to give **dGDP** as white solid (654 mg, 1.02 mmol, yield = 40%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.23-1.30 (t, *J* = 7.32, 27H), 2.53-2.65 (m, 1H), 2.70-2.87 (m, 1H), 3.13-3.24 (q, *J*=7.32, 18H), 4.08-4.15 (m, 2H), 4.25-4.32 (m, 1H), 4.66-4.73 (m, 1H), 6.35 (t, *J* = 6.59, 1H), 8.48 (s, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80 MHz) δ (ppm): -6.64 (d, *J* = 23.2Hz), -10.90 (d, *J* = 23.19Hz).

ESI-MS: calculated $C_{10}H_{14}N_5O_{10}P_2$ (M – H) 426.02, observed 426.30.

5.1.18 Synthesis of acyclovir diphosphate **ACVDP**



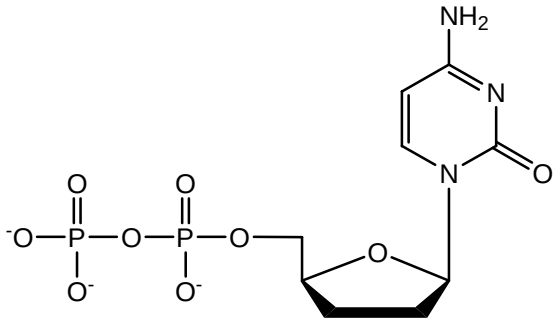
The procedure described above for **29** was followed for the preparation of acyclovir diphosphate (**ACVDP**) using acyclovir monophosphate triethylammonium salt (1.01 g 2 mmol) instead of **23**, to give **ACVDP** as white solid (984 mg, 1.42 mmol, yield = 70%).

^{1}H-NMR (D_2O, pH 8.5, 200MHz) δ (ppm): 1.23-1.30 (t, *J* = 7.3 Hz, 27H), 3.14-3.25 (q, *J* = 7.3 Hz, 18H), 3.77-3.81 (m, 2H), 4.04-4.12 (m, 2H), 5.56 (s, 1H), 7.98 (s, 1H).

^{31}P-NMR (D_2O, pH 8.5, 80MHz) δ (ppm): -6.50 (d, *J* = 22.1), -10.77 (d, *J* = 22.1).

ESI-MS: calculated $C_8H_{12}N_5O_9P_2$ (M - H) 384.01, observed 384.20.

5.1.19 Synthesis of 2'3'-dideoxycytidine-5'-diphosphate **ddCDP**

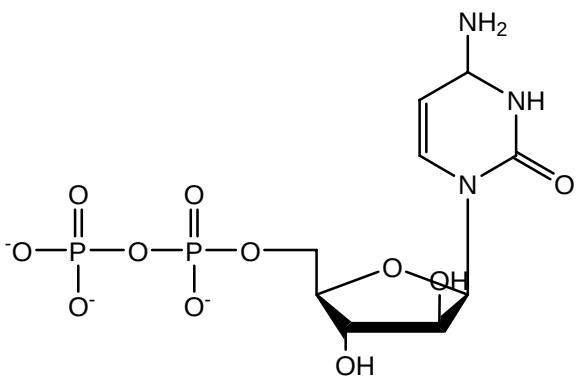


The procedure described above for **29** was followed for the preparation of 2',3'-dideoxycytidine-5'-diphosphate (**ddCDP**) using 2'3'-dideoxycytidine-5'-monophosphate triethylammonium salt (105 mg, 0.2 mmol) instead of **23**, to give **ddCDP** as white solid (100mg, 0.14 mmol, yield = 70%).

1H-NMR (D2O, pH 7.5, 200MHz) δ (ppm): 1.23-1.30 (t, J = 7.32, 18H), 1.62-1.96 (m, 2H), 2.12-2.37 (m, 2H), 3.13-3.24 (q, J=7.32, 12H), 3.75-3.80 (m, 1H), 3.92-4.02 (m, 1H), 4.11-4.21 (m, 1H), 5.85-5.88 (m, 2H), 7.81-7.85 (d, J=7.32, 1H).

31P-NMR (D2O, pH 7.5, 80 MHz) δ (ppm): -4.51:-6.47 (br s), -8.40:-9.40 (br s).

5.1.20 Synthesis of arabinocytosine-5'-diphosphate **AraCDP**

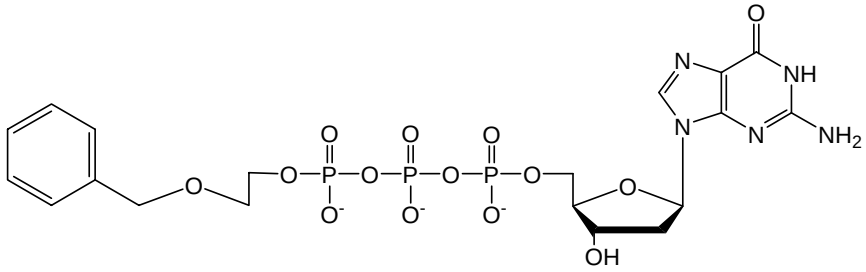


The procedure described above for **29** was followed for the preparation of arabinocytosine-5'-diphosphate (**AraCDP**) using arabinocytosine-5'-monophosphate triethylammonium salt (1.08g, 2 mmol) instead of **23**, to give **AraCDP** as white solid (900 mg, 1.25 mmol, yield = 62.5%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.23-1.30 (t, *J* = 7.32, 27H), 3.13-3.24 (q, *J*=7.32, 18H), 3.98-4.07 (m, 1H), 4.13-4.26 (m, 3H), 4.34-4.42 (m, 1H), 6.01-6.10 (br d, *J* = 6.70, 1H), 6.18 (d, *J* = 5.86 Hz, 1H), 7.86 (d, *J* = 5.86 Hz, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80 MHz) δ (ppm): -6.01 (d, *J* = 21.9 Hz), -10.51 (d, *J* = 21.9 Hz).

5.1.21 Synthesis of 2-benzyloxyethanoyl 2'-deoxy-5'-guanosyltriphosphate **Ph-TP-dG**



The tributylammonium salt of **dGMP** and **29** were prepared by first converting their sodium salt or triethylammonium salt in pyridinium salt by chromatography on a Dowex 50W-X8 column.

A solution of water (5 ml) and 714 mg of **dGMP** (2 mmol) was loaded onto a Dowex 50W-X8 (pyridinium form). The column was eluted with 50% aqueous methanol. The eluent was evaporated under reduced pressure to dryness, and then 464 µl of tributylamine (2 mmol) and 10 ml water were added. After 30 minutes the mixture was evaporated to dryness. The resulting residue was subjected to 3 x 10 ml evaporation from anhydrous pyridine and 2 x 5ml evaporation from anhydrous DMF.

550 mg of **29** triethylammonium salt (1 mmol) was dissolved in 10 ml water loaded on a Dowex 50W-X8 column and eluted with water/methanol (1:1). The solution was concentrated under vacuum, and then 500 µl tributylamine (2 mmol) and 10 ml water/methanol (1:1) were added. After 30 minutes under stirring the reaction mixture was evaporate to dryness, the residue was dried by repeated addition and evaporation of anhydrous pyridine (3 x 7.5 ml) and anhydrous DMF (2 x 7.5 mL).

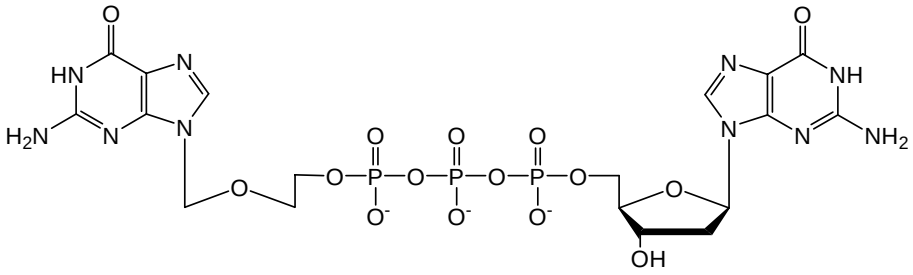
The anhydrous tributylammonium salt of **29**, 10 ml DMF and 800 mg 1,1'-carbonyldiimidazole (5 mmol) in 5 mL of DMF were mixed in a Schlenk tube under anhydrous conditions. After 2h stirring at room temperature on molecular sieves, 400 µl

methanol (7 mmol) and 30 minutes later the anhydrous tributylammonium salt of **dGMP** in 14 ml of DMF were added. After additional 14 h stirring, the precipitate of the reaction mixture was removed by centrifugation. At the solution was added 100 mL of water and extracted with CHCl3 (3 x 50mL). Then the water solution was evaporated to dryness, dissolved in minimal volume of water and loaded on DEAE-cellulose column. The column was eluted with linear gradient (0.05-0.5M) of triethylammonium bicarbonate buffer (pH 7.5). Ph-TP-dG-containing fractions were pooled and concentrated to dryness. The residual bicarbonate was eliminated by two sequential evaporations from 20 ml methanol, and the residual material was converted into its sodium salt using Dowex 50W-X8 resin, after converting the latter from its H+ form to its sodium form by washing with four bed volumes of NaOH 1M, then washing with water until pH neutrality. The resulting preparation was dried under vacuum to a dry yellow powder (200 mg, 0.83 mmol, yield = 28%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 2.36-2.47 (m, 1H), 2.61-2.75 (m, 1H), 3.67 (t, *J* = 4.40 Hz, 2H), 4.05-4.21 (m, 5H), 4.44 (s, 2H), 4.65-4.71 (m, 1H), 6.18 (t, *J* = 6.59, 1H), 7.27-7.37 (m, 4H), 8.05 (s, 1H).

^{31}P-NMR (D2O, pH 7.5, 80MHz) δ (ppm): -10.95 (d, *J* = 18.31Hz), -11.29 (d, J=18.31), -22.75 (t, *J* = 18.31Hz).

5.1.22 Synthesis of Acycloguanosyl 2'-deoxy-5'-guanosyltriphosphate **ACV-TP-dG**



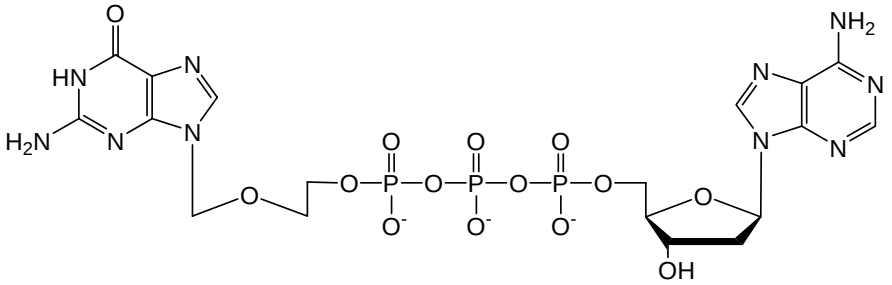
The procedure described above for **Ph-TP-dG** was followed for the preparation of **ACV-TP-dG** using 2'-deoxyguanosine-5'-monophosphate disodium salt (**dGMP**, 1 g, 2.8 mmol) and **ACVDP** tributylammonium salt (194 mg, 0.28 mmol) instead of **29**, to give **ACV-TP-dG** sodium salt as yellow powder (650 mg, 0.83 mmol, yield = 59%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 2.35-2.48 (m, 1H), 2.65-2.79 (m, 1H), 3.70-3.74 (m, 2H), 4.05-4.20 (m, 5H), 4.65-4.71 (m, 1H), 5.39 (s, 1H), 6.16-6.23 (t, *J* = 6.6 Hz, 1H) 7.84 (s, 1H), 7.99 (s, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): -11.25 (d, *J*=19.5 Hz), -11.35 (d, *J* = 19.5), -23.13(t, *J* = 19.5 Hz).

ESI-MS: calculated $C_{18}H_{24}N_{10}O_{15}P_3$(M - H) 713.6, observed 713.20.

5.1.23 Synthesis of Acycloguanosyl 2'-deoxy-5'-adenosyltriphosphate **ACV-TP-dA**

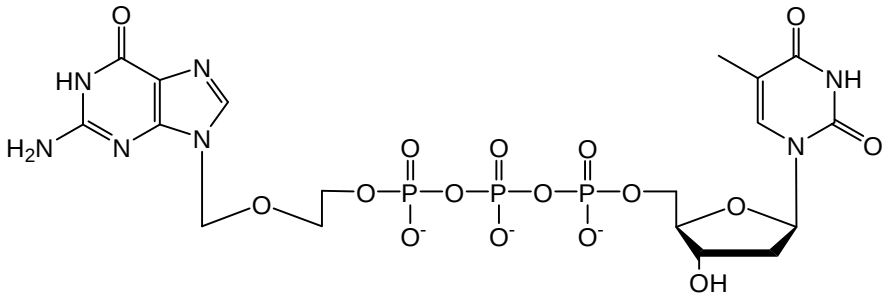


The procedure described above for **Ph-TP-dG** was followed for the preparation of **ACV-TP-dA** using 2'-deoxyadenosine-5'-monophosphate disodium salt (**dAMP**, 165 mg, 0.5 mmol) instead of **dGMP** and **ACVDP** tributylammonium salt (194 mg, 0.28 mmol) instead of **29**, to give **ACV-TP-dA** sodium salt as yellow powder (82 mg, 0.11 mmol, yield = 39%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 2.42-2.53 (m, 1H), 2.62-2.75 (m, 1H), 3.61-3.65 (m, 2H), 3.97-4.19 (m, 5H), 4.64-4.71 (m, 1H), 5.29 (s, 1H), 6.31-6.38 (t, *J* = 6.6 Hz, 1H) 7.70 (s, 1H), 8.05 (s, 1H), 8.34 (s, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): -11.53 (d, *J* = 19.5 Hz), -11.71 (d, J=19.5 Hz), -23.52 (t, *J* = 19.5 Hz).

5.1.24 Synthesis of Acycloguanosyl 5'-thymidyltriphosphate **ACV-TP-T**

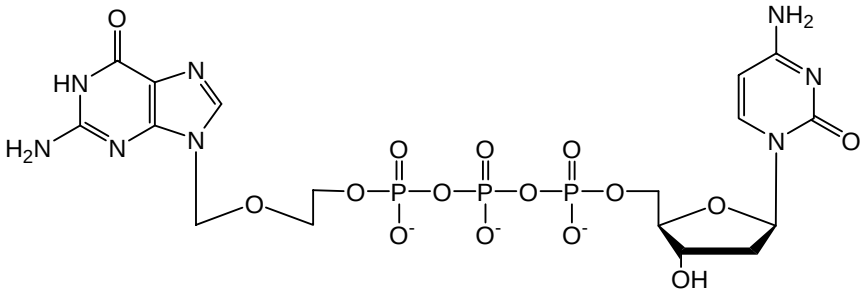


The procedure described above for **Ph-TP-dG** was followed for the preparation of **ACV-TP-T** using thymidine-5'-monophosphate disodium salt (**TMP**, 183 mg, 0.5 mmol) instead of **dGMP** and **ACVDP** tributylammonium salt (194 mg, 0.28 mmol) instead of **29**, to give **ACV-TP-T** sodium salt as yellow powder (100 mg, 0.13 mmol, yield = 46%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.95 (s, 3H), 2.31-2.40 (m, 1H), 3.83-3.89 (m, 2H), 4.12-4.29 (m, 5H), 4.61-4.68 (m, 1H), 5.59 (s, 1H), 6.31-6.38 (t, *J* = 6.59, 1H), 7.66 (s, 1H), 7.79 (s, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): -11.30:-11.64 (d, *J*=19.50), -11.59:-11.83 (d, *J*=19.50), -23.32: -23.80 (t, *J*=19.50).

5.1.25 Synthesis of Acycloguanosyl 5'-cythydyltriphosphate **ACV-TP-dC**

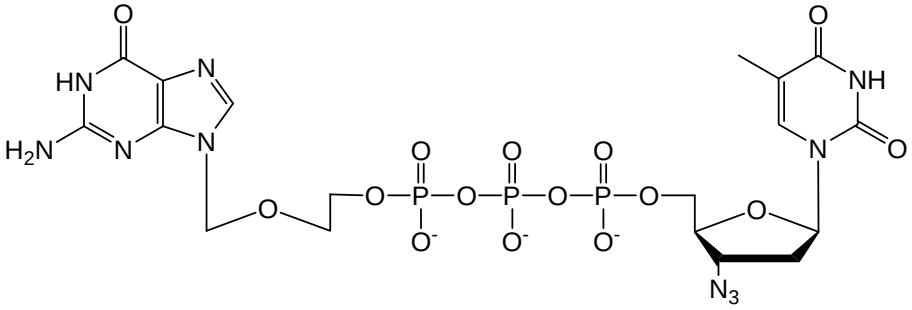


The procedure described above for **Ph-TP-dG** was followed for the preparation of ACV-**TP-dC** using 2'-deoxycythidine-5'-monophosphate disodium salt (**dCMP**, 76 mg, 0.25 mmol) instead of **dGMP** and **ACVDP** tributylammonium salt (87 mg, 0.14 mmol) instead of **29**, to give **ACV-TP-dC** sodium salt as yellow powder (41 mg, 0.13 mmol, yield = 39%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 2.13-2.26 (m, 1H), 2.30-2.42 (m, 1H), 3.75 (br t, *J* = 4.39 Hz, 2H), 4.05-4.15 (m, 5H), 4.50-4.55 (m, 1H), 5.48 (s, 2H), 5.97 (d, *J* = 7.32Hz, 1H), 6.21 (t, *J* = 6.59, 1H), 7.81 (d, *J* = 7.32 Hz, 2H), 7.88 (s, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): -11.16 (d, *J* = 19.53 Hz), -11.35 (d, *J* = 18.31 Hz), -22.91 (dd, *J*= 19.53, 18.31 Hz).

5.1.26 Synthesis of Acycloguanosyl-3'-azidothymidine-5'-triphosphate **ACV-TP-AZT**



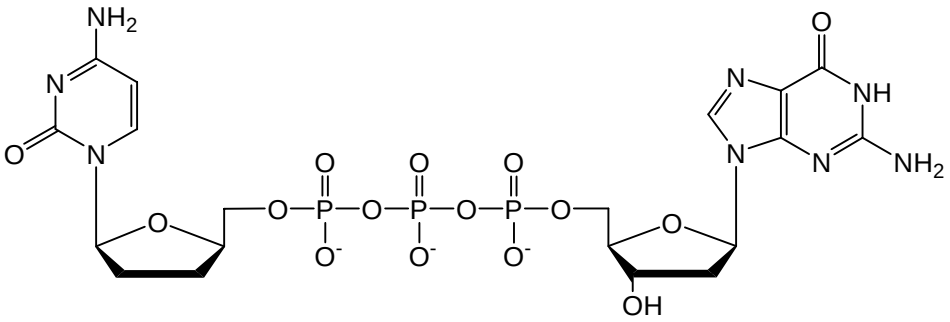
The procedure described above for **Ph-TP-dG** was followed for the preparation of **ACV-TP-AZT** using 3'-azidothymidine-5'-monophosphate triethyammonium salt (**AZTMP**, 90 mg,

0.16 mmol) instead of **dGMP** and **ACVDP** tributylammonium salt (80 mg, 0.12 mmol) instead of **29**, to give **ACV-TP-AZT** sodium salt as yellow powder (30 mg, 0.04 mmol, yield = 33%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.91 (s, 3H), 2.36-2.52 (m, 2H), 3.72-3.83 (m, 2H), 4.05-4.24 (m, 4H), 4.52-4.63 (m, 1H), 5.43 (s, 2H), 6.27 (t, *J* = 7.3 Hz, 1H), 7.75 (s, 1H), 7.88 (s, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): -11.50 (d, *J*=18.3 Hz), -11.89 (d, *J*=18.3 Hz), -23.33 (t, *J* = 18.3 Hz).

5.1.27 Synthesis of 2',3'-dideoxycytidine-2'-deoxy-5'-guanosyltriphosphate **ACV-TP-ddC**

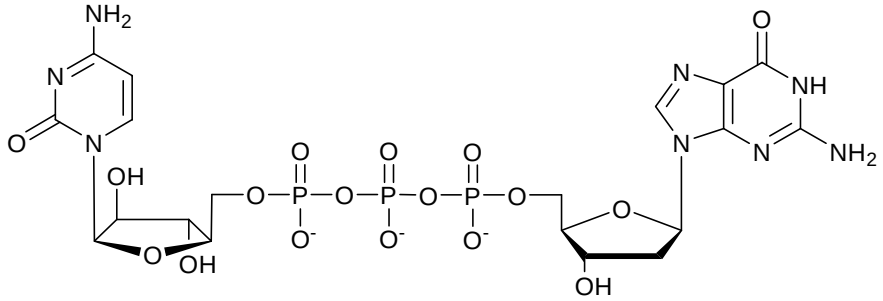


The procedure described above for **Ph-TP-dG** was followed for the preparation of **ddC-T-PdG** using 2',3'-dideoxycytidine-5'-diphosphate triethylammonium salt (**ddCDP**, 100 mg, 0.14 mmol) instead of **29** triethylammonium salt, and 2'deoxyguanosine-5'-monophosphate disodium salt (dGMP, 98 mg, 0.28 mmol), to give **ddC-TP-dG** sodium salt as yellow powder (20 mg, 0.04 mmol, yield = 28%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 1.74-2.11 (m, 2H), 2.35-2.75 (m, 4H), 3.70-3.85 (m, 2H), 3.92-4.02 (m, 1H), 4.11-4.21 (m, 2H), 4.52-4.63 (m, 1H), 5.50 (s, 2H), 6.06-6.27 (m, 2H), 7.85 (d, *J*=7.3 Hz, 1H), 7.95 (s, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): -11.25 (d, *J*=18.3, 2P), -22.75 (t, *J*=18.3 Hz).

5.1.28 Synthesis of arabinocythydyl 2'-deoxy-5'-guanosyltriphosphate **AraC-TP-dG**



The procedure described above for **Ph-TP-dG** was followed for the preparation of **AraC-TP-dG** using 2'-deoxyguanosine-5'-monophosphate disodium salt (**dGMP**, 1.45 g, 4.76 mmol) and **AraCDP** tributylammonium salt (1.54 g, 2.4 mmol) instead of **29**, to give **AraC-TP-dG** sodium salt as yellow powder (760 mg, 0.95 mmol, yield = 39.6%).

^{1}H-NMR (D_2O, pH 7.5, 200MHz) δ (ppm): 2.19-2.31 (m, 1H), 2.38-2.51 (m, 1H), 3.58-3.65 (m, 1H), 3.94-4.10 (m, 6H), 4.45-4.50 (m, 2H), 5.96 (t, 1H), 6.05-6.10 (m, 1H), 7.78 (s, 1H), 7.85-7.90(m, 1H).

^{31}P-NMR (D_2O, pH 7.5, 80MHz) δ (ppm): -11.32 (d, J = 18.31 Hz, 2P), -22.84 (dd, J = 18.31 Hz, 1P).

5.2 Biological tests

5.2.1 Cell Cultures.

The human cell lines used in the study, SW480 colon carcinoma cells, their relative lymph node metastatic SW620 cells were obtained from the European Collection of Cell Cultures (ECACC), COLO320DMF colon carcinoma cells, COLO741 metastatic colon carcinoma cells, HT1080 human fibrosarcoma cells, RKO colon carcinoma cells were obtained from National Institute for Cancer Research (ICLC), CCD-18Co normal colon cell lines were obtained from ATCC, HeLa A1 cervical cancer cells were obtained from Swiss Institute for Experimental Cancer Research (ISREC), HL60 Human acute promyelocytic leukemia cells from Department of Experimental Pathology and Oncology of Florence. SW480, SW620, HT1080, RKO, COLO741 cells were grown in RPMI 1640 medium with L-glutamine (2 mM), 10% FCS, penicillin (100.000 units/liter), streptomycin (100 mg/liter), COLO320DMF, HeLa A1 in DMEM medium with L-glutamine (2 mM), 10% FCS, penicillin (100.000 units/liter), streptomycin (100 mg/liter), CCD-18Co in EMEM medium medium with NEAA

(0.1 mM), L-glutamine (2 mM), 10% FCS, penicillin (100.000 units/liter), streptomycin (100 mg/liter). Cells cultures were maintained at 37° C in a humid atmosphere of 5% CO_2 and 95% air. Cells were grown until 60-70 % confluence, and the experiments performed after 4-8 passages.

5.2.2 Analysis of cell proliferation and viability

Normal and tumour cells were seeded in 96 well plates at a 15000 cells/well density, 24 h after seeding medium was changed and the prodrugs was added for additional 24 h. Cell viability was assessed measuring intracellular ATP content using Promega CellTiter-Glo® Luminescent Cell Viability Assay. The experiment was run according to manufacturer protocol, luminescence was recorded with a Wallac multiplate reader (Perkin-Elmer). Cell proliferation was assessed measuring BrdU incorporation in DNA synthesis (Cell proliferation ELISA, BrdU, Roche). BrdU was added to each well 6 h before the end of the experiment. The assay was run according to manufacturer protocol luminescence was recorded with a Wallac multiplate reader (Perkin-Elmer).

5.2.3 Mice tests

Animal studies for ACV-TP-dG and AraC-TP-dG on nu/nu (nude mice), Balb/c e C57BL/6 mice were carried out at the Academy of Science of the Czech Republic, Division of Immunology and Gnotobiology. The studies of AraC-TP-dG on NOD/SCID mice (nude mice) were carried out in Florence at the Department of Experimental Pathology and Oncology.

6 PUBLICATIONS

6.1 Prodrugs activated by RNA-dependent DNA-polymerases, and their therapeutic uses.

Bertini, Ivano; Luchinat, Claudio; Quattrone, Alessandro; Calamante, Massimo; Mordini, Alessandro

Publication number: WO200613203
Application number: WO2005EP53765 20050802

Herein described are prodrugs activated by RNA-dependent DNA-polymerases, such as telomerase and retroviral reverse transcriptases, and their use for the preparation of pharmaceutical compositions, to be used for the treatment of solid tumours, of haematological tumors, of precancerous states and of diseases caused by infection with retroviruses.

6.2 A New Class of Cytotoxic Prodrugs. Synthesis and in vitro Evaluation

(Submitted)

A New Class of Cytotoxic Prodrugs. Synthesis and *in vitro* Evaluation

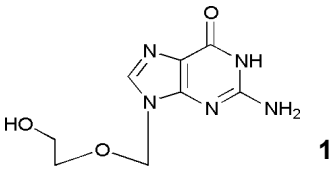
Ivano Bertini,*[a,b] Massimo Calamante,[a,c] Claudio Luchinat,[a,d] Alessandro Mordini,[e] Alessandro Provenzani,[a,f] and Alessandro Quattrone[a,d]

Receipt/Acceptance Data **[DO NOT ALTER/DELETE THIS TEXT]**
Publication data **[DO NOT ALTER/DELETE THIS TEXT]**
DOI: 10.1039/b000000x [DO NOT ALTER/DELETE THIS TEXT]

A library of acyclovir-containing triphosphates has been designed and synthesized aiming at a selective release of the cytotoxic agent inside tumor cells.

Introduction

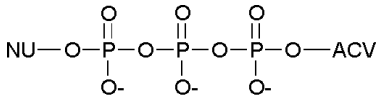
It is well known that the nucleoside analogue acyclovir (9-(2-hydroxyethoxymethyl)guanine, **ACV** (**1**), and its related compounds, owe their pharmacological anti-herpetic activity to a phosphorylation process which takes place in virally infected cells[1].



1

This phosphorylation is catalyzed by a herpes-specific thymidine kinase activity. The resulting monophosphate derivative (**ACV**-MP) is toxic to human cells, as it is further phosphorylated by cellular kinases to the diphosphate (**ACV**-DP) and then the triphosphate (**ACV**-TP). The latter is incorporated into DNA by DNA polymerases and acts as a chain terminator leading to death of the virus-infected cell. **ACV** itself is of course able to penetrate the cell membrane, while **ACV**-MP, **ACV**-DP and **ACV**-TP are not, due to their higher charge[2], so that they must be produced inside the cell.

While the selective exploitation of a viral activity to generate the toxic phosphorylated **ACV** derivatives *in situ* makes **ACV** itself a very successful antiviral drug, it occurred to us that the toxic potential of **ACV**-MP, **ACV**-DP or **ACV**-TP could also be exploited to target other diseases – such as cancer – if one could base their production *in situ* not on a kinase-dependent phosphorylation but on a hydrolysis of a phosphorylated, inactive prodrug. We therefore conceived a class of molecules constituted by **ACV** (or any of its analogues) attached to a nucleoside triphosphate **NU**-TP (**2**).

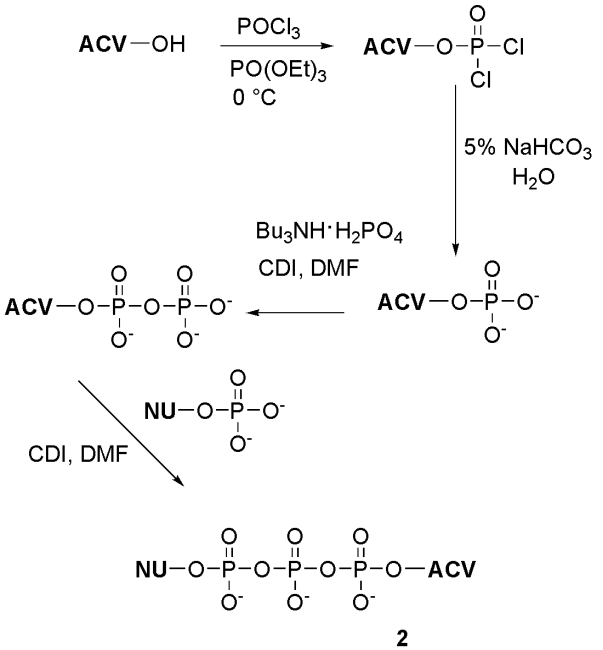


2

Such molecules should be more easily internalized in cells than the free **ACV** mono- di- and triphosphates due to the presence of a base moiety at each end, and should be easily hydrolyzed by enzymes such as phosphatases, DNA polymerases and telomerases, yielding primarily **ACV**-MP or **ACV**-DP. All these enzyme activities are lower in most normal cells than in cancer cells, or even absent.[3,8] With these premises, we faced the problem of accessing a small library of molecules with general formula (**2**).

Synthesis of triphosphates (2).

All the triphosphates of general formula (**2**) have been prepared according to the same experimental procedure. The **ACV**-DP was prepared in two steps by a first chemical phosphorylation with $POCl_3$[4,5] , in triethylphosphate at 0 °C to give the corresponding phosphorodichloridrate which is then hydrolyzed and eluted through a DEAE-cellulose column with triethylammonium bicarbonate in order to obtain the **ACV**-MP as triethylammonium salt.



Scheme 1. Synthesis of triphosphate (**2**)

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† Electronic Supplementary Information (ESI) available: cell cultures and analysis; synthesis of triphosphates (**2**) and characterization. See http://dx.doi.org/10.1039/b000000x/

The latter was then transformed into the tributylammonium salt by elution through a Dowex 50W-X8 (pyridinium form resin), followed by treatment with tributylammine in methanol/water (1:1) and then further phosphorylated with tributylammonium ortophosphate in the presence of 1,1'-carbonyldiimidazole to afford **ACV**-DP[5,6]. Finally, the tributylammonium salt of the resulting **ACV**-DP was condensed[7] with the **NU**-monophosphate (usually commercially available) in DMF, again using 1,1'-carbonyldiimidazole as the activator[5,6].

All the reactions were carried out using the tributylammonium salt, while the purifications was performed on a DEAE-cellulose column with a triethylammonium bicarbonate solution to give the corresponding phosphates as triethylammonium salts. All the final compounds were then converted into the corresponding sodium salts by elution through a Dowex 50W-X8 (sodium form resin).

Table 1 contains all the substrates synthesized and tested so far.

Table 1[a] Nucleoside triphosphate-acyclovir derivatives produced in the present research.

Entry	**Nu**-MP[2]	**ACV**-TP-**NU** (yield)
1	dG-MP	**ACV**-TP-dG (59%)
2	dA-MP	**ACV**-TP-dA (39%)
3	T-MP	**ACV**-TP-T (46%)
4	dC-MP	**ACV**-TP-dC (39%)

[a]ACV= acyclovir, ACV-DP has been prepared in 41% yield; [2]dG= 2'-deoxyguanosine; dA= 2'-deoxyadenosine; T= thymidine; dC= 2'-deoxycytidine; MP= monophosphate; DP= diphosphate; TP= triphosphate; all monophosphate are commercially available

Biological activity of triphosphates (2)

It is important to note first that in order to be active, the prodrug needs to retain its original structure which allows it to cross the cell membrane. Indeed when we treated SW620 cells (colon rectal carcinoma cells) with the fragments **ACV**-MP or **ACV**-DP, no toxicity was detected, indicating that the hydrolyzed fragments are not able to enter the cell. Addition of lipids is effective in helping the membrane crossing in the case of **ACV**-MP and **ACV**-DP, while the **ACV**-TP-**NU** compounds show the same toxicity both in the presence and in the absence of lipids. This clearly indicates that the cytotoxicity of our prodrugs is due to their structural features that allow them to enter the cell and to release the active drug once inside.

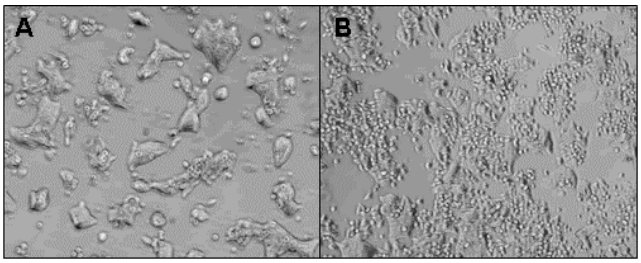

Fig.1 (A) SW620 colo-rectal carcinoma cells tested with ACV-TP-dG; (B) Control SW620 colo-rectal carcinoma cells .

All compounds in our library were then tested on cell coltures of different colo-rectal carcinoma cell lines, showing a slower growth of cells treated with the prodrug (Fig. 1A) in comparison with the control experiments (i.e. not treated cells, Fig. 1B).

Figures 2A and 2B show the results in term of vitality and proliferation (see supporting material) of COLO320 cells (colo-rectal carcinoma) when submitted to treatment with the prodrugs constituted by acyclovir and 2'-deoxyguanosine (dG), 2'-deoxyadenosine (dA), thymidine (T) and 2'-deoxycytidine (dC) as the **NU** fragment. It is quite evident that all the prodrugs tested show toxicity against cancer cells, and it is interesting to note that the activity at high (1 mM) concentration is related to the group **NU** used, according to the following order: dG > T > dA > dC.

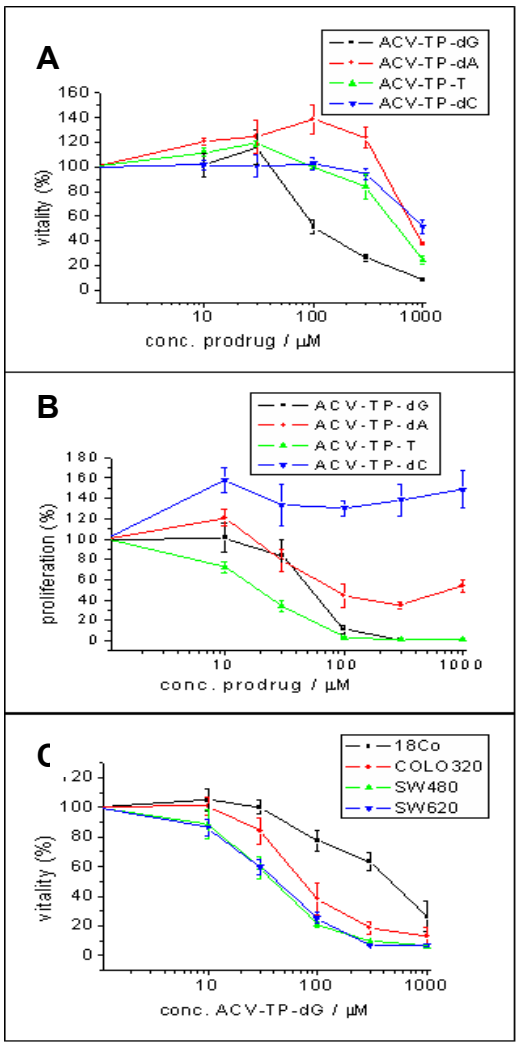

Fig.2 Cellular vitality (A) and proliferation (B) of COLO320 cells using different NU groups. Cellular vitality at different concentration of prodrug ACV-TP-dG in healty cell line and 3 cancer cells line. A) 18Co: normal colon epithelial cell line; b) COLO320: metastatic colorectal tumor cell line; c) SW480: colorectal tumor cell line; d) SW620: metastatic colorectal tumor cell line.

Prodrug **ACV**-TP-dG was then tested on a series of cell coltures of different cancer types of colo-rectal carcinoma and normal colon epithelial cell line, showing that its cytotoxicity is higher for cancer

cells than healthy ones by about an order of magnitude at 300 μM concentration (Fig. 2C). This suggests that the release of the active substance may indeed be due to one or more enzyme activities that are higher in such cells.

The present experimental results suggest that the activity of the prodrugs follows the order which should be expected if telomerase was the active enzyme in the process. Telomerase uses series of TTAGGG to build telomeres[8]. Therefore, at each cycle, telomerase should release three cytotoxic compounds in the case of **ACV**-TP-dG, two for **ACV**-TP-T, only one for **ACV**-TP-dA and none for **ACV**-TP-dC. Most somatic human cells do not produce telomerase, which is on the contrary highly expressed in cancer cells. While a telomerase contribution to the toxicity of the present prodrugs to cancer cells is an attractive possibility, its quantitation should await further experimental work and is outside the scope of the present research.

Conclusions

We have designed and synthesized a small library of acyclovir containing triphosphates aiming at the development of new active prodrugs. The latter are selectively toxic against cancer cells, thus showing that i) the newly designed molecules can penetrate the cell membrane and that ii) the drug is selectively liberated in cells affected by cancer. These findings hold promises towards a new approach in the therapeutic treatment of tumors.

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7 Conclusions

A new approach in the therapeutic treatment of tumors based on telomerase activity of cancer cells has been attempted. A library of triphosphate prodrugs recognized by telomerase enzymes has been synthesized and the activity of all compounds tested with cellular coltures of different cancer types showing promising activity. Every compound tested exhibits toxicity on the cancer cell lines used. Several evidences, particularly the cytotoxicity of the prodrugs with the same **CT** portions and different **TS** groups (Figure 31), the results with healthy cells (Figure 33) and the results with HeLa cell lines (Figure 37) support our hypothesis.

The tests with liposome gave unexpected results. The high charge of our prodrugs should prevent them to cross the cellular membrane thus decreasing their toxicity. We have seen instead similar value both with liposome and without liposome. This means that the molecule can pass through the cellular membrane without aid of liposome probably due to an active transport mechanism. On the other hand we have found that the monophosphate or diphosphate of the cytotoxic compound can not cross the cellular membrane without liposome. This is important because it shows that if the prodrug is hydrolized out of the cell it cannot be toxic without liposome.

All these evidences are in agreement with our hypothesis. The prodrug can cross the membrane of tumor cells and it is then recognized by the telomerase which hydrolizes it into 2'-deoxyguanosine monophosphate and the diphosphate bound to the molecule that is responsible for the cytotoxicity of the drug. Despite this positive evidence, no test allowed us to get a molecular proof validating our hypotheses. It cannot therefore be excluded that also DNA-polymerase and phosphatase (not only telomerase) are involved in hydrolysis of the prodrug. A further step of the present work can be the development of an analytic method that could help finding the missing molecular proof.

The modification of triphosphate leads indeed to a more toxic **AraC-TP-dG** compound, thus showing that more active prodrugs can be obtained by a careful choice of new cytotoxic portion **CT.** Moreover this compound showed an *in vivo* activity towards leukemia which can be compared with the antileukemia drugs AraC. The next step is to increase the activity of these compounds changing, for example, the **CT** portions.

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