Novel Analogues of 5-Fluorouracil – Synthesis, X-ray Crystallography, and Cytotoxic Effects in Normal Human Peripheral Blood Lymphocytes and Colon Adenocarcinoma HT 29

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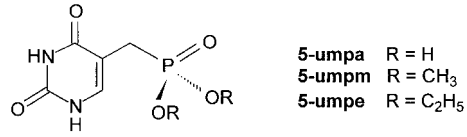
The aim of many studies is to discover new chemicals as potential antitumor agents. We have prepared novel analogues of 5-fluorouracil (5-Fu): 5-uracilmethylphosphonic acid (5-umpa), dimethyl 5-uracilmethylphosphonate (5-umpm), diethyl 5-uracilmethylphosphonate (5-umpe) and a K^+ (K^+/5-umpa, **1**) and Na^+ (Na^+/5-umpa, **2**) complex of 5-umpa. Complexes **1** and **2** have infinite chain structures built up by H-bonded pairs of 5-umpa$^-$ and $\{(H_2O)K(\mu\text{-}OH_2)_3K(OH_2)\}^{2+}$ and $\{(H_2O)_2Na(\mu\text{-}OH_2)_2Na(OH_2)_2\}^{2+}$ entities. The alkali metal ions bind directly to the exocyclic oxygen atoms O(2) and O(4) of the nucleobase moiety and indirectly, i.e. through hydrogen-bond interactions involving metal-coordinated water molecules, to the phosphonate group. The new derivatives were tested for their antiproliferative and cytotoxic effects on normal human peripheral blood lymphocytes and HT 29 cancer lines in vitro using the tetrazolium salt (MTT) assay. The IC_{50} values were evaluated (the drug concentration inhibiting 50 % of the cell growth after 72 h exposure of cells to the drug). The results indicate that 5-umpa, 5-umpm, 5-umpe, and the K^+ and Na^+ complexes of 5-umpa do not exert toxic effects on regular lymphocytes and on the colon adenocarcinoma HT 29 cell line ($IC_{50} > 1$ mM), in comparison with 5-Fu. However, an atypical course of survival curves was observed after treatment of HT 29 cells with 5-umpa, 5-umpm and the Na^+ complex of 5-umpa. These observations suggest that it is very important to continue the studies in different biological systems in vitro and in vivo using various experimental protocols.

Introduction

For many years 5-fluorouracil (5-Fu) has been widely used in cancer chemotherapy, especially in the treatment of colorectal, liver, ovarian, head and neck, and lung and breast carcinomas.[1–4] This fluorinated analogue of the natural pyrimidine base uracil can be used alone in monotherapy or in combination with other cytotoxic drugs (e.g. methotrexate, cisplatin) or with agents that are themselves not toxic but that modulate antitumor activity of 5-Fu (e.g. leucovorin).[3,5–10] Unfortunately, clinical application of this drug is limited by many undesirable effects, such as myelosuppresion (leucopenia, thrombocytopenia), gastrointestinal symptoms (nausea, vomiting, stomatitis, diarrhea), neurotoxicity (ataxia of trunk or extremities, unsteady gait, slurred speech, nystagmus), cardiotoxicity, and hyperpigmentation of skin.[1] Due to this scientists continually search for new less toxic and more effective compounds.

We have discovered novel phosphonate derivatives of uracil: 5-umpa (5-uracilmethylphosphonic acid), 5-umpm (dimethyl 5-uracilmethylphosphonate), 5-umpe (diethyl 5-uracilmethylphosphonate), and a K^+ and Na^+ complex of 5-umpa (Scheme 1).[11]



Scheme 1. Schematic structures of the uracil phosphonate derivatives.

Recently, the first three compounds 5-umpa, 5-umpm, and 5-umpe have been shown to prolong the survival time of mice with lymphoid leukemia 1210 when used in combination with cisplatin and as individual agents.[12] These findings prompted studies on the complexing properties with respect to platinum[13] and biologically relevant metal ions.[14,15]

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The spectroscopic investigation and X-ray structure determination of 5-umpa, 5-umpm, and 5-umpe were described previously.[11,16] Solution studies on complex formation between the uracil derivative 5-umpa and alkaline earth metal ions have been reported recently and showed that Ca^{2+} and Mg^{2+} interacted directly with the phosphonate oxygen atom, while the neutral nucleobase residue did not participate in complex formation.[15] In the present study, two complexes of 5-umpa with Na^+ and K^+ ions are described. Coordination of alkali metal ions to nucleobase derivatives having negatively charged phosphonate groups also has some relevance to the interaction of alkali metal ions with nucleic acids in biological systems. Due to their function as counterions for the charge neutralization of the sugar-phosphate backbone of DNA and RNA, alkali metal ions are essential for the stability of nucleic acids. In principle, they can interact with nucleic acids through direct coordination or through hydrogen bonding through aqua ligands; the potential binding sites are the negatively charged phosphate groups and the nucleobase donor atoms, in particular, exocyclic carbonyl oxygen atoms. Recently, direct binding of Na^+ ions in the minor groove of DNA, e.g. to N(3) of adenine, O(2) of thymine or cytosine, has been proposed on the basis of high-resolution X-ray analyses of B-DNA fragments.[17] Furthermore, coordination of Na^+ and K^+ to the exocyclic oxygen atoms of nucleobases, such as O(6) of guanine and O(4) of thymine or uracil, has been found to be crucial for the formation of multistranded nucleic acid arrangements, e.g. guanine, uracil or thymine quartets.[18–22] Several studies have been devoted to the preparation of models for alkali-metal-ion–nucleic-acid interactions. These mostly involve the use of neutral model nucleobases that lack phosphate groups as alternative binding sites for the alkali metal ions.[14,15,23–25] Although 5-umpa differs from the natural uracil nucleotide, it can nevertheless give some further insight into the coordination mode in complexes of alkali metal ions and nucleic acids.

Here we report on the solid-state structures of $[\{K(H_2O)(\mu\text{-}OH_2)_{1.5}\}_2(5\text{-umpa}^-)_2]_n$ (**1**) and $[\{Na(H_2O)_2(\mu\text{-}OH_2)\}_2(5\text{-umpa}^-)_2]_n$ (**2**), where Na^+ and K^+ bind directly to the exocyclic oxygen atoms O(2) and O(4) of the nucleobase part and interact indirectly, i.e. through hydrogen-bond interactions involving metal-coordinated water molecules, with the phosphonate group.

The first step in estimating biological activity of new chemical compounds is to study their cytotoxicity in vitro. In view of this, we used the ability of viable cells to convert the yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphentyltetrazolium bromide] to purple crystals of formazan by mitochondrial dehydrogenases.[26]

Results and Discussion

X-ray suitable crystals of the potassium and sodium complexes of 5-umpa were obtained from aqueous solutions of 5-umpa upon adjusting the pH to 8.9 with KOH or NaOH. Single-crystal X-ray analysis (Table 5) of the potassium complex **1** reveals an infinite chain structure built up by H-bonded pairs of 5-umpa$^-$ and $\{(H_2O)K(\mu\text{-}OH_2)_3\text{-}K(OH_2)\}^{2+}$ entities, in which two K^+ ions are triply bridged by water molecules (Figure 1 and Table 1, Scheme 2).

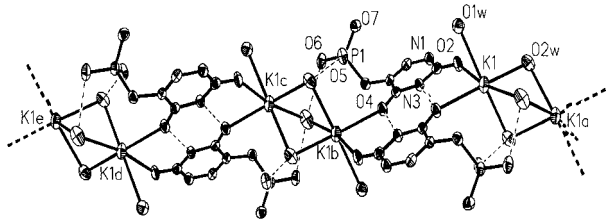
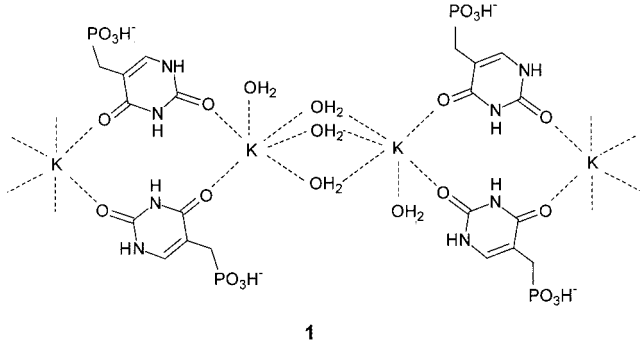


Figure 1. Infinite chain observed in the K^+ complex of 5-umpa showing H-bonding interactions.

Table 1. Bond lengths [Å] and angles [°] in **1** and **2**.

	1	**2**
M(1)–O(2)	2.719(3)	2.429(2)[d]
M(1)–O(4)	2.761(2)[a]	2.423(2)
M(1)–O(1w)	2.873(3)	2.470(2)
M(1)–O(2w)	2.710(3)	2.463(2)
M(1)–O(2w)	2.813(3)[b]	2.405(2)[c]
M(1)–O(3w)	2.801(3)	2.376(3)
O(2)–M(1)–O(4)	81.78(7)[a]	88.92(7)[d]
O(2)–M(1)–O(2w)	90.02(7)	81.81(7)[c,d]
O(2)–M(1)–O(3w)	167.38(6)	101.31(8)[d]
O(1w)–M(1)–O(4)	81.99(7)[a]	96.40(8)
O(2w)–M(1)–O(3w)	77.75(7)	89.95 (8)
O(2w)–M(1)–O(2w)	75.52(9)[b]	88.25(7)[c]
O(2w)–M(1)–O(4)	169.58(8)[a]	170.38(7)[c]
O(3w)–M(1)–O(4)	110.72(6)[a]	93.83(8)

[a] –1/2 –*x*, –1/2 – *y*, 1 – *z*. [b] –1 – *x*, *y*, 1/2 – *z*. [c] –*x*, 1 – *y*, 2 – *z*. [d] –*x*, 1 – *y*, 1 – *z*.



Scheme 2. Formula diagram of **1**. H-bonding interactions are omitted for clarity.

The phosphonate group of the uracil derivative is monodeprotonated as expected on the basis of the pK_a value of 5-umpa (7.15 ± 0.01) and as evident from the P–O bond lengths.[15] Two P–O bonds [P(1)–O(5) and P(1)–O(6)] are of equal length within the standard deviations [1.499(2) and 1.506(2) Å], and these bond lengths are slightly longer than those found for P=O double bonds (1.449–1.489 Å).[30] The P(1)–O(7) bond of 1.576(2) Å is significantly longer and compares well with the average bond length of a P–O single bond, so that the proton can be assumed to be bound to

O(7).[30] This interpretation is also consistent with the H-bonding pattern described below. Direct binding of K^+ ions to the nucleobase moiety through the exocyclic carbonyl oxygen atoms O(2) and O(4), as well as hydrogen bonding between K^+-bound water and the phosphonate group is observed. Each K^+ is coordinated in a distorted octahedral manner to four water molecules, three of which form bridges to the neighboring K^+ ion, and two carbonyl oxygen atoms; the latter are *cis* to each other. The pairs of 5-umpa$^-$ ligands are connected through direct interaction with K^+ and through two H-bonds of normal length that involve N(3) and O(4) [O(4)···N(3) 2.864(4) Å, –1/2 – *x*, –1/2 – *y*, 1 – *z*]. The H-bonded umpa$^-$ ligands that are located around a twofold axis of symmetry are exactly coplanar. Bond lengths and angles in the uracil ring show no unusual features for this heterocyclic system. Additional H bonding occurs through the bridging water ligands O(2w) and O(3w) and the phosphonate oxygen atoms O(5) and O(6); the O(2w)···O(5) and O(3w)···O(6) distances are 2.707(3) and 2.931(3) Å, respectively. These intrastrand hydrogen-bond formations give rise to eight-membered macrochelates, as indicated in Figure 1. Besides the intrastrand interactions, interstrand H bonding, i.e. between neighboring chains, through the terminal and bridging aqua ligands is observed. This involves phosphonate groups as well as exocyclic carbonyl oxygen atoms [O(2)] (Table 2). Thus, extensive hydrogen bonding is realized in the crystal packing. The shortest H-bond [2.614(3) Å] is formed between two mono-deprotonated phosphonate groups of adjacent chains [O(5)···O(7), –*x*, –*y*, 1 – *z*].

Table 2. Hydrogen-bonding interactions [Å] in **1**.

O(2w)···O(5)[a]	2.707(3)	O(3w)···O(6)[a]	2.931(3)
O(4)···N(3)[b]	2.864(4)	O(1w)···O(2)[c]	2.819(3)
O(1w)···N(1)[d]	2.833(3)	O(2w)···O(6)[e]	2.826(3)
O(1w)···O(6)[f]	2.823(4)	O(5)···O(7)[f]	2.614(3)

[a] –1/2 + *x*, –1/2 – *y*, –1/2 + *z*. [b] –1/2 – *x*, –1/2 – *y*, 1 – *z*. [c] –1/2 – *x*, –1/2 + *y*, 1/2 – *z*. [d] *x*, –1 + *y*, *z*. [e] –1/2 + *x*, 1/2 – *y*, –1/2 + *z*. [f] –*x*, –*y*, 1 – *z*.

The basic structure of the sodium complex of 5-umpa **2** is identical to that of **1**. Figure 2 shows a section of the infinite chain formed by pairs of 5-umpa$^-$ ligands connected through two N(3)···O(4) H-bonds and $\{(H_2O)_2Na(\mu\text{-}OH_2)_2Na(OH_2)_2\}^{2+}$ moieties. For the P–O bond lengths, the same trend is observed as in the case for **1** [P(1)=O(7) 1.507(2) Å, P(1)=O(5) 1.524(2) Å and P(1)–O(6)H 1.559(2) Å]. Again, the alkali metal ions bind directly to the exocyclic carbonyl oxygen atoms O(2) and O(4) and interact indirectly through aqua ligands with the phosphonate oxygen atoms (Table 1 and Table 3) leading to eight-membered macrochelates as shown in Figure 2 and Scheme 3.

However, in contrast to **1**, only one intramolecular hydrogen bond is formed between a terminal water ligand and O(6) of the phosphonate group [2.931(3) Å]. Interestingly, this H-bond involves the phosphonate oxygen atom that is likely to be protonated, considering the P(1)–O(6) bond length of 1.559(2) Å. Compared with the conformation of the phosphonate group observed in **1**, the phosphonate

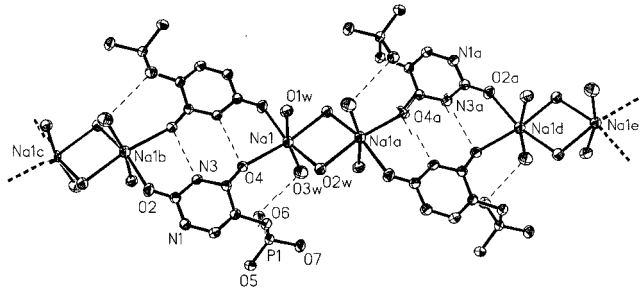
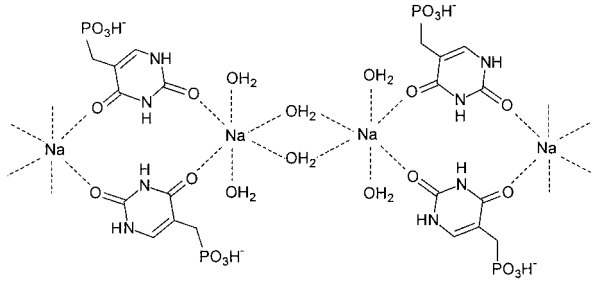


Figure 2. Infinite chain observed in the Na^+ complex of 5-umpa showing H-bonding interactions.

Table 3. Hydrogen-bonding interactions [Å] in **2**.

O(1w)···O(7)[a]	2.701(3)	O(3w)···O(6)	2.936(3)
O(4)···N(3)[b]	2.845(3)	O(1w)···O(2w)[c]	3.167(3)
O(1w)···O(3w)[c]	2.876(3)	O(1w)···N(1)[d]	2.781(3)
O(2w)···O(2)[d]	2.841(3)	O(2w)···O(4w)	2.858(3)
O(3w)···O(4w)[e]	2.785(3)	O(4w)···O(7)	2.735(3)
O(4w)···O(7)[f]	2.834(3)	O(5)···O(6)[g]	2.551(3)

[a] *x*, –1 + *y*, *z*. [b] –*x*, 1 – *y*, 1 – *z*. [c] –*x*, 1 – *y*, 2 – *z*. [d] 1 – *x*, 1 – *y*, 1 – *z*. [e] –1 + *x*, *y*, *z*. [f] –*x*, 2 – *y*, 2 – *z*. [g] –*x*, 2 – *y*, 1 – *z*.



2

Scheme 3. Formula diagram of **2**. H-bonding interactions are omitted for clarity.

group seems to be pointed away from the $\{(H_2O)_2Na(\mu\text{-}OH_2)_2Na(OH_2)_2\}^{2+}$ entity. This allows a short interstrand H-bond between O(7) and O(1w) at *x*, –1 + *y*, *z*. The differences in the H-bonding details (i.e. intra- versus interstrand) in **1** and **2** may be connected to the fact that the average K–O bond length (2.78 Å) in **1** is 0.35 Å longer than the average length of a Na–O bond in **2** (2.43 Å). Furthermore, the different number of bridging and terminal water ligands, as well as the presence of further uncoordinated, interstitial water molecules in **2**, lead to major differences in the crystal packing of **1** and **2**. In particular, the uncoordinated water molecule O(4w) generates an extended network of H-bonding interactions, as depicted in Figure 3. Adjacent chains are stacked along the *x* axis, however, the distance of approximately 5 Å rules out any significant stacking interactions between the uracil rings. The stacks are stabilized by hydrogen bonding between the bridging water ligand O(2w) and the carbonyl oxygen atom O(2) at 1 – *x*, 1 – *y*, 1 – *z*. Neighboring stacks are connected through hydrogen-bonding interactions between terminal O(1w) and the phosphonate group [O(1w)···O(7) 2.701(3) Å, *x*, –1 + *y*, *z*]. In addition, strong H-bonds with a length of 2.551(3) Å

are formed between adjacent mono-deprotonated phosphonate groups, these are even shorter than the analogous ones in **1**.

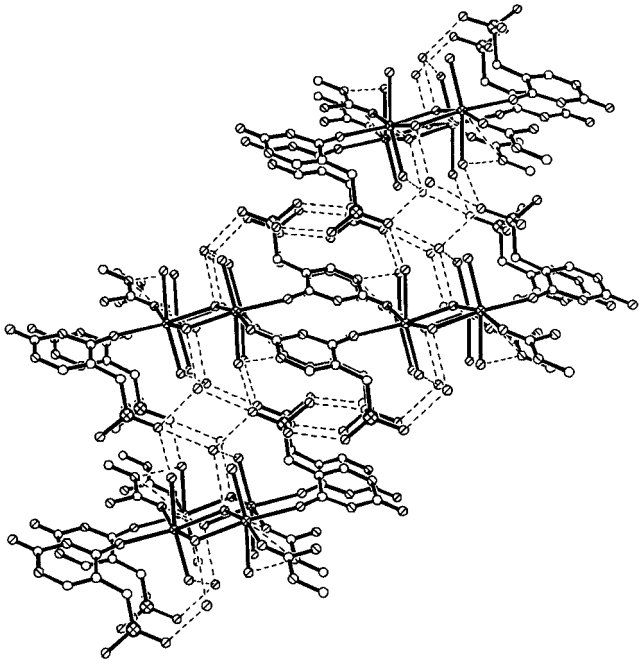


Figure 3. Hydrogen-bonding pattern in the crystal packing of **2**.

Recent solution studies have shown that the phosphonate oxygen atoms were the primary target for alkaline earth metal ions, while the uracil residue did not participate in complex formation when it was in its neutral form.[16] Likewise – although with some exceptions[31] – cocrystallization of Mg^{2+} with neutral nucleobases usually gives structures in which the $[Mg(H_2O)_6]^{2+}$ cation interacts indirectly through the aqua ligands with the nucleobase.[32] By contrast, the nucleobase residue seems to be the primary target of alkali metal ions as evident from the direct binding observed in **1** and **2**, and in other examples with neutral nucleobases.[25]

The observation of direct binding to the carbonyl oxygen atoms of uracil and indirect binding to the phosphonate oxygen atoms can be considered relevant to the interaction of alkali metal ions with natural nucleotides that contain nucleobase residues and negatively charged phosphate groups as alternative binding sites.

Survival curves for regular lymphocytes and HT 29 cells exposed to different concentrations of 5-Fu, 5-umpa, 5-umpm, 5-umpe, Na^+/5-umpa, K^+/5-umpa – and IC_{50} values calculated on grounds of the mathematical analysis of these curves– are presented in Figure 4A–4F (lymphocytes), Figure 5A–5F (HT 29), and in Table 4.

Data for 5-umpe and 5-umpm are not presented in Table 4 due to the fact that the concentrations used (1–10 mM) only caused the growth of the lymphocytes to slow down up to 85% (Figure 4C, Figure 4D). Likewise, in cells treated with Na^+/5-umpa, only the highest concentration caused an evident viability decrease to about 50%; however, this value was never exceeded (Figure 4E). Thus, it was impossible to establish IC_{50} from these survival curves.

The IC_{50} values for-5 Fu, 5-umpa, and K^+/5-umpa are 1.72 mM, 5 mM, and 4.15 mM, respectively, and their relation is as follows: 5-Fu < K^+/5-umpa < 5-umpa (Figure 4A, 4B, 4F).

The IC_{50} values are similar for K^+/5-umpa and 5-umpa, but they are nearly twice that obtained for 5-Fu. All IC_{50} values obtained after treatment of regular lymphocytes with 5-Fu and its novel analogues are higher than 1 mM, which suggests the almost lack of cytotoxic effects of these compounds – this is the most relevant and desired feature of every compound that is considered as a new tumor chemotherapeutic drug.[3,36]

However, the data given in Table 4 show that new analogues of 5-Fu also exhibited no activity in killing HT 29 tumor cells. Only model 5-Fu was very effective against HT 29 tumor cells; the IC_{50} factor was 2.9 μM.

In fact, we would like to point out that it is difficult to establish mutual relationships between the new 5-Fu analogues with respect to their effect on HT 29 tumor cells due to the atypical course of each survival curve. Therefore, it seems reasonable to separately analyze the effects exerted by each particular compound. As mentioned above, 5-Fu is very active against HT 29 tumor cells (Figure 5A). However, even small (0.5 μM) doses of 5-umpa and 5-umpm induced a rapid decrease in the viability of the HT 29 cells to 60–65%. A further increase in the dose slowly diminished the HT 29 cells viability, and finally IC_{50} was reached at 2.16 mM for 5-umpa and 1.58 mM for 5-umpm (Figure 5B, Figure 5C). It was impossible to establish IC_{50} for Na^+/5-umpa. The survival curve for this compound was very atypical. Low concentrations of Na^+/5-umpa, 0.2 μM and 1 μM, caused a rapid loss of the viability of the HT 29 tumor cells to 65% and 51%, respectively. A further dose increase did not affect the viability of the HT 29 cells, which was oscillating near 50% (Figure 5E). The survival curve for K^+/5-umpa was more typical, IC_{50} was 3.31 mM, whereas a dose of 1 μM diminished the viability to 75% (Figure 5F). Compound 5-umpe appeared to be ineffective against the HT 29 cells. Only at the highest concentration did the cell viability decrease to 55% (Figure 5D).

Anticancer chemotherapy usually involves the administration of various chemical compounds, which immediately kill, or take control of transformed cells. Unfortunately, the success of chemotherapy strongly depends on evoked, undesirable effects. The majority of currently used chemotherapeutics do not display particular anticancer specificity, and affect both tumor and nontumor tissues. Present in vitro and in vivo studies point out that many presently applied compounds exhibit mutagenic, teratogenic, and carcinogenic activity.[33]

Our study suggests that 5-Fu and its new analogues show insignificant cytotoxic effects on regular human peripheral blood lymphocytes. These findings might be of help in applying the drugs characterized below as potential anticancer compounds in chemotherapy. According to the cytotoxic and genotoxic studies of Tice et al.,[34] the compounds

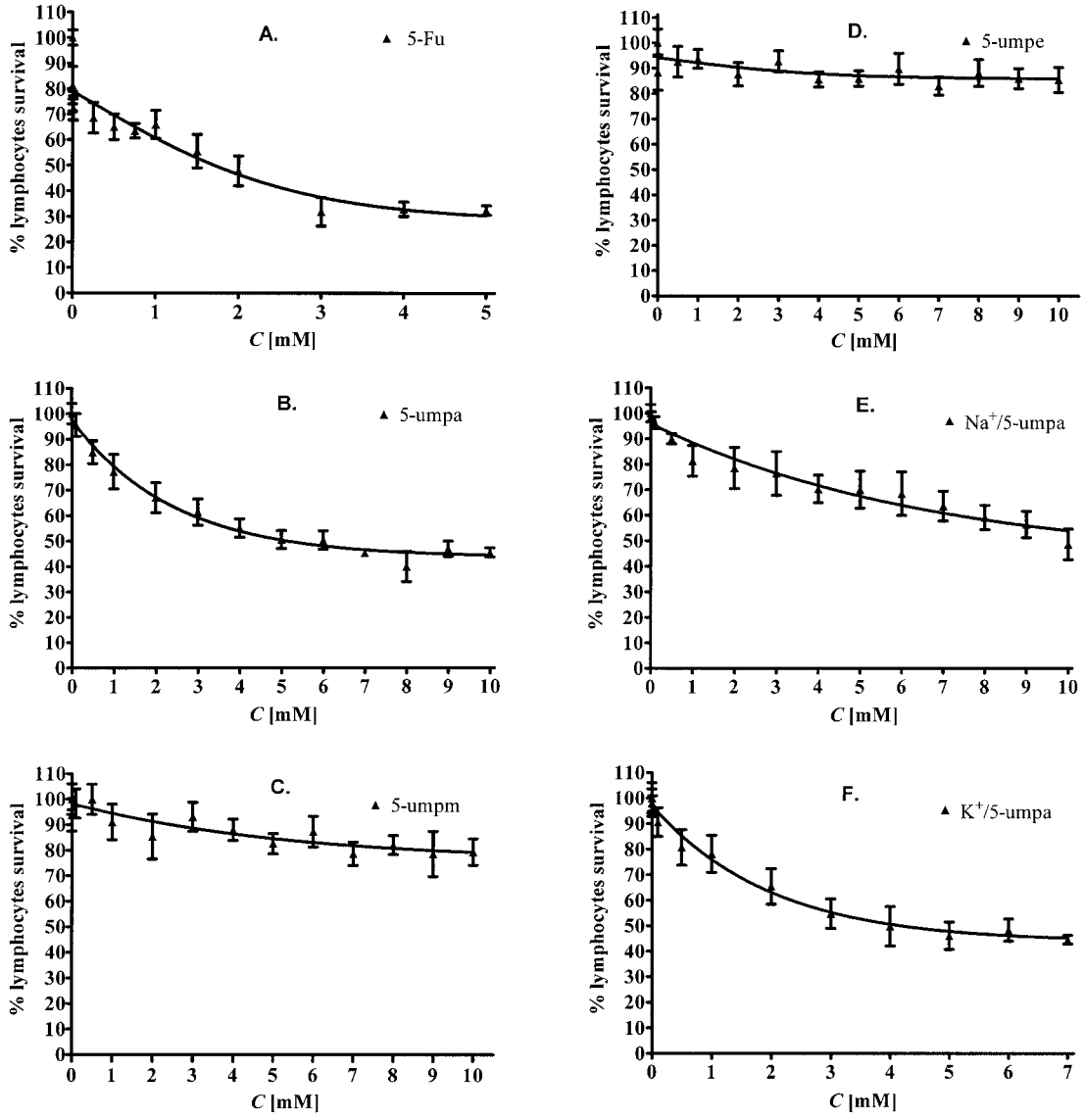


Figure 4. Dose-dependent growth inhibition curves for human peripheral blood lymphocytes after exposure to: 5-Fu **(A)**, 5-umpa **(B)**, 5-umpm **(C)**, 5-umpe **(D)**, Na^+/5-umpa **(E)**, K^+/5-umpa **(F)**. Lymphocytes were treated with various concentrations of the tested compounds for 72 h (37 °C, 5% CO_2). After incubation, the antiproliferative effects of these compounds were examined by MTT assay. These results represent the mean ± s.d. of three replicates from at least six separate experiments.

showing noticeable effects with a concentration of over 10 mM can be recognized as nontoxic; thus, we postulate that Na^+/5-umpa, 5-umpm and 5-umpe remain harmless for human lymphocytes.

The results obtained after exposure of HT 29 colorectal adenocarcinoma cell lines to new 5-Fu analogues seem to be interesting despite the fact that 5-Fu exerts the strongest aversive activity. These results are similar to those reported by Mader et al.[35] and Wiebke et al.[10]

IC_{50} was not assessed for each compound, however, we would like to point out that, in particular, after the treatment with 5-umpa, 5-umpm or Na^+/5-umpa, rapid loss of viability of the HT 29 cells was observed already after small doses of up to 1 μM. A further increase in the dose did not significantly affect the viability decrease. We cannot forget that DNA repair is an accomplice in cancer therapy resistance. It influences the chemosensitivity of tumor cells toward DNA-reactive cytotoxic drugs.[37] Probably, the effectiveness of the repair mechanisms in HT 29 cells strongly contributed to this phenomenon, and results in the limited tolerance to toxic activity of the applied compounds. However, these mechanisms were not fully successful, which may be in favor of the new 5-Fu analogues. It may be assumed that not only the dose but also the expose time should be taken into consideration in toxicity assessment of the new analogues of 5-Fu. Further studies are needed to confirm this hypothesis. In summary, the above results seem to be incredibly interesting because a question can be put forward: is the key element in searching for efficient chemotherapeutics the high toxicity of the compound examined

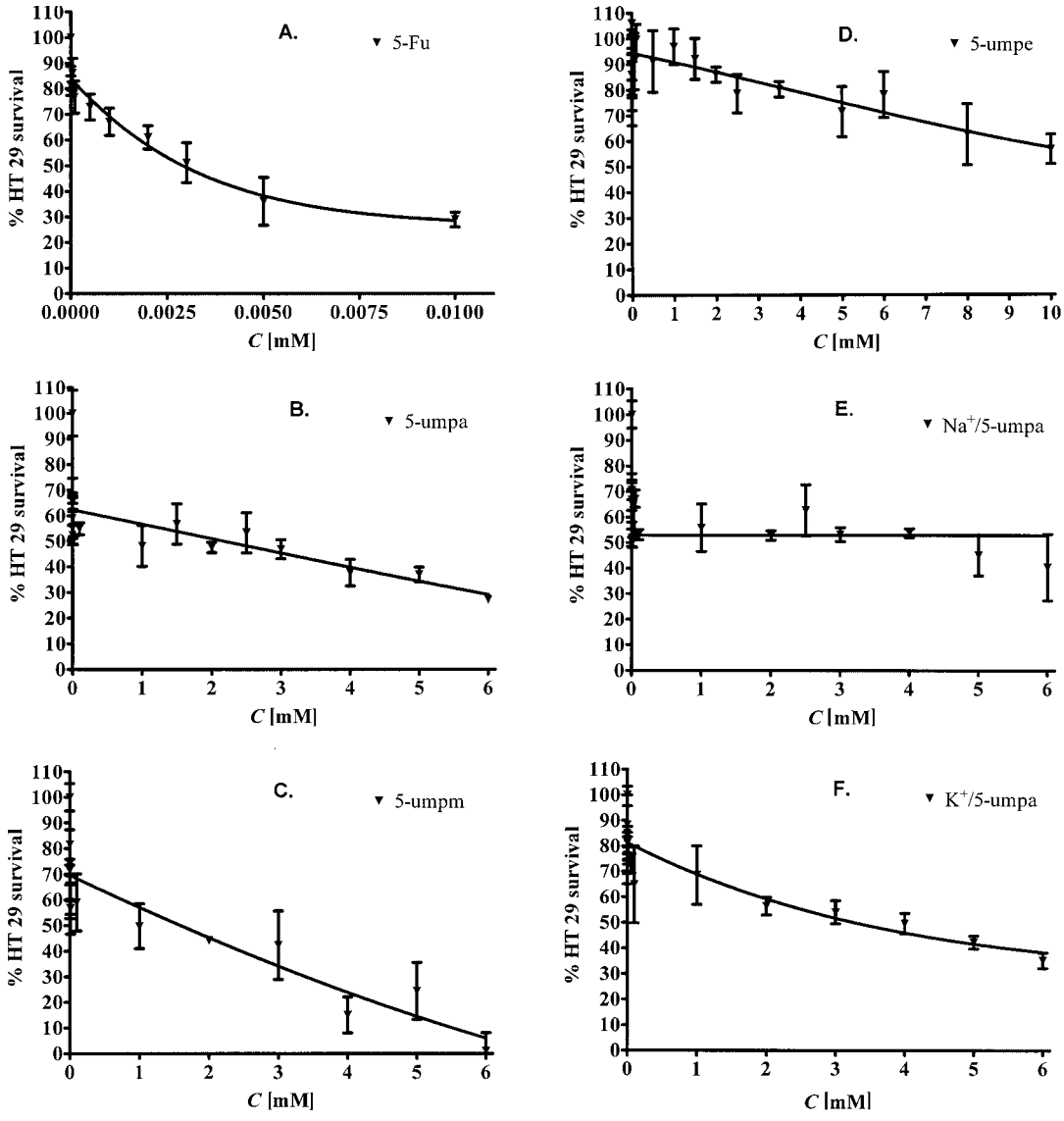


Figure 5. Dose-dependent growth inhibition curves for colon adenocarcinoma cell lines HT 29 after exposure to: 5-Fu **(A)**, 5-umpa **(B)**, 5-umpm **(C)**, 5-umpe **(D)**, Na^+/5-umpa **(E)**, K^+/5-umpa **(F)**. Treatment with various concentrations of the tested compounds for 72 h (37 °C, 5% CO_2). After incubation of HT 29 in 96-well plates, the antiproliferative effects of these compounds were examined by MTT assay. These results represent the mean ± s.d. of three replicates from at least six separate experiments.

Table 4. Cytotoxicity of 5-Fu and its new analogues in normal human peripheral blood lymphocytes and colon adenocarcinoma cells HT 29 in vitro after exposure for 72 h. IC_{50} values were established as drug concentrations that cause 50% inhibition of cell growth after 72 h incubation.

Drug	IC_{50}	
	Lymphocytes	HT 29
5-Fu	1.72 mM	0.0029 mM
5-umpa	5 mM	2.16 mM
5-umpm	–[a]	1.58 mM
5-umpe	–[a]	–[a]
Na^+/5-umpa	–[a]	–[b]

[a] Decrease in the viability of the cells to 80% for 5-umpm and 5-umpe, but to nearly 50% after exposure to the highest concentration of Na^+/5-umpa. [b] Rapid loss of cells viability at low concentrations (0.5–1 μM) to 50–65%, higher concentrations did not further decrease cell viability.

with respect to tumor cells, or the low toxicity towards healthy cells after application of small doses? We opt for the latter, especially, since it promotes searching for adjuvants that exhibit a synergistic effect in the efficient extermination of tumor cells.

Our results are of significance if the studies undertaken by Ochocki and Graczyk,[12] who investigated synergism of new analogues of 5-Fu and cisplatin in vivo in leukemia mouse 1210 (so-called "*platinum pyrimidine blues*"), are referred to. The authors demonstrated that 5-umpe did not exert any cytotoxic effect, whereas umpm, in conjunction with cisplatin, was very effective against L1210 leukemia cells. Our in vitro studies carried out recently confirmed the results of our antecedents with respect to 5-umpe; however, the same studies revealed a nontoxic character of 5-umpm and confirmed its efficiency in the elimination of tumor cells.

5-umpa seems to deserve more attention. Its carcinotoxicity towards colorectal adenocarcinoma cells was observed already at low concentrations. In vivo studies demonstrated the lethal effect of this compound. It is strongly recommended that the synergism of 5-umpa with cisplatin is investigated, with the use of lower doses for more accurate cytotoxic and genotoxic assessment.

The lack of experimental data for Na^+/5-umpa and K^+/5-umpa results from the fact that these analogues have been prepared recently. Notwithstanding the fact that further studies have to be carried out, our first results demonstrate that these compounds, especially Na^+/5-umpa, display a certain ability to destroy HT 29 colorectal adenocarcinoma cells.

Conclusions

In conclusion, the results of our in vitro experiments conducted with the use of 5-umpa, Na^+/5-umpa, and 5-umpm are very interesting and provide evidence for their potential practical application as anticancer drugs. The results obtained are the basis for further studies to establish, for example, their mechanism of action, genotoxicity, and synergism with other drugs. The results will be useful for the selection of compounds that are the most effective chemotherapeutics whilst simultaneously exhibiting the lowest toxicity to healthy cells, and they can be used during the process of their registration as anticancer drugs. They may also indicate new approaches in searching for novel ways of synthesizing analogues for already existing drugs.

Experimental Section

General Remarks: The peripheral blood was obtained from the Blood Bank in Lodz, Poland, whereas the colon adenocarcinoma HT 29 cell line was purchased from the Centre of Oncology in Gliwice, Poland.

Compound 5-Fu was purchased from Sigma–Aldrich CO (St. Louis, MO, U.S.A). The new phosphonate derivatives: 5-umpa, 5-umpm, 5-umpe, or K^+/5-umpa and Na^+/5-umpa were obtained from the Department of Bioinorganic Chemistry, Medical University, Lodz, Poland.

5-uracilmethylphosphonic acid, dimethyl 5-uracilmethylphosphonate, and diethyl 5-uracilmethylphosphonate were prepared as described previously.[11] Infrared spectra of KBr pellets were measured with an ATI MATTSOM IFS FT spectrometer.

$K(C_5H_6N_2O_5P)(H_2O)_{2.5}$ (1): 5-umpa (103 mg, 0.5 mmol) was dissolved in water (20 mL) and adjusted to pH 8.9 with 1 N KOH. Slow evaporation of the solvent at room temperature gave colorless needles of **1** for X-ray analysis. Yield: 116 mg (90%). Drying of **1** in vacuo gave $K(C_5H_6N_2O_5P)\cdot(H_2O)_{0.75}$. Selected IR data: 3553 (br), 3070 (br), 2923 (br), 2847 (br), 1710 (vs), 1460 (br), (m), 1440 (m), 1327 (m), 1134 (m), 997 (m), 913 (s), 503 (s) cm^{-1}. $K(C_5H_6N_2O_5P)\cdot(H_2O)_{0.75}$: calcd. C 23.42, H 3.29, N 10.93; found C 23.31, H 2.93, N 10.87.

$Na(C_5H_6N_2O_5P)(H_2O)_4$ (2): 5-umpa (103 mg, 0.5 mmol) was dissolved in water (20 mL) and adjusted to pH 8.9 with 1 N NaOH. Slow evaporation of the solvent at room temperature gave colorless plates of 2. Yield: 108 mg (92%). Drying of **2** in vacuo gave $Na(C_5H_6N_2O_5P)\cdot(H_2O)_{0.33}$. Selected IR data: 3512 (br), 3060 (br), 2923 (br), 2852 (br), 1685 (vs), 1460 (br), (m), 1421 (m), 1245 (s), 1143 (m), 1022 (m), 908 (s), 517 (s) cm^{-1}. $Na(C_5H_6N_2O_5P)\cdot(H_2O)_{0.33}$: calcd. C 25.43, H 2.71, N 12.04; found C 25.66, H 2.87, N 11.97.

Fresh stock solutions of all compounds were prepared in water [Sigma–Aldrich CO (St. Louis, MO, U.S.A)] and were heated at 37 °C until the compounds dissolved. Before use, the stock solutions were cooled and diluted in a culture medium; they were then used to investigate the cytotoxicity effects in vitro.

PBS (phosphate buffered saline) MTT, SDS (lauryl sulfate), DMF (*N*,*N*-dimethylformamide), DMSO (dimethyl sulfoxide) were purchased from Sigma–Aldrich CO (St. Louis, MO, U.S.A). The culture medium for lymphocytes consisted of RPMI 1640 medium supplemented with 15% FBS (fetal bovine serum), 1% penicillin–streptomicin solution stabilized, 1% L-glutamine, and 1% PHA (phytohemagglutinin), whereas the culture medium for the HT 29 cell line contained of RPMI 1640 with Glutamax, 10% FBS, 1% penicillin–streptomicin solution stabilized, and 1% MEM non-essential amino acid solution. For trypsinization of adherent HT 29 cells, trypsin-EDTA was used. PHA, RPMI with Glutamax, FBS, and trypsin-EDTA were purchased from Gibco Laboratories Life Technologies, INC (Grand Island, New York, U.S.A.); the remaining reagents were purchased from Sigma–Aldrich CO (St. Louis, MO, U.S.A).

X-ray Crystallographic Study: Crystal data for compounds **1** and **2** were collected at room temperature with an Enraf–Nonius-KappaCCD diffractometer (KappaCCD package, Nonius, Delft, The Netherlands, 1997) using graphite-monochromated Mo-K_α radiation (λ = 0.71069 Å) (Table 5). For data reduction and cell refinement, the programs DENZO and SCALEPACK were used.[27] The structures were solved by direct methods and subsequent Fourier syntheses, and refined by full-matrix least-squares on F^2 with the SHELXTL PLUS and SHELXL-93 programs.[28] All non-hydrogen atoms were refined anisotropically. Carbon- and nitrogen-bound hydrogen atoms were placed at calculated positions and given isotropic thermal parameters equivalent to 1.2 times those of the atom to which they are attached. CCDC-251795 for **1** and CCDC-251794 for **2** contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.′

Cell Culture and MTT Assay: The peripheral blood was obtained from six healthy, nonsmoking donors of both sexes. Lymphocytes were isolated by centrifugation in gradient of density of Histopaque 1077 (Sigma–Aldrich CO, St. Louis, MO, U.S.A) (15 min, 100×*g*). Mitogen PHA was used for stimulation of proliferation of lymphocytes which exhibit a doubling time of approximately 30 h. Lymphocytes were counted and then seeded in 96-well microtitre plates (Nunc Brandt Products, U.S.A.) at a density of 10^5cells/well with a final volume of 100 μL. The monolayer of colon adenocarcinoma HT 29 cells in 25 cm^2 flasks was treated with trypsin-EDTA, and then the cells were seeded in 96-well microtitre plates at a density of 6×10^3 cells/well with a final volume 200 μL. The cultures of the HT 29 tumor cells show a doubling time of about 30–40 h.

After a 24-h incubation in a 5% humidified incubator at 37 °C, the cells were treated with different concentrations of 5-Fu and its new analogues. After a 72-h incubation, 20 μL of MTT solution (5 mg/mL in sterile PBS) was added to each well, and the plates were incubated for a further 2 h.

Table 5. Crystallographic data for **1** and **2**.

	1	**2**
Formula	$K(C_5H_6N_2O_5P)(H_2O)_{2.5}$	$Na(C_5H_6N_2O_5P)(H_2O)_4$
Molecular mass	289.23	300.14
Crystal system	monoclinic	triclinic
Space group	*C*2/*c*	$P\bar{1}$
a [Å]	16.906(1)	6.385(1)
b [Å]	6.626(1)	9.404(1)
c [Å]	20.654(1)	10.841(1)
α [°]	–	83.01(1)
β [°]	104.89(1)	78.46(1)
γ [°]	–	71.74(1)
V [Å^3]	2236.0(4)	604.5(1)
Z	8	2
$\rho_{calcd.}$ [g cm^{-3}]	1.718	1.649
Independent reflections	2048	2403
Observed reflections [$I > 2\sigma(I)$]	1453	1876
R_1, wR_2 [$I > 2\sigma(I)$][a,b]	0.043, 0.131	0.047, 0.151
R_1, wR_2 (all data)	0.066, 0.151	0.079, 0.211

[a] $R_1 = \Sigma||F_o| - |F_c||/\Sigma|F_o|$. [b] $wR_2 = [\Sigma w(F_o^2 - F_c^2)^2/\Sigma w(F_o^2)^2]^{1/2}$; $w^{-1} = \sigma^2(F_o^2) + (aP)^2 + bP$; $P = (F_o^2 + 2F_c^2)/3$ with $a = 0.0898$ for **1**, 0.1074 for **2**, $b = 0.0000$ for **1** and 0.0600 for **2**.

The purple formazan crystals produced were dissolved in a mixture of 20%SDS/50%DMF (120 µL/well, 24 h) in the case of the lymphocytes, and in DMSO in the case of the HT 29 cell line (100 µL/well, 5 min).

The plates were agitated on a plate shaker for 5 min, and the absorbance of each well was measured at 580 nm using a Stat Fax–2100 Awareness Technology Inc. microplate reader.[29]

The sensitivity of the cells to the tested compounds was shown as percentage of the survival of the cells (an untreated control value – 100% in relation to drugs concentration values). The cytotoxic effects were expressed as the IC_{50} value, which is the concentration of the drug required to reduce cell growth to 50% relative to the control. Those parameters were estimated on the basis of the analysis of the cell growth curves using the GraphPad Prism 4 computer program. All results were presented as mean ± s.d. of three replicates from six to twelve independent experiments.

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