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# Phosphorylation of dGMP analogs by vaccinia virus TMP kinase and human GMP kinase

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ABSTRACT

Vaccinia virus thymidylate kinase, although similar in sequence to human TMP kinase, has broader substrate specificity and phosphorylates (*E*)-5-(2-bromovinyl)-dUMP and dGMP. Modified guanines such as glyoxal-dG, 8-oxo-dG, $O^6$-methyl-dG, $N^2$-ethyl-dG and $N^7$-methyl-dG were found present in cancer cell DNA. Alkylated and oxidized dGMP analogs were examined as potential substrates for vaccinia TMP kinase and also for human TMP and GMP kinases. Molecular models obtained from structure-based docking rationalized the enzymatic data. All tested nucleotides are found surprisingly substrates of vaccinia TMP kinase and also of human GMP kinase. Interestingly, $O^6$-methyl-dGMP is the only analog specific for the vaccinia enzyme. Thus, $O^6$-Me-dGMP could be useful for designing new compounds of medical interest either in antipoxvirus therapy or in experimental combined gene/chemotherapy of cancer. These results also provide new insights regarding dGMP analog reaction with human GMP kinase and their slow recycling by salvage pathway nucleotide kinases.



## Introduction

Poxvirus infections in human are slowly re-emerging, mainly due to monkeypox virus infecting human [1]. As smallpox was eradicated in the late 1970s, today's population is not immunized against poxviruses. A few compounds such as cidofovir and ST246 are efficient pharmacologic antipox agents [2]. Vaccinia virus strains resistant to cidofovir have already been identified, raising the requirement for novel drugs [3]. Vaccinia virus, the prototype of poxviruses, encodes enzymes for DNA precursors synthesis, including thymidine and thymidylate kinases, expressed in the early phase of infection cycle, and is non-essential to virus growth [4,5]. Vaccinia virus thymidine kinase (TK) behaves like human cytosolic TK, but can also accept deoxycytidine as a substrate [6,7]. Vaccinia virus thymidylate kinase (Vacc-TMPK) phosphorylates TMP, dUMP and bulkier derivatives such as dGMP and 5(E)-5-(2-bromovinyl)-dUMP (BVdU) [8,9]. Such a broad specificity was already shown for herpes simplex virus type 1 thymidine kinase (HSV1-TK) with acyclic guanosine analogs (acyclovir, ganciclovir) [10] and thymidine analogs (brivudin, BVdU) [11]. These derivatives are now used as anti-herpes drugs and, together with HSV1-TK, in experimental combined gene/chemotherapy of cancer and as safety gene in cell therapy. Nucleoside analogs phosphorylated by poxvirus kinases and not by host cell kinases can likely find medical applications, particularly in the case of oncolytic viruses *i.e.,* poxvirus strains targeting tumours [12]. Vacc-TMPK is 42% identical to human TMP kinase (hTMPK) but both enzymes differ in subunit association and active site geometry [8,9]. The Vacc-TMPK structure in a complex with BVdUMP explains the large base pocket resulting from the unique orthogonal subunits arrangement instead of the anti-parallel configuration in human enzyme. Thus, other dGMP analogs could be substrates for Vacc-TMPK. Several

*Abbreviations:* BVdU, (E)-5-(2-Bromovinyl)-2′-deoxyuridine; dThd, 2′-deoxythymidine; dT (M, D, T), P thymidine 5′-mono, di, triphosphate; HSV, herpes simplex virus; hTMPK, human TMP kinase; hGMPK, human GMP kinase; TK, thymidine kinase; Vacc-TMPK, Vaccinia virus thymidylate kinase; VZV, varicella-zoster virus.

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chemical modifications of guanine have been described in oxidized or alkylated DNA, such as glyoxal-dG and 8-oxo-dG or $O^6$-methyl-dG, $N^2$-ethyl-dG and $N^7$-methyl-dG [13]. They are mutagen and lethal, leading to alterations in gene expression and cell signalling [14]. If these compounds are phosphorylated by Vacc-TMPK, they may play a role in gene-therapy of cancer. These bases are removed from DNA by DNA-repair enzymes, such as 8-oxo-Gua DNA glycosidase and $O^6$-Methyl-DNA methyl transferase [14,15], that should be inhibited during such therapies. Here several alkylated (a methyl group on $O^6$ and $N^7$ or an ethyl group on $N^2$) and oxidized (8-oxo- and glyoxal) dGMP analogs are explored, as well as the C-nucleotide 9-deaza-2′-dGMP. C-nucleotides are not substrates for N-hydrolases such as Rcl and are presumably more stable *in vivo* [16]. These nucleotide analogs are studied as specific substrates for Vacc-TMPK, hTMPK and human GMP kinase (hGMPK), the latter being expressed for the first time in *Escherichia coli* as an active enzyme. A variety of dGMP analogs surprisingly act as efficient substrates for Vacc-TMPK. A comprehensive explanation for the observed enzyme specificities is provided by structure-based docking studies.

## Materials and methods

*Chemicals.* All modified 2′-deoxyguanosine 5′-monophosphate analogs were synthesized by Jena Biosciences (Jena, Germany) upon request.

*Cloning and expression of Vacc-TMPK, human TMP and GMP kinase.* Cloning, expression and purification of both Vacc-TMPK and hTMPK have been described previously [8,17]. The 591 bp corresponding to the *gmk* gene coding for the hGMPK was amplified by PCR [18] using the human heart Quick-clone cDNA (BD Biosciences Clontech) as the matrix. The synthetic oligonucleotides used for amplification were 5′-GGAATTC**CATATG**TCGGGCCCCAGGCCTG-3′ and 5′-CGC**GGATC C**TCAGGCGCCGGTCCTTTGAG-3′. During amplification, NdeI and BamHI restriction sites (in bold letters in the oligonucleotide sequences) were created at both ends of the amplified fragments. After digestion by NdeI and BamHI, the amplified fragments were inserted into the pET28a plasmid (Novagen, Inc.) digested with the same enzymes. The DNA inserts were sequenced using the double-stranded dideoxynucleotide sequencing technique [19] in order to check for the absence of any mutational events in the course of amplification. The plasmid harbouring the human *gmk* gene was named pHL40-5.

Recombinant hGMPK was expressed and purified as reported for Vacc-TMPK [8]. The protein appeared to be >95% pure as judged by SDS–PAGE gels (Fig. S1). No loss in activity was observed after 3 months at −20 °C. The protein absorbance was calculated by the content of aromatics in the unfolded protein ($A_{280}$ = 0.345 for 1 mg/mL hGMPK).

*TMP kinase and GMP kinase enzymatic assays.* Both kinase activities were measured at steady state using the classical coupled spectro-photometric assay as described [8,20]. The initial rates were calculated on the basis of one ADP generated during the reaction and expressed in μmol $min^{-1}$. At least two saturation curves were measured for each compound and resulting parameters were calculated as the mean of these values. Curve-fitting was performed using KALEIDAGRAPH (Synergy Software, Reading, PA, USA) for a hyperbolic progress curve.

*Structure-based docking modelling.* The $O^6$-Me-dGMP coordinates were searched on pubchem (http://pubchem.ncbi.nlm.nih.gov) and checked in PRODRG [21]. The analog was then placed in the active site by manual nucleotide superimposition onto dTDP from the dTDP-VaccTMPK complex structure (pdb id 2v54) followed by energy minimization using CNS [22].

The hGMPK structure was build by homology modelling from the mouse GMPK structure (pdb id 1lvg) using Modeller software [23]. The model should be highly reliable since mouse GMPK and hGMPK present 88% identity and 93% homology. Resulting figures were created in PyMOL (http://pymol.sourceforge.net/).

## Results

### *Reaction of dGMP analogs with recombinant vaccinia virus and human TMP kinases*

Alkylated and oxidized dGMP analogs shown in Fig. 1 were all recognized as substrates by Vacc-TMPK except for 9-deaza-dGMP. As shown in the saturation curves (Fig. 2 and Table 1), $O^6$-Me-dGMP, $N^2$-ethyl-dGMP and 8-oxo-dGMP were well-tolerated with rate constants of phosphotransfer ($k_{cat}$ from 0.28 to 0.58 $s^{-1}$) and Michaelis constants ($K_m$ in the 0.2–0.4 mM range) similar to dGMP. $N^7$-Me-dGMP was found to be a really poor substrate but glyoxal-dGMP as fast as dUMP and TMP. These data resulted in the catalytic efficiencies presented in Table 1 and Fig. 3 (230–7100 $M^{-1}s^{-1}$).

In contrast, hTMPK was unable to phosphorylate GMP, dGMP nor any of the dGMP analogs, even though the catalytic parameters of the viral and human enzymes for their natural substrates TMP and dUMP were similar [8].

### *Recombinant human GMP kinase activity with natural substrates*

In order to compare with hGMPK, the gene *gmk* has been cloned and the protein over-expressed and purified. Prior to this work, expression of hGMPK in *E. coli* or production by cell-free translations from the human *gmk* gene resulted in inactive enzyme [24]. In a GST expression vector, the enzyme has been characterized by nanoelectrospray mass spectrometry, but without kinetic analysis [25]. Here recombinant His-tagged hGMPK was successfully expressed in *E. coli* and highly purified to a final concentration of 30 mg/L of cell culture medium (Fig. S1). In the presence of ATP, hGMPK accepted GMP as a substrate with parameters ($K_m$ = 0.05 mM, $k_{cat}$ = 500 $s^{-1}$, $k_{cat}/K_m$ = $10^7$ $M^{-1}s^{-1}$) that were close to those of dGMP (Table 1). No evidence of inhibition by high concentrations of GMP was observed (result not shown), in contrast to the yeast enzyme [26]. Our data are in agreement with the properties of hGMPK purified from human erythrocytes [27,28]. Additionally, hGMPK was found unable to phosphorylate TMP (Fig. 3).

### *Reaction of recombinant human GMP kinase with dGMP analogs*

The presence of a methyl group at positions 6 or 7 of dGMP was deleterious for hGMPK activity: $O^6$-Me-dGMP and $N^7$-Me-dGMP were not substrates (Table 1, Fig. 3). $N^2$-ethyl-dGMP, 8-oxo-dGMP and 9-deaza-dGMP were found to be poor substrates with low affinities ($K_m$), very low $k_{cat}$ and catalytic efficiencies in the 100 $M^{-1}s^{-1}$ range, similar to the reaction rates of PMEA and [*R*]PMPA with human AMP kinases [29]. This result opens the possibility for slow recycling of modified nucleotides after excision from DNA, as observed for other modified nucleotides [14,30]. Glyoxal-dGMP is a substrate for hGMPK (at a $k_{cat}/K_m = 3 \times 10^5$ $M^{-1}s^{-1}$) that was proved to be 20 times less efficient as dGMP, as also found with Vacc-TMPK (Table 1, Fig. 3).

The catalytic efficiencies of these compounds for the three investigated enzymes have been compared on the histogram shown in Fig. 3. Taken together (Fig. 3), these data showed that $O^6$-Me-dGMP was the only compound specifically phosphorylated by Vacc-TMPK with a pronounced efficiency ($k_{cat}/K_m$ = 1200 $M^{-1}s^{-1}$).

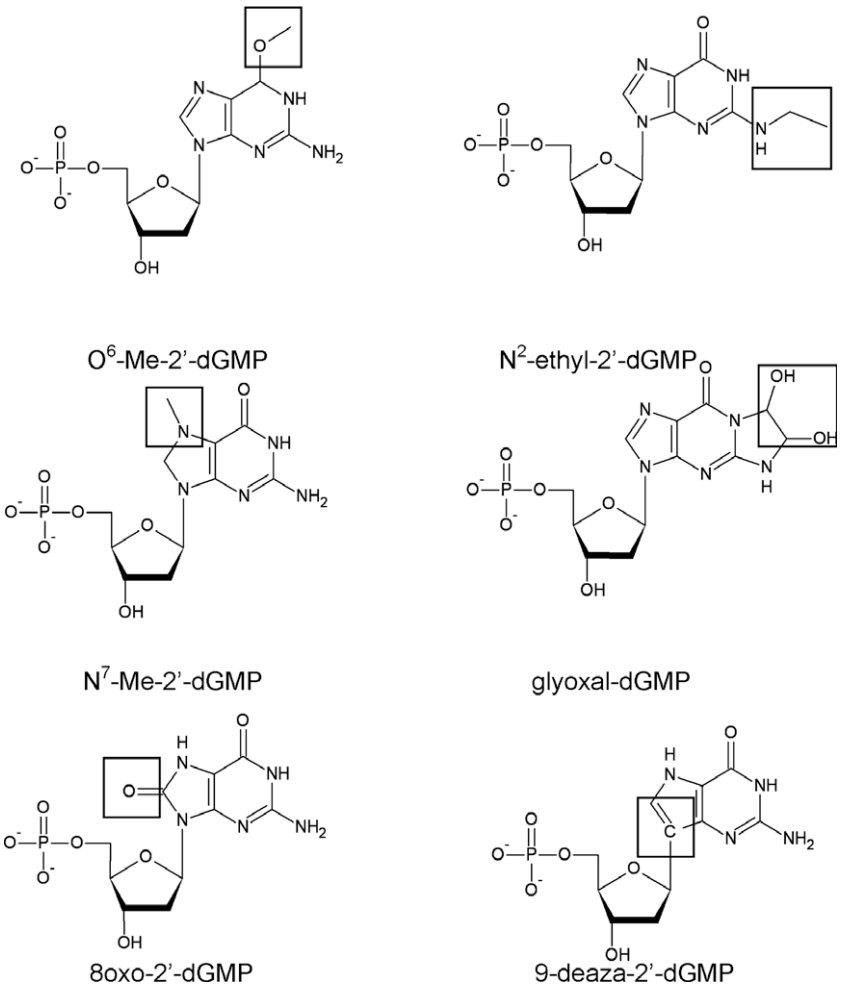


**Fig. 1.** Formulae of dGMP analogs. A black square underlines dGMP modifications.

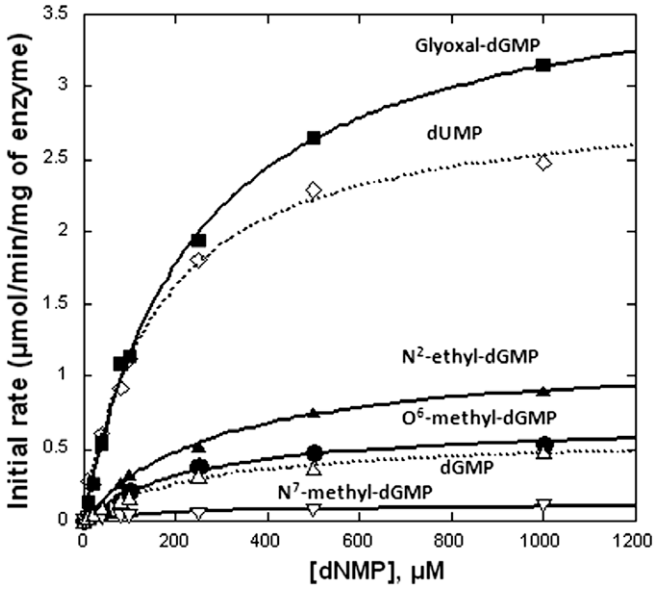


**Fig. 2.** Reaction of Vacc-TMPK with dGMP, dUMP and dGMP analogs. Saturation curve of Vacc-TMPK with (■) glyoxal-dGMP, (▲) $N^2$-ethyl-dGMP, (●) $O^6$-methyl-dGMP, (▽) $N^7$-methyl-dGMP compared to (◇) dUMP and (△) dGMP. The experiments were carried out in the presence of 1 mM ATP and 5 mM $Mg^{2+}$ using the standard coupled assay.

*Structure-based molecular docking of dGMP analogs in the Vacc-TMPK and hGMPK active sites*

Although NMP kinases have a conserved 3D-structure, their NMP binding sites differ in residues accounting for substrate specificities. Vacc-TMPK subunits were shown to orientate differently at the interface near the active site [9]. When comparing vaccinia and human enzymes the His to Asn and Thr to Ala substitutions observed at amino acid positions 65 and 102, respectively, and the Asn65 side chain flipping in Vacc-TMPK created an extra cavity that can readily accommodate bulkier basis, in particular $O^6$-Me-guanine (Fig. 4A). Such a cavity does not exist in the human enzyme, explaining the absence of interaction of dGMP and dGMP analogs with hTMPK. In the modelled $O^6$-Me-dGMP bound to Vacc-TMPK, the oxygen at position 6 is H-bonded with Arg72. The viral enzyme-enlarged cavity likely accommodates several modifications, in particular an ethyl at position 2 and the larger glyoxal group, bound to N1 and the amino group carried by C2 of the guanine ring. In contrast, the replacement of N9 by a carbon atom, which prevents any measurable reaction, causes the N7 nitrogen protonation, therefore preventing its recognition by Arg72 and its binding to the active site.

Yeast, mouse and *E. coli* GMPK structures have been solved in the presence of nucleotides and present a strong analogy to other nucleoside monophosphate kinases [31]. The very high sequence similarity between mouse and human GMPK (88% identity, 93% homology) assures that the model of the hGMPK structure created

**Table 1**
Kinetic parameters of recombinant Vacc-TMP kinase and hGMPK with dGMP analogs.

| | Vacc-TMP kinase | | | hGMPK | | |
|---|---|---|---|---|---|---|
| | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($M^{-1}s^{-1}$) |
| TMP | 2.2 ± 0.2[a] | 20 ± 2[a] | $10^5$[a] | nd | nd | nd |
| dUMP | 1.2 ± 0.3[a] | 130 ± 20[a] | 9200[a] | | | |
| dGMP | 0.58 ± 0.10[a] | 240 ± 20[a] | 2400[a] | 210 ± 30 | 35 ± 5 | $6 \times 10^6$ |
| BVdUMP | 0.86 ± 0.10[b] | 190 ± 20[b] | 4500[b] | nd | nd | nd |
| $O^6$-methyl-dGMP | 0.28 ± 0.04 | 240 ± 20 | 1200 | nd | nd | nd |
| $N^2$-ethyl-dGMP | 0.48 ± 0.04 | 280 ± 30 | 1700 | 0.023 ± 0.04 | 180 ± 40 | 130 |
| Glyoxal-dGMP | 1.6 ± 0.1 | 230 ± 20 | 7100 | 150 ± 50 | 430 ± 90 | $3 \times 10^5$ |
| 8-oxo-dGMP | 0.36 ± 0.04 | 400 ± 50 | 910 | 0.04 ± 0.01 | 400 ± 100 | 100 |
| Deaza-dGMP | nd | nd | nd | 0.08 ± 0.01 | 1400 ± 500 | 60 |
| $N^7$-methyl-dGMP | 0.05 ± 0.01 | 220 ± 30 | 230 | 0.07 ± 0.01 | 2300 ± 300 | 30 |
| GMP | nd | nd | nd | 500 ± 100 | 50 ± 10 | $10^7$ |

nd, non-detectable.
[a] from [8].
[b] from [9].

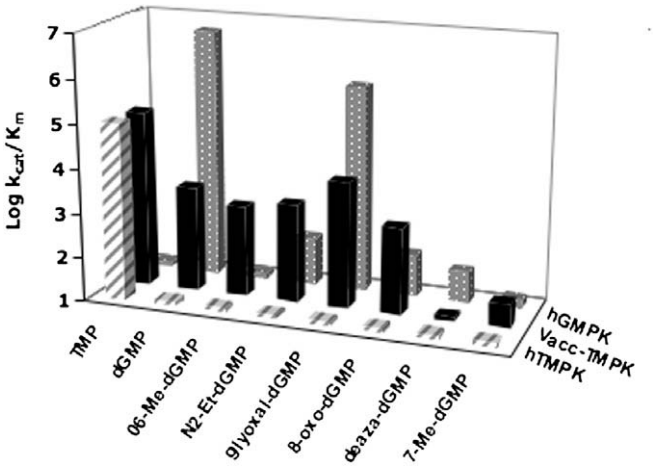


**Fig. 3.** Comparison of the phosphotransfer efficiencies for dGMP analogs with hTMPK, Vacc-TMPK and hGMPK. Kinetic parameters were measured at the steady state for the three recombinant enzymes as described. Catalytic efficiencies were calculated from the ratio $k_{cat}/K_m$ and expressed in $M^{-1}s^{-1}$. Along *z* axis, gray stripes: hTMPK; black, Vacc-TMPK; white dots: hGMPK. Along the *X* axis, are shown standards (TMP and dGMP) and dGMP analog names.

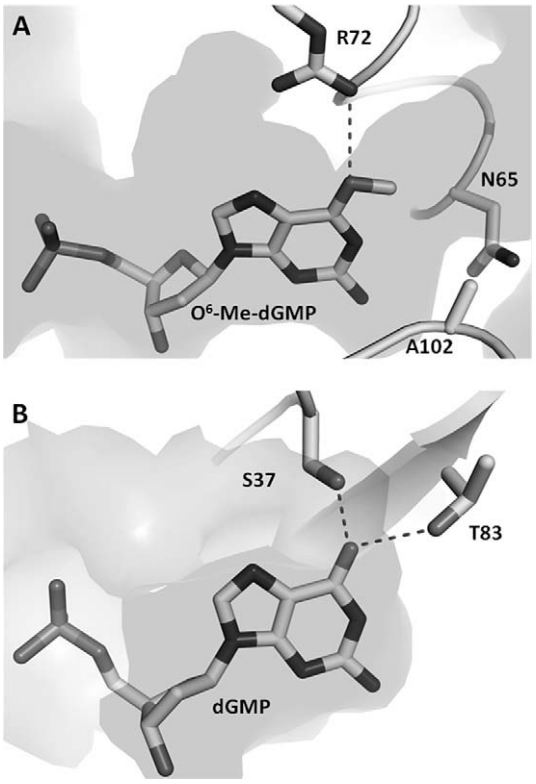


**Fig. 4.** Modelling $O^6$-Me-dGMP at the active site of Vacc-TMPK and hGMPK. (A) $O^6$-Me-dGMP was docked at the active site of Vacc-TMPK (pdb id 2v54). The observed extra cavity between Asn65 and Ala102 (grey surface), absent in hTMPK [9] can accommodate the methyl group (light grey). (B) Homology model of the hGMPK structure based on the mouse GMPK structure (pdb id 1lvg) with bound dGMP. The $O^6$ is H-bonded to Ser37 and Thr83 and there is no room in the active site pocket (grey surface) to accommodate a methyl group.

by homology based on the mouse structure is very close to reality (Fig. 4B). $O^6$-Me-dGMP was docked at the active site of the modelled hGMPK. The conserved Ser37 and Thr83 interact with the guanine carbonyl at position 6. They leave no room in the active site to accommodate an extra-methyl group at position 6. These residues also play a major role in the specificity between AMP and GMP. Moreover when 6-thioguanine, another guanine derivative with $O^6$ replaced by a sulfur atom was used as an anticancer agent, resistance was found in hGMPK at the level of Ser37 [32], underlining the importance of this serine residue in the enzyme structure.

## Discussion

Our results provide new insights concerning the recognition of dGMP analogs by hGMPK. In contrast to DNA repair and excision of incorporated oxidized and alkylated dG, little information is available on their potential anabolism (re-phosphorylation) to the triphosphate form. There is no reported evidence that modified dG are substrates for nucleotide salvage kinases with the exception of 8-oxo-dGDP which is converted to 8-oxo-dGTP by NDP kinase [33]. GMPK from cell extracts was previously found unreactive for 8-oxo-dGMP [34] in contrast to the activity, although weak, observed here using recombinant enzyme. Also surprising is the high reactivity of glyoxal-dGMP with hGMPK. The diphosphate derivative is probably an excellent substrate for NDP kinase, an enzyme with a large base specificity [35]. However, glyoxal and methylglyoxal are used in several foodstuffs [36] and may not be totally innocuous for health.

The main point brought by our study is the specific reaction of $O^6$-Me-dGMP with VaccTMPK: neither hTMPK nor hGMPK were able to phosphorylate it, opening the way to the design of a new anti-poxvirus compound, $O^6$-Me-dGMP or a $O^6$-Me-dGMP analog that could be phosphorylated and incorporated in DNA. The pres-

ence of $O^6$-Me-dG in DNA has been found to cause lethal DNA lesions [37]. When accumulating in nuclear and mitochondrial DNAs, lesions promote DNA instability and trigger cell apoptosis [14,15]. $O^6$-Me-dG specifically activated in cells infected by poxviruses could be of medical interest in antiviral and anticancer chemotherapy. A poxvirus TMP kinase gene could be used as a safety gene in parallel to the herpes TMP kinase suicide-gene coupled to ganciclovir [38]. We propose to use a $O^6$-Me-dGMP derivative with a phosphonate (instead of a phosphate group) in a pro-nucleotide approach as for PMEA-dipivoxil for example. The pro-drug approach was shown to achieve nucleotide delivery into cells, bypassing the limitations of intracellular nucleotide formation from their nucleoside precursors and improving antiviral potency [39,40]. Preliminary cellular studies showed that $O^6$-Me-dG was not cytotoxic on Hela cells and human lymphocytes, but could not reveal the anti-vaccinia virus activity of $O^6$-Me-dG on HEL cells infected by vaccinia virus (result not shown). Several reasons may explain this lack of activity such as poor cellular availability of $O^6$-Me-dG or lack of $O^6$-Me-dG conversion into a monophosphate metabolite. All these features could be overcome by the pronucleotide approach where a stable phosphonate (analogous to $O^6$-Me-dGMP) carries lipophilic groups. Such studies are currently ongoing in the laboratory.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.089.

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