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## <sup>43</sup>Ca<sup>2+</sup> – Paramagnetic impact on DNA polymerase beta function as it relates to a molecular pharmacology of leukemias

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### ABSTRACT

Human acute myeloblastleukemia HL60 cells overexpresses a beta - type DNA polymerase (EC 2.7.7.7) which is found to be operated by magnetic isotope effect (MIE) of Calcium once the Mg<sup>2+</sup> ions replaced with the stable <sup>43</sup>Ca<sup>2+</sup> isotopes inside the enzyme catalytic sites. The isotopes mentioned are the only paramagnetic species of the Calcium isotopic set with a 0.135 natural abundance value and the negative 7/2 nuclear spin providing a nuclear magnetic moment equal to 1.317 Bohr magnetons. As compared to the Mg<sup>40</sup>Ca substitution, a 2.25-fold enzyme inhibition has been shown to prove the <sup>43</sup>Ca-MIE dependent mode of the catalysis turning down. This <sup>43</sup>Ca – promoted enzyme hyper - suppression leads to a residual synthesis of shorted DNA fragments that counts 25 – 35 nucleotides in length contrasting with the 180n – 210n DNA produced by either intact or <sup>40</sup>Ca – loaded polymerase. Being occurred simultaneously with a marked MIE – promoted enzyme inhibition, this fact itself makes possible to consider these short (“size-invalid”) DNA segments hardly efficient in the DNA base – excision repair. The latter is a survival factor in leukemic cells where the DNAPolβ was found overexpressed. That confirms a concept considering the DNAPolβ a legitimate target for antitumor agents since its inhibition deprives the malignant cell from a DNA base – excision repair in neoplasma. A possible trend making role of these data for molecular pharmacology of cancers is in a focus.

**Keywords:** magnetic isotope effect (MIE), acute myeloblast leukemia (AML), DNA Polymerase Beta (DNAPolB), <sup>40</sup>Ca and <sup>43</sup>Ca isotopes, HL-60 cells

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### INTRODUCTION

DNA Polymerase Beta (DNAPolB, EC 2.7.7.7) is a key player in the DNA base-excision repair [1,2]. Most of the variable DNAPolB species were found to be over – expressed in many malignant tumors [3,4,5,6]. Moreover, this over – expression of DNAPolB is highly linked to the survival of the human acute myeloblastleukemia (AML) cells and the poor prognosis of leukemic patients [7-11].

Taking into account a known fact of the cell/tumor – specific structural diversity of the DNAPolB superfamily [2,12], some efforts were made to employ DNAPolB inhibitors as the anti – cancer agents [9,10,13]. In other words, enzymes of this group might be considered the legitimate targets for cytostatics as long as the latter are sufficient to

meet such pharmacophore related requirements as the low toxicity, high selectivity and the efficiency of the enzyme inhibitory activity [14].

One of the obstacles on a route of the DNAPolB – targeting pharmacophores administration is their toxicity [9,13]. Being  $Mg^{2+}$  - dependent metalloenzymes (two  $Mg^{2+}$  ions loosely coordinated inside two separate catalytic sites), DNAPolB species were found to be controllable by a so called magnetic isotope effects (MIE) of the non – toxic bivalent metal ions,  $^{25}Mg$ , and  $^{67}Zn$  [15]. This nuclear spin selective path of the enzyme activity regulation has been revealed for a number of metalloenzymes [16,17,18,19]. The mechanism beyond involves a singlet – triplet conversion of the ion – radical intermediates formed during the enzymatic intermolecular transfer of phosphate [16,19]. As a result, these spin - possessing (magnetic) ions,  $^{25}Mg^{2+}$  and  $^{67}Zn^{2+}$ , work as the far better suppressors for DNAPolB reaction compared to the spinless (non – magnetic) ions of the very same metals [15]. Noteworthy, these inhibitors are no doubt the non – toxic ones [20].

Furthermore, the endo – osmotic inter – enzyme substitution of endogenous magnesium with other magnetic bivalent metal ions like  $^{43}Ca^{2+}$  or  $^{67}Zn^{2+}$  leads to an essential MIE expressed in several kinases directed phosphate transfer enzymatic reactions – in both pure enzyme tests and leukemia cell cultures [18,20]. In a later case, some porphyrine – based amphiphilic nano – cationites were used to deliver the magnetic ions into the cell, regardless the ion concentration gradient vector [20,21,22].

It would be safe to say, therefore, that the MIE – promoting pharmacophores could engage the DNAPolB molecular targets in anti – cancer therapies. This itself requires a firm biochemical, to be exact - chemico – enzymological, background.

Since the blasticleukemias, acute myeloblast ones (AML) including, are known for their DNAPolB over – expression [5,6,10,11], it makes sense to investigate the MIE pharmacological potential using enzyme isolated from these peculiar cancer cells. Earlier, we have purified and detail – characterized the unique tumor – specific DNAPolB over – expressed in AML/HL-60 cells [6]. In a present study, we're evaluating the  $^{43}Ca$ -MIE impact on this enzyme function with a special respect to further perspectives in anti – leukemia therapy pre – clinical developments.

## MATERIALS AND METHODS

### *Enzyme*

The enzyme samples, DNA Polymerase Beta (DNAPolB, EC 2.7.7.7), were previously purified from the AML/HL-60 cell chromatin according to [6].

### *Labeled products*

$^{40}CaO$  and  $^{43}CaO$  species, A grade, 96.8% and 98.7% isotopic purity, respectively, were purchased from Gamma Lab AS (Spain). Tritium labeled DNA precursor, [Methyl-1,2- $^3H$ ]dTTP with a specific activity of 90 – 120 Ci/mmol, NET520A, was purchased from New England Nuclear, Inc., USA.

### *Reagents and disposal materials*

DNase-free agarose (Helicon Co., UK); ethidium bromide (AppliChem AG, Sweden); single strand DNA markers kit, 30n – 550n (SibEnzyme Ltd, Russia); AccuPrep Genomic DNA Extraction Kit (Bioneer Ltd, Korea); ethanol, acetone, HCl, salts and buffers, all of A grade, were purchased from Serva GmbH (Germany). 20CR fiberglass filters (Molli-pore Co., France) and RX5 [3H]sensitive autoradiography films (Fuji Corp, Japan) were employed.

### *Enzyme pre – incubation loading with exogenous Ca isotopes*

To convert  $^{40}CaO$  and  $^{43}CaO$  into chloride salts, a conventional acidic treatment has been performed first [18]. Once the isotopic-pure salt sample solution adjusted to a stock 1.0 M concentration by the flame atomic absorption spectrophotometry (LQ600 AAA System, Shimadzu Corp, Japan), a routine incubation mixture has been composed as described by [15]. This mixture was employed then to replace (endo – osmotic ion substitution, EOIS) the enzyme endogenous magnesium with an exogenous  $Ca^{2+}$  under the following conditions: 15 mM Tris – HCl (pH 8.0) / 20 mM  $CaCl_2$  / 1.5 mM EDTA / 60 – 80 microgram pure enzyme per 1.0 mL / +37 $^{\circ}C$ , 2 hrs [15]. To monitor the enzyme – bound Calcium level values estimated in pg Ca per 1.0 mg protein, the fiberglass filter retained acetone – precipitated pellets (acetone washed, 100 mL/cm $^3$ ) were analyzed by X-ray fluorescent spectrometry in SL420 XF Analytical System, Bruker GmbH, Germany [18,23]. For protein measurements, a Bradford – like routine colorimetric procedure has been applied [6,24].

***DNApolB catalytic activity measurements***

To detect and quantify the enzymespecific catalytic activity, our original technique has been employed [6]. The resulted activity values were expressed in amounts of the labeled DNA precursor incorporated into the enzyme – directed nascent single strand DNA sequences corrected to 1.0 mg pure enzyme, i.e. [<sup>3</sup>H]cpmDNA/mg protein. 220LX Liquid Scintillation Counter (Wallac OY, Finland) employed. The protein ultramicroamounts were estimated according to [25]. The post – incubation mixtures were subjected to a quantitative extraction of the DNA ultramicroamounts using an AccuPrep Genomic DNA Extraction Kit (Bioneer, Korea) as described by [26], for further DNApolB activity and DNA size detection electrophoresis tests. In diluted solutions, the DNA ultramicroamount measurements were performed according to [6].

***DNA electrophoresis***

A standard 1.7% agarose gel electrophoretic technique has been employed [27], modification: [6]. Prior to the samples supply, the routine colorimetric DNA measurements were performed [28]. To observe an immediate result, the ethidium bromide treated unfixed gels were studied in the ImageQuant LAS4000 UV Scanner – Visualizer, GE HealthCare Life Sciences, Inc., USA[6]. The gel [<sup>3</sup>H]radioautographs were registered as described in [24].

***Statistics***

The experiment data repetition preciseness as well as a significance of the experiment / control data differences were elucidated by a non – parametric standard technique for *n* lower than 6 [29]. The data were processed in HP700E analytical module (Hewlett Packard, Inc., USA) using the VaryLab-6 software package (Litekh Ltd, Russia).

**RESULTS AND DISCUSSION**

In a number of previously conducted studies, a universal mode of the nuclear spin dependent effects (MIE) expressing in several metalloenzyme directed reactions was clearly proven and specified [15,16,17,18,19,20,21,22]. Thus, the DFT-method provided simulation models show why magnetic and non – magnetic bivalent metal ions act differently once involved into the phosphate carrying enzymatic catalysis [17,18,19]. A wide diversity of the phosphate transferring metalloenzymes, including the HL-60 DNApolB, was found operable (controllable) by those above mentioned magnetic isotopes [15,19] which makes a scheme presented in Figure 1 a quite legitimate way to interpret the <sup>43</sup>Ca-MIE/DNApolB interaction we're about to describe.

Being a self – sufficient kind of illustration, this scheme (Figure 1) deserves, nonetheless, a brief comment. The magnetic (<sup>43</sup>Ca) nuclei induced singlet – triplet conversion of the ion – radical pair is a key element of this quantum mechanical mechanism [16,19]. Consequently, this non – nucleophilic, very fast ion – radical reaction is to desynchronize the orchestrated work of two separate metal – possessing DNApolB catalytic sites. This, in turn, might deprive one of them of an appropriate functional interaction with a second one[15]. This statement is in accordance with the data on unequal ion accessibility of different catalytic sites in enzyme studied [2,4,12,30].

However, there is one attention catching point in the <sup>43</sup>Ca-MIE we observed which is a simultaneous (a) enzyme inhibition and (b) an essential decrease of a maximum size of the DNA fragments produced, from nearly 200*n* to about 30*n* (Figures 2 and 3). A marked difference between the results obtained in <sup>40</sup>Ca (non – magnetic) and <sup>43</sup>Ca (magnetic) isotope tests is enough to exclude any doubt regarding the MIE nature of the phenomenon we've seen here [16,19].

Noteworthy, this is the first report ever on the MIE – promoted changes in lengths of DNA blocks processed by DNApolB, a target for magnetic ions. Fast UV – EtBr visualization of key results (Figure 2) was then completed with the detailed radiometric tests conducted in both DNA electrophoresis gel autoradiography and the enzyme catalytic activity measurements (Figure 3).

Taking into account that the DNApolB synthesized DNA segment length was found to be one of the critical parameters for the DNApolB – directed DNA repair and, hence, to the cell survival [1,2,4,5,12], we might assume a certain pharmacological potential beyond the data presented in Figures 1 – 3. To our opinion, this assumption makes sense due to a known fact of the DNApolB over – expression occurred in most leukemic cells [3,8,9,14] including the HL-60 ones [6].

Looking at this biochemical data through the “pharmacology spectacles”, we're facing two major problems, both related to the task of making the leukemic DNApolB a real molecular target for magnetic metal ions. First, the latters must be administered using a targeted delivery system of some sort. Second, the *in situ* intracellular ferromagnetic ions content (Fe<sup>2+</sup>, first of all) must be considered as long as a target compartment (organella, cell, tissue) is known.

Concerning the targeted delivery of bivalent metal ions, some low toxic nano – cationites were qualified as the efficient  $Me^{2+}$  - carriers in both *in vitro* and *in vivo* studies. Thus,  $^{25}Mg^{2+}$  ions were selectively delivered towards myocardiocytes and lymphocytes in rats owing to nano – cationites based on water – soluble porphyrine adducts of fullerene- $C_{60}$ ; the porphyrine – affine signaling mitochondria membrane proteins were playing a role of traps catching and then retaining these pharmacophores [21,22,31,32]. Several oligoheterocyclicaminothiolates were also found efficient to provide the cell plasmatic membranes and nuclei permeability for  $Ca^{2+}$ ,  $Mn^{2+}$ ,  $Cu^{2+}$  and  $Zn^{2+}$  ions in leukemic cells *in vitro* [33]. All these and related medicinal nano – particles are to release the cations they're loaded with in response to a slight metabolic acidosis, so common for the most malignant tumors known [21,22,32,33].

In our previous studies, we have revealed that MIE are hardly inducible in iron – rich mammalian tissues (spleen, liver) as compared to the iron – poor ones [23,34]. Obviously, these were the easily predictable results. There is no way for  $^{43}Ca$ -MIE to get expressed in a “heavily iron – polluted” environment [19]. That's why it is important to find out a general regularity of such a dependence as this:  $MIE = f[Ca^{2+}]$ . This is what our findings (Figure 4) are all about. The regularity shown here is worthy of being used to correct pharmacodynamics patterns in further  $^{43}Ca$ -MIE studies.

A non – Markoff population dynamics model allows to predict a cytostatic efficiency for  $^{43}Ca^{2+}$  - loaded PMC16 nano – particles (porphyrincyclohexylfullerene), cation releasing pharmacophore [22]. Such cytostatic effect was observed by [20] in experiments with  $[^{67}Zn]_4$ PMC16 carriers in normal lymphocytes and human leukemia cell lines.

To summarize the results presented in Figures 1 – 4, we may propose a biochemical path for the DNAPolB engaging MIE of  $^{43}Ca$ . As a matter of fact, this scheme (Figure 5) is based on the ion – radical concept of the phosphate – transferring metalloenzyme function which involves a numerous DFT – calculations and experimental data [15,17,18,19,20,23,31,32,34]. As seen from this chart, the only path for the dNTP-oxyradical decay in a  $^{43}Ca^{2+}$  - possessing DNAPolB is a fast and irreversible ion – radical move leading to the nascent DNA chain growth (compare Figures 1 and 5).

Considering that one of the  $Mg^{2+}$  ions belonging to bicationic DNAPolB molecule is hardly accessible to the endo – osmotic isotope substitution, i. e. to the enzyme intermolecular metal ion replacement procedure [1,2,4,12,30], it would be reasonable to expect a maximum  $Ca^{2+}$  - enzyme incorporation level to be close to 50%.

As we have confirmed (Figures 6 and 7), about a half of endogenous enzyme – bound magnesium was indeed replaced with calcium in our experiments. Keeping the intra-enzyme metal substitution conditions optimal (see *Materials and Methods*), we realized that not more than 54 – 58% of the protein – bound magnesium was in fact replaced with  $^{43}Ca^{2+}$  (Figure 6). Two hour long incubation time scale was found optimal to reach the ion exchange (replacement) equilibrium point in 20 mM  $CaCl_2$  media (Figure 7). Most likely, the  $^{43}Ca$ -MIE we observed had indeed caused by dis-synchronization of functioning of two metal – containing enzyme catalytic sites which is a consequence of the insertion of magnetic  $^{43}Ca^{2+}$  into just one – the only accessible one – of the above mentioned two metal possessing domains.

Last not least, DNAPolB makes a contribution to the cell chromatin stability/flexibility transitions. Being located in both deep and superficial strata of chromatin [5,7,9,10,14,33], this enzyme fits the requirements we have for a molecular target accessibility for magnetic metal ions delivered by an appropriate ion – releasing pharmacophore.

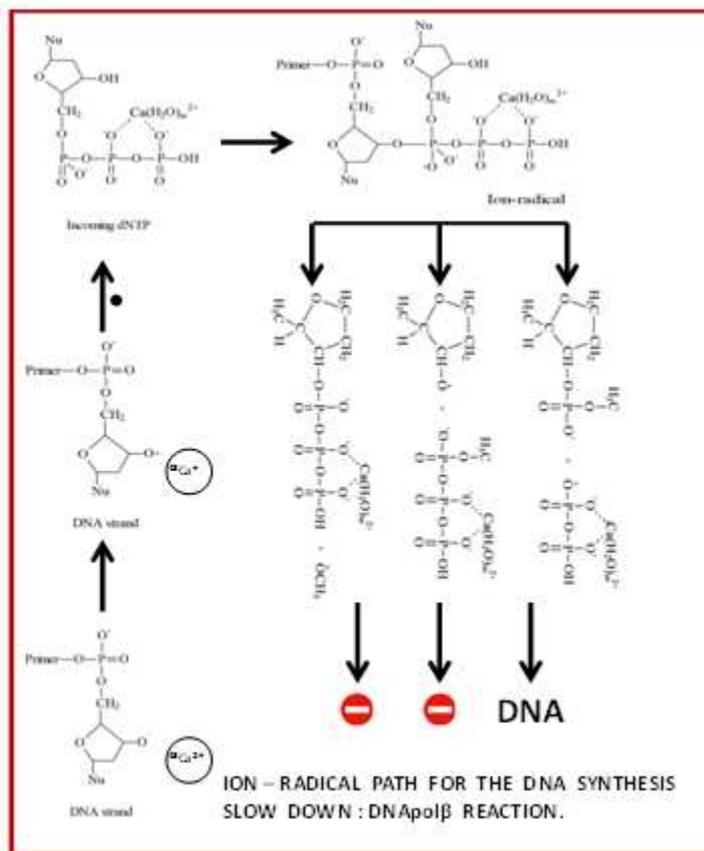


Figure 1. ION – RADICAL PATH FOR THE DNA SYNTHESIS SLOW DOWN :DNAPolβ REACTION

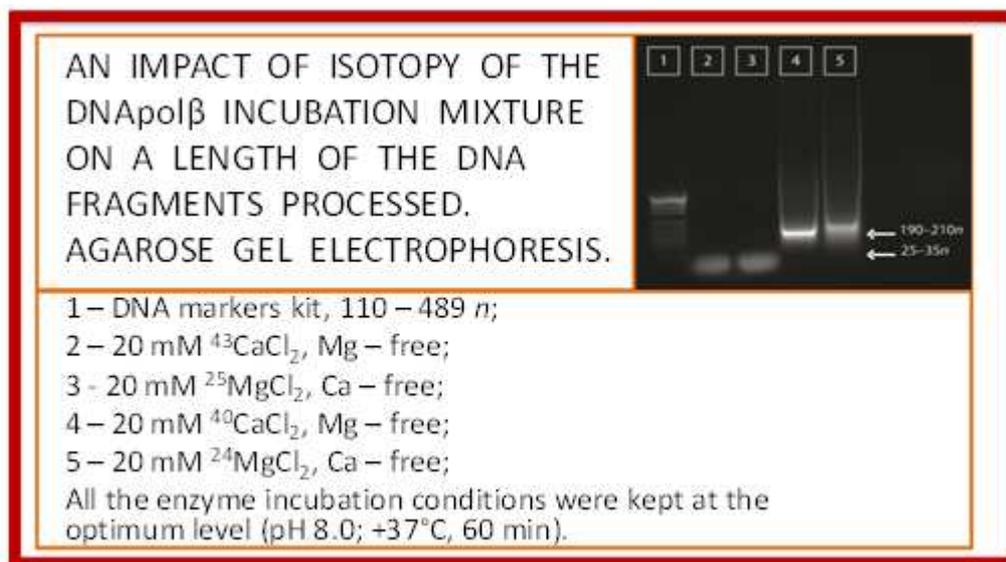


Figure 2. AN IMPACT OF ISOTOPIY OF THE DNAPolβ INCUBATION MIXTURE ON A LENGTH OF THE DNA FRAGMENTS PROCESSED. AGAROSE GEL ELECTROPHORESIS

1 – DNA markers kit, 110 – 489 n; 2 – 20 mM  $^{43}\text{CaCl}_2$ , Mg – free; 3 - 20 mM  $^{25}\text{MgCl}_2$ , Ca – free; 4 – 20 mM  $^{40}\text{CaCl}_2$ , Mg – free; 5 – 20 mM  $^{24}\text{MgCl}_2$ , Ca – free;

All the enzyme incubation conditions were kept at the optimum level (pH 8.0; +37°C, 60 min).

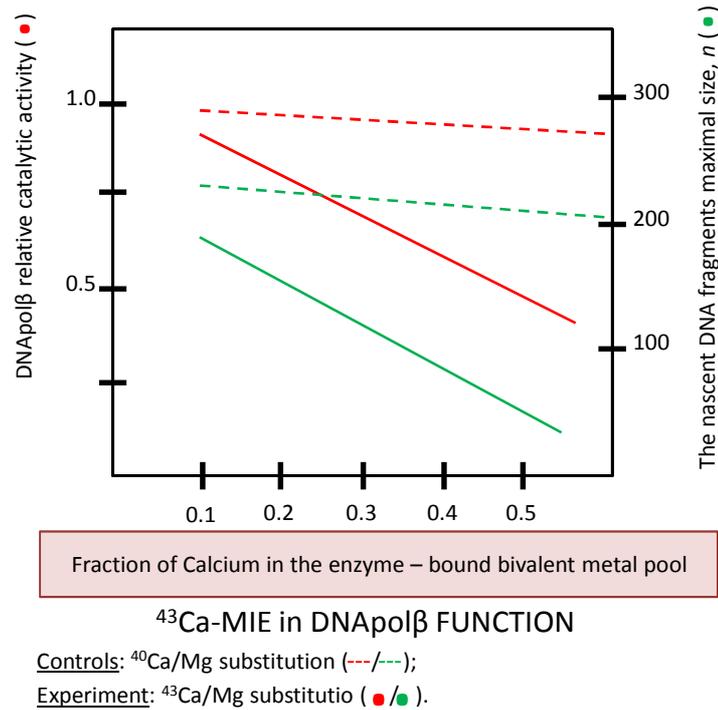


Figure 3. FRACTION OF CALCIUM IN THE ENZYME – BOUND BIVALENT METAL POOL

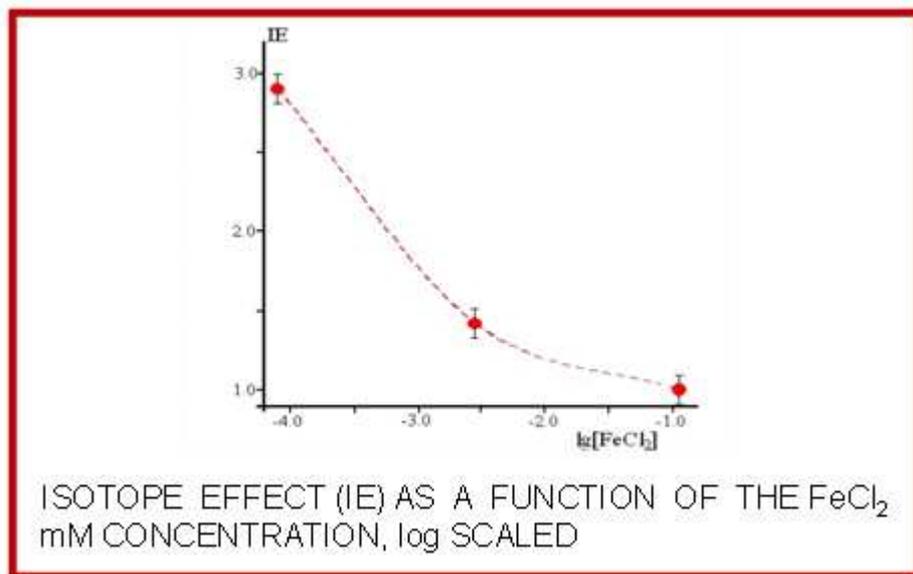


Figure 4. ISOTOPE EFFECT (IE) AS A FUNCTION OF THE FeCl<sub>2</sub> mM CONCENTRATION, log SCALED

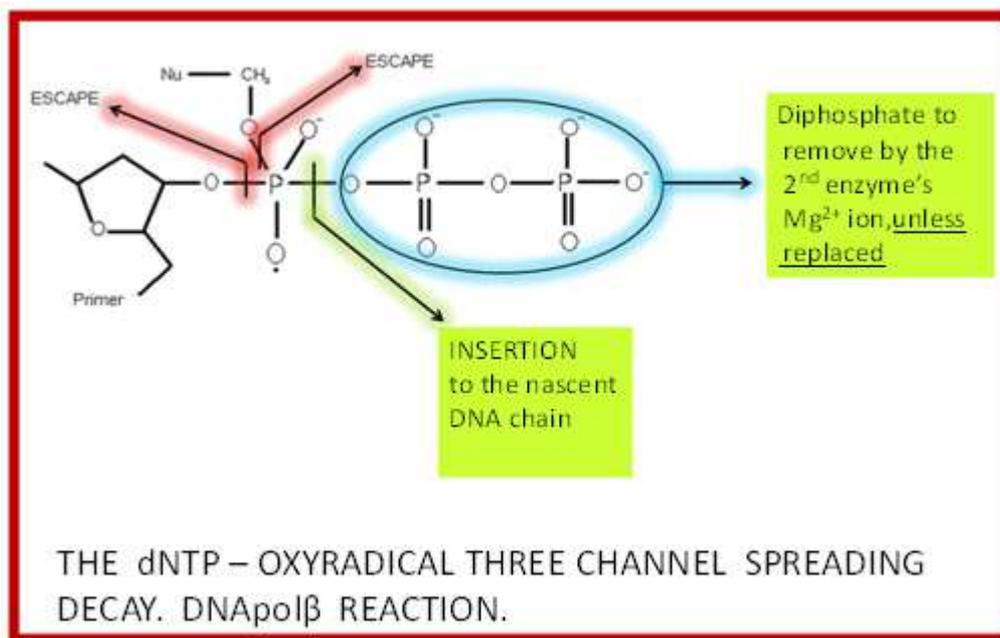


Figure 5. THE dNTP – OXYRADICAL THREE CHANNEL SPREADING DECAY. DNAPolβ REACTION

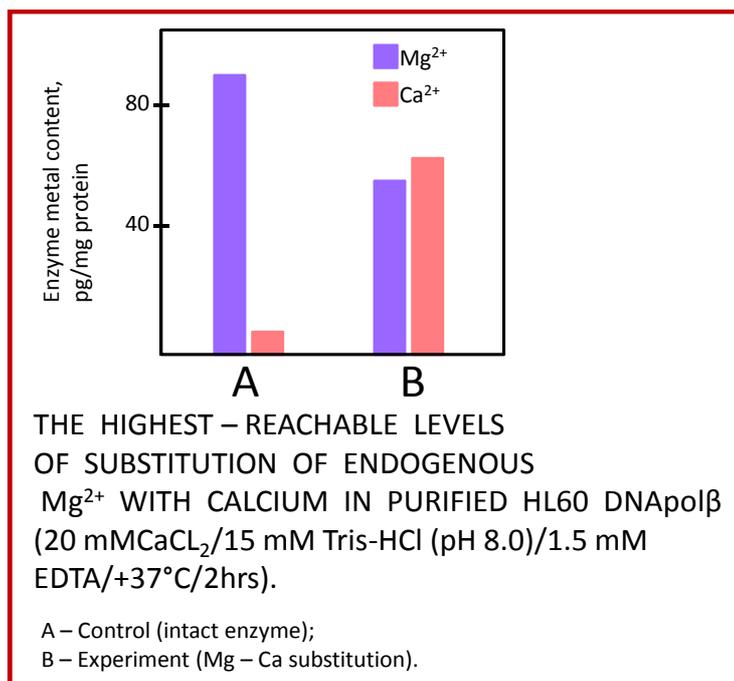
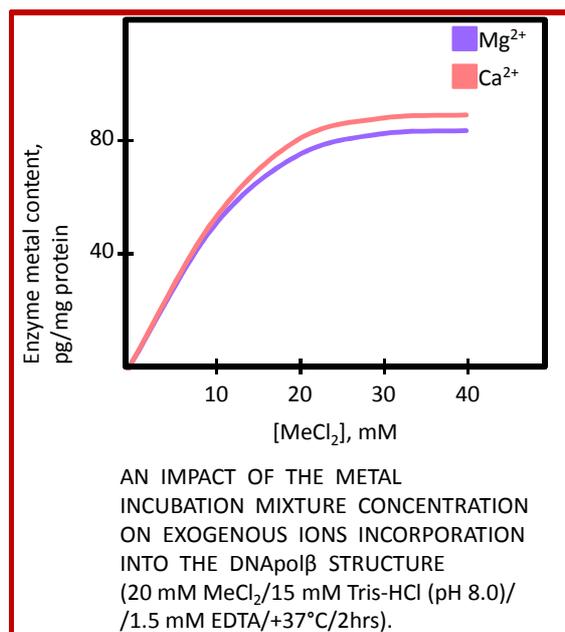


Figure 6. THE HIGHEST – REACHABLE LEVELS OF SUBSTITUTION OF ENDOGENOUS Mg<sup>2+</sup> WITH CALCIUM IN PURIFIED HL60 DNAPolβ (20 mM CaCl<sub>2</sub>/15 mM Tris-HCl (pH 8.0)/1.5 mM EDTA/+37°C/2hrs).  
A – Control (intact enzyme); B – Experiment (Mg – Ca substitution).



**Figure 7. AN IMPACT OF THE METAL INCUBATION MIXTURE CONCENTRATION ON EXOGENOUS IONS INCORPORATION INTO THE DNAPolβ STRUCTURE (20 mM MeCl<sub>2</sub>/15 mM Tris-HCl (pH 8.0)/ /1.5 mM EDTA/+37°C/2hrs).**

### CONCLUSION

The Ca – Mg substitution occurred inside the AML/HL-60 chromatin DNAPolB catalytic sites leads to a sharp inhibition of this enzyme once the magnetic <sup>43</sup>Ca isotope got engaged. This phenomenon had caused by the <sup>43</sup>Ca-related magnetic isotope effect known for its ion – radical mechanism specified earlier by [15,16,17,18,19]. Moreover, the <sup>43</sup>Ca-MIE manifests itself not only in the DNAPolB inhibition but in a residual synthesis of abnormally short, DNA-repair hardly sufficient, “invalid” DNA fragments. Since the isotope substitution performed was efficiently managed in the simple endo – osmotic pressure tests, the data presented are worthy of further study on their pharmacological potential. This potential derives also from the DNAPolB properties making it a promising candidate to a role of the molecular target for <sup>43</sup>Ca<sup>2+</sup> - inhibitor in pre-clinical safe cytostatic developments.

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