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# Ribonucleotide reductase and thymidine phosphorylation: two potential targets of azodicarbonamide

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**Abstract**

Azodicarbonamide tested as an anti-HIV agent was reported to expulse zinc from viral zinc-cysteine factors and to inhibit calcium mobilization machinery. It has structural analogy with hydroxyurea that inhibits ribonucleotide reductase and could also act on this target. Azodicarbonamide was therefore tested for its capacity to modulate deoxyribonucleotides triphosphate pools alone or in combination with other agents in the lymphoblastic SUP-T1 cell line susceptible to HIV infection. The deoxyribonucleotides triphosphate were evaluated by an enzymatic assay using sequenase. Two hours exposure of SUP-T1 cells to 100 μM azodicarbonamide induced a 50% reduction of each deoxyribonucleotide triphosphate. Among other inhibitors of nucleotide metabolism (hydroxyurea, methotrexate and thymidine), hydroxyurea only reproduces the effect of azodicarbonamide. This suggests, but does not demonstrate directly, that azodicarbonamide inhibits ribonucleotide reductase activity. The combination of azodicarbonamide with each of these inhibitors affected particularly the dCTP pool. During this study it was also suggested that azodicarbonamide could interfere with thymidine phosphorylation. Thymidine phosphorylating activity was measured with $^{3}$H-thymidine as substrate. In acellular preparations, azodicarbonamide also non-competitively inhibits thymidine phosphorylating activity. This effect was not reproduced by hydroxyurea. Thus, *in vitro* azodicarbonamide decreases the intracellular pool of deoxyribonucleotide and thymidine phosphorylation.


*Keywords:* Azodicarbonamide; Ribonucleotide reductase; Thymidine phosphorylation

## 1. Introduction

Azodicarbonamide (1,1′-azobisformamide) has the structural formula: $H_2N–CO–N=N–CO–NH_2$. In literature, this compound is sometimes shortened under the group of three letters ''ADA''. This abbreviation however can carry to confusion with that used for adenosine deaminase. We therefore used the abbreviation AZDA. Azodicarbonamide but not its major metabolite biurea inhibits *in vitro* ($IC_{50}$ = 5 μg/mL or 43 μM) the replication of various strains of HIV-1 virus by unknown mechanisms. Azodicarbonamide does not act (a) on the binding or fusion of the virus to the target cell; (b) on reverse transcription activity; (c) on the incorporation of proviral DNA; (d) on transactivation when the Long Terminal Repeat (LTR) gene of the virus is expressed; (e) on HIV-1 protease activities [1]. Azodicarbonamide inhibits dose-dependently the responses of human $CD4^+$ T lymphocytes stimulated either by monoclonal antibodies against CD3 (OKT3) and CD28 or by allogeneic dendritic cells through a direct action on the calcium mobilization machinery [2]. *In vivo*, its administration to mice blunted their response to polyclonal T-cell activation induced by OKT3 and resulted in delayed rejection of skin allografts. It also prevents the progression of human $CD4^+$ T lymphocytes into the G1 phase of the cell cycle, inhibits their blastogenesis, down-regulates their membrane expression of CD25 and CD69, and decreases the transcription of cytokines genes. Addition of the calcium ionophore A23187 restores T cell proliferation in the presence of azodicarbonamide. Furthermore, azodicarbonamide acts synergistically with cyclosporin A to inhibit $CD4^+$ T cell proliferation [3]. It is a specific inhibitor of the zinc fingers proteins of the HIV virus through the

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*Abbreviations:* AZDA, azodicarbonamide (1,1′-azobisformamide); DMSO, dimethylsulfoxide; dNTP, deoxynucleoside-5′-triphosphate; SUP-T1, T cell lymphoblastic lymphoma (human); HIV, human immunodeficiency virus.

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ejection of the zinc atom enclosed in the virus nuclear capsid protein NCp7, by an electrophilic attack of the sulfur atoms of the zinc coordinating cysteine residues [4,5]. This effect is rapid, irreversible and limited to the zinc finger model $Cys–(X)_2–Cys–(X)_4–His–(X)_4–Cys$. Azodicarbonamide is currently evaluated in phase I/II clinical trials conducted in patients with advanced AIDS, and increased $CD4^+$ T cell percentage, increased the $CD4^+/CD8^+$ T cell ratio and decreased plasma RNA viral load from baseline [6]. It could also, by analogy with hydroxyurea [7] alter the synthesis of deoxynucleotides and thus, by reducing substrate concentrations impair DNA synthesis. This was evaluated in the present study.

## 2. Materials and methods

### 2.1. Cells

The lymphoblastic SUP T-1 cell line was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, L-glutamine, sodium pyruvate, essential and non-essential aminoacids.

### 2.2. Preparation of azodicarbonamide solutions

Stock solutions of azodicarbonamide were prepared in dimethylsulfoxide (DMSO) and diluted in the culture medium. In all the assays, the final concentration of DMSO was 0.1% that was added in the controls.

### 2.3. Determination of the deoxynucleotides

The assay was based on the DNA polymerase-catalyzed incorporation of deoxyribonucleotides (dNTP) into DNA. When one of three dNTP present in excess was radiolabeled, the amount of radioactivity incorporated into DNA was proportional to the amount of the fourth, limiting dNTP. The amount of dNTP was determined by enzymatic assay using Sequenase enzyme and the template primers described by Gao *et al*. [8] on a cell pellet ($5 \times 10^6$ cells) extracted with 1 mL 60% methanol; the extract incubated for 3 min at 95° was cooled on ice, filtered on Millex-GS filters and stored at −80°. Briefly, the reaction mixture contained 0.05 U Sequenase, 50 mM Tris–HCl, pH 7.5, and 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.25 μM template primer, 2.5 μM $^3$H-dATP (23 Ci/mmol for dCTP, dTTP, and dGTP determinations) or 2.5 μM $^3$H-dTTP (15 Ci/mmol, for dATP determination). Extract from cells (5 μL) was then added to 45 μL of the reaction mixture. The reaction was carried out at 26° for 20 min, followed by spotting 40 μL of the reaction mixture onto Whatman DE81 paper. The filters were extensively washed three times with 5% $Na_2HPO_4$ (10 min per wash), rinsed with distilled water, then with 95% ethanol and finally dried before counting.

Templates:

for dATP determination, 5′-AAATAAATAAATAAA-TAAATGGCGGTGGAGGCGG-3′;
for dCTP determination, 5′-TTTGTTTGTTTGTTT-GTTTGGGCGGTGGAGGCGG-3′;
for dGTP determination, 5′-TTTCTTTCTTTCTTT-CTTTCGGCGGTGGAGGCGG-3′;
for dTTP determination, 5′-TTATTATTATTATTAT-TAGGCGGTGGAGGCGG-3′;
universal primer: 5′-CCGCCTCCACCGCC-3′.

The enzymatic assay of dNTP had a high sensitivity to dNTP (0.1 pmol) and therefore can be performed on $1 \times 10^4$ cells. The extraction/assay method was reproducible (typical SEM for a triplicate determination was 2.8% of the mean) and gave high recoveries (greater than 80%), that were determined by comparing standard dNTP values tested alone and in presence of cell samples.

### 2.4. Determination of the nucleoside diphosphate and triphosphate pools

The cellular extracts were analyzed by ion-exchange high pressure liquid chromatography. Separation was made on column Hypersil 5 μm APS 2 $NH_2$ (250 mm × 4.6 mm) with a flow rate of 1 mL/min. The mobile phase consisted in the mixture of buffer A ($KH_2PO_4$ 5 mM, $CH_3CN$ 3%, pH 2.65) and buffer B ($KH_2PO_4$ 0.5 M, KCl 1 M, $CH_3CN$ 3%, pH 3.25) according to the linear gradient 0–100% of B in 100 min [9,10]. The absorbance was measured at 254 nm. Peak identification, as well as quantification of the different ribonucleoside diphosphates and triphosphates, was made from their UV absorption spectra and retention time compared with standards.

### 2.5. Thymidine phosphorylation

The thymidine phosphorylation was evaluated by the thymidine kinase assay according to Sherley *et al*. [11]. It is based on the measure of the dTMP production from thymidine and does not distinct between the thymidine kinase 1 and 2. SUP-T1 cells ($5 \times 10^6$ cells/mL) were lysed in 10 mM Tris–HCl (pH 7.5), 250 mM sucrose, 160 mM KCl, 1.5 mM $MgCl_2$, 50 mM ε-amino-n-caproic acid and 0.5% Nonidet P-40 and immediately tested. The thymidine kinase reaction mixture contained 0.19 M Tris–HCl (pH 7.5), 1.9 mM ATP, 1.9 mM $MgCl_2$, 22 μM [*methyl*-$^3$H]thymidine (910 mCi/mmol), 1% bovine serum albumin, 3 mM phosphocreatine, 0.54 U creatine kinase, 10 mM NaF, and cellular extract, in a final volume of 0.1 mL. The assay mixture was incubated at 37° for 30 min. The reaction was linear for at least 4 hr, provided 10% of the substrate was still present after the reaction. The reaction was stopped by spotting 50 μL of the reaction mixture on a Whatman DE81 paper disc, then immediately dropping it in alcohol (10 mL/disc) and washing three

times with alcohol. The disc was dried and inserted into a vial containing 5 mL of scintillation solution (PCS, Amersham/Searle). Counting of radioactivity was performed in a Beckman scintillation counter.

### 2.6. Statistical analysis

Statistical analysis (mean, SEM) were performed by Student's *t*-test using Graph Pad Prism software.

## 3. Results

### 3.1. dNTP levels in SUP-T1 cells

During the exponential growth of SUP-T1 cells the deoxynucleotide levels were $1.63 \pm 0.2$, $0.46 \pm 0.02$, $0.62 \pm 0.1$, $1.82 \pm 0.2$ pmol/million cell for dATP, dGTP, dCTP, and dTTP, respectively (mean ± SEM of four experiments made in duplicate).

### 3.2. Effect of azodicarbonamide on dNTP pools in SUP-T1 cells

Exposure of SUP-T1 cells for 2 hr to 50 and 100 μM azodicarbonamide induced a general reduction of the four deoxynucleotides of $37 \pm 3\%$ (mean ± SEM) and $50 \pm 2\%$, respectively (Table 1) without affecting significantly the absolute nucleoside triphosphate pool and the NTP/NDP ratio (Table 1). After a 24 hr exposure to azodicarbonamide, the depletion was still significant but blunted (Table 2). During this period, azodicarbonamide had no detectable effect on cell counts (data not shown).

Table 1
Effect of AZDA 100 μM on NTP pool, NTP/NDP ratio and dNTP pool after 2 hr exposure

| | Control | AZDA 100 μM |
|---|---|---|
| NTP (pmol/$10^6$ cells) | | |
| ATP | 1323 ± 133 | 1307 ± 128[*] |
| GTP | 704 ± 96 | 691 ± 62[*] |
| CTP | 442 ± 72 | 486 ± 40[*] |
| UTP | 952 ± 49 | 1079 ± 159[*] |
| NTP/NDP ratio | | |
| ATP/ADP | 3.1 ± 0.3 | 3.0 ± 0.2[*] |
| GTP/GDP | 6.0 ± 1.6 | 5.8 ± 0.4[*] |
| CTP/CDP | 4.3 ± 1.3 | 4.5 ± 0.4[*] |
| UTP/UDP | 4.4 ± 0.5 | 4.4 ± 0.7[*] |
| dNTP (pmol/$10^6$ cells) | | |
| dATP | 1.63 ± 0.2 | 0.83 ± 0.3[***] |
| dGTP | 0.46 ± 0.02 | 0.24 ± 0.4[***] |
| dCTP | 0.62 ± 0.1 | 0.28 ± 0.5[**] |
| dTTP | 1.82 ± 0.2 | 0.86 ± 0.4[***] |

AZDA, azodicarbonamide. Mean ± SEM. (Four to five independent experiments, two determinations per sample.)
[*] Non-significant: $P > 0.05$ vs. control (by Student's *t*-test on unpaired values).
[**] $0.01 < P < 0.05$ vs. control (by Student's *t*-test on unpaired values).
[***] $0.001 < P < 0.01$ vs. control (by Student's *t*-test on unpaired values).

Table 2
Effect of hydroxyurea, methotrexate or thymidine alone or in combination with AZDA 100 μM on dNTP pool of SUP-T1 cells

| | AZDA 100 μM | % of control | | | |
|---|---|---|---|---|---|
| | | dATP | dCTP | dGTP | dTTP |
| After 2 hr exposure | | | | | |
| – | + | 51 ± 10[***] | 45 ± 10[***] | 53 ± 12[**] | 47 ± 10[***] |
| HU 100 μM | – | 59 ± 10[***] | 48 ± 9[****] | 69 ± 13[**] | 71 ± 12[**] |
| | + | 65 ± 7[**] | 46 ± 7[***] | 79 ± 9[*] | 74 ± 4[***] |
| MTX 10 μM | – | 93 ± 6[*] | 66 ± 8[**] | 68 ± 7[***] | 62 ± 3[****] |
| | + | 74 ± 2[****] | 33 ± 6[****] | 50 ± 5[****] | 45 ± 4[****] |
| Thymidine 1 mM | – | 80 ± 1[****] | 42 ± 7[****] | 305 ± 14[****] | 208 ± 16[****] |
| | + | 58 ± 5[****] | 19 ± 3[****] | 204 ± 20[***] | 140 ± 15[**] |
| After 24 hr exposure | | | | | |
| – | + | 66 ± 11[**] | 62 ± 4[****] | 70 ± 8[***] | 66 ± 9[***] |
| HU 100 μM | – | 53 ± 7[****] | 65 ± 9[***] | 57 ± 10[***] | 54 ± 9[***] |
| | + | 53 ± 6[***] | 47 ± 4[****] | 54 ± 0.5[****] | 60 ± 3[****] |
| MTX 10 μM | – | 139 ± 8[***] | 44 ± 6[****] | 24 ± 2[****] | 23 ± 3[****] |
| | + | 108 ± 8[*] | 17 ± 3[****] | 13 ± 3[****] | 19 ± 4[****] |
| Thymidine 1 mM | – | 136 ± 5[****] | 21 ± 3[****] | 362 ± 9[****] | 298 ± 19[****] |
| | + | 93 ± 8[*] | 9 ± 0.5[****] | 190 ± 38[*] | 159 ± 16[**] |

AZDA, azodicarbonamide; HU, hydroxyurea; MTX, methotrexate. Mean ± SEM (three to six independent experiments, two determinations per sample).
[*] Non-significant:$P > 0.05$ vs. control (by Student's *t*-test on unpaired values).
[**] $0.01 < P < 0.05$ vs. control (by Student's *t*-test on unpaired values).
[***] $0.001 < P < 0.01$ vs. control (by Student's *t*-test on unpaired values).
[****] $P < 0.001$ vs. control (by Student's *t*-test on unpaired values).

### 3.3. Comparative effects of azodicarbonamide, hydroxyurea, methotrexate and thymidine on dNTP levels and their combination with azodicarbonamide

100 μM hydroxyurea reduced the four deoxyribonucleotides (Table 2). The 30–50% reduction after 2 hr exposure was maintained during 24 hr. Exposure for 2 hr to 10 μM methotrexate, reduced significantly the levels of dTTP, dCTP and dGTP, but not dATP; after 24 hr of exposure, the depletion was even more marked. After 2 hr of exposure to thymidine there was a 60% reduction of dCTP and a large increase in dGTP (two times) and dTTP (two times); after 24 hr, the observed effects were accentuated.

Combination of hydroxyurea and azodicarbonamide led to a reduction in the four deoxynucleotides comparable to that observed for each product alone and they were thus not additive. The combination of methotrexate and azodicarbonamide led to an accentuation of the dNTP changes observed with methotrexate alone and to a small but significant reduction in dATP observed after 2 hr of incubation only. When thymidine and azodicarbonamide were given together, the reduction in dCTP was significantly more pronounced and there was also a decrease in dTTP and dGTP concentration. As exogenous thymidine by-passed ribonucleotide reductase step, the effect on dTTP was surprising and we hypothesized that azodicarbonamide could prevent the formation of dTTP through thymidine kinase inhibition (see below).

Table 3
Proliferation of SUP-T1 cells after 24 hr exposure to hydroxyurea, to methotrexate or to thymidine in combination with AZDA 100 μM

| | AZDA 100 μM | Ratio: $conc_{24\ hr}/conc_0$ |
|---|---|---|
| Control | – | 1.70 ± 0.08 |
| | + | 1.62 ± 0.05* |
| HU 100 μM | – | 1.33 ± 0.02*** |
| | + | 1.24 ± 0.07*** |
| MTX 10 μM | – | 1.06 ± 0.04**** |
| | + | 0.99 ± 0.03**** |
| Thymidine 1 mM | – | 1.09 ± 0.03**** |
| | + | 1.10 ± 0.06*** |

Mean ± SEM (three independent experiments). AZDA: azodicarbonamide; HU: hydroxyurea; MTX: methotrexate. DMSO is present in all the solutions at a final concentration of 0.1%.

* Non-significant: $P > 0.05$ vs. control (by Student's $t$-test on unpaired values).

*** $0.001 < P < 0.01$ vs. control (by Student's $t$-test on unpaired values).

**** $P < 0.001$ vs. control (by Student's $t$-test on unpaired values).

Contrary to azodicarbonamide, hydroxyurea, methotrexate and thymidine had a significant effect on the proliferation of SUP-T1 cells (Table 3). There was no detectable synergistic effect between azodicarbonamide and one of these three inhibitors in the time schedule followed (Table 3).

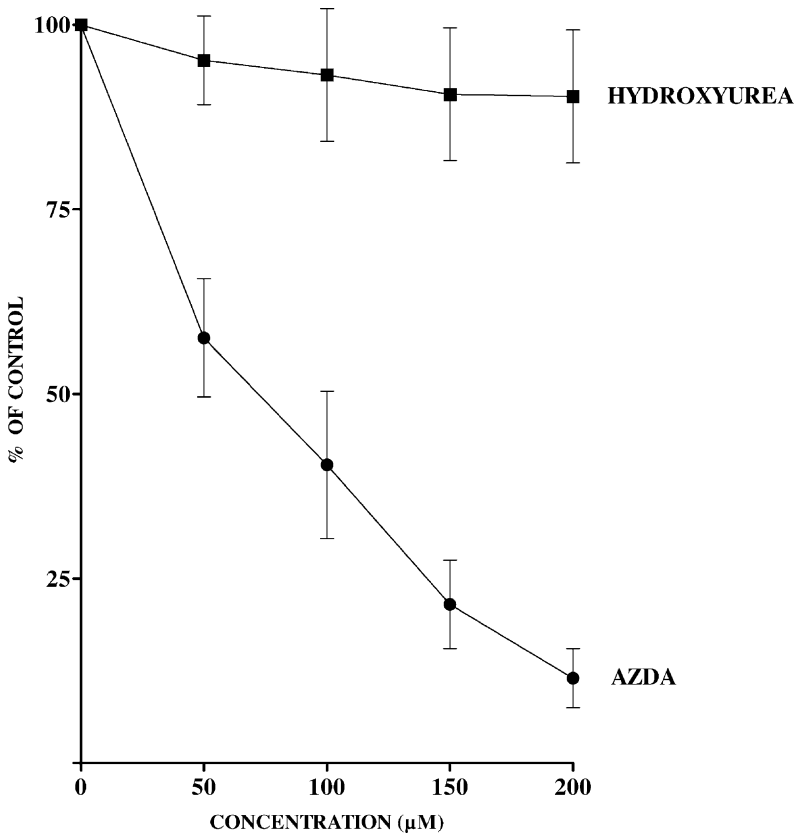


Fig. 1. Effect of azodicarbonamide (●) and hydroxyurea (■) on thymidine phosphorylating activity. Results represent means ± SEM of five to seven independent experiments performed in triplicate.

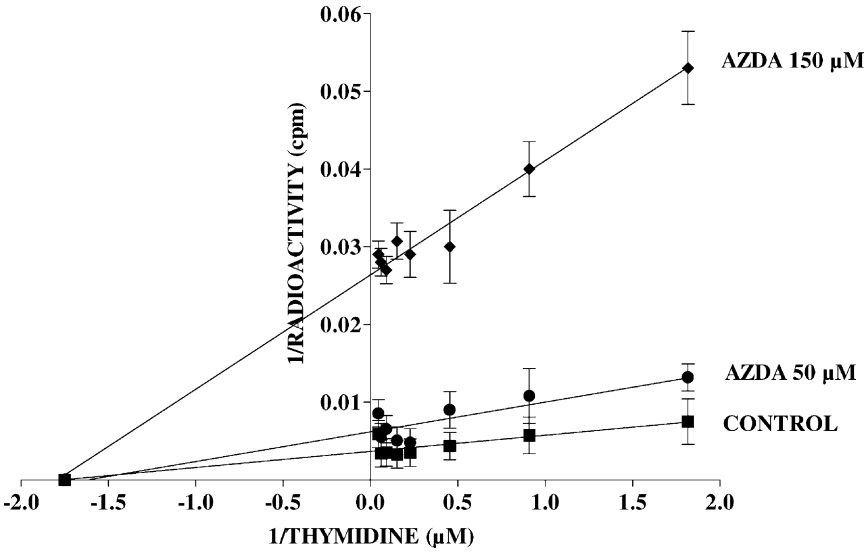


Fig. 2. Double-reciprocal plot of the effect of azodicarbonamide on thymidine phosphorylating activity of SUP-T1 cell. Azodicarbonamide at 0 µM (■), 50 µM (●) and 150 µM (◆). Results represent means ± SEM of three independent experiments.

### *3.4. Effect of azodicarbonamide on thymidine phosphorylating activity of SUP-T1 cells*

Incubation of cellular extracts with 50–200 µM azodicarbonamide for 15 min at 37° induced a dose dependent decrease in the thymidine phosphorylating activity from SUP-T1 cytosol. The $IC_{50}$ value was estimated to 70.5 µM. Hydroxyurea had no effect (Fig. 1). When two azodicarbonamide concentrations were tested in the presence of increasing concentrations of substrate, the values were compatible with a non-competitive inhibition. As we did not obtain these results (see Section 4) on pure enzyme but on a mixture of ''thymidine phosphorylating activities'' we did not evaluated a $K_i$ value (Fig. 2).

## 4. Discussion

Azodicarbonamide is currently under clinical investigation as an anti-HIV drug [1–6]. Due to its structural analogy with hydroxyurea, we hypothesized that it could also act similarly, that means by inhibiting ribonucleotide reductase that supplies the dNTP required for cell and virus replication. Hydroxyurea inhibits HIV-1 replication *in vitro* and when combined with 2′,3′-dideoxyinosine (ddI), induces a significant antiretroviral synergy appreciated at the plasma HIV-1 RNA levels [12–14]. The present results, although indirect, support the hypothesis of a similar mechanism of action for azodicarbonamide:

It reduced similarly the pools of the four deoxyribonucleotides without affecting significantly the pools of the four ribonucleosides diphosphates and triphosphates.

At concentrations that did not affect cell viability azodicarbonamide and hydroxyurea reduced similarly the dNTP pools and their effects were not additive.

When given in the presence of drugs that affect the deoxyribonucleotide levels by a mechanism different from ribonucleotide reductase inhibition, it accentuated their effect: (a) methotrexate that inhibits dihydrofolate reductase and the *de novo* purine synthesis decreases the intracellular concentration of dTTP causing an increase of the dATP pool by changing substrate specificity of the ribonucleotide reductase [15]; azodicarbonamide markedly amplifies the methotrexate induced deoxynucleotide reduction. (b) Exogenous thymidine induces an increase in dTTP which inhibits the production of dCTP and stimulates the synthesis of dGTP and dATP; addition of azodicarbonamide reduces spectacularly the four deoxynucleotides in the presence of thymidine; the effect is particularly pronounced on the dCTP pool and the marked increase of dTTP pool was blunted. These experiments based on the intracellular levels of the deoxynucleotides indirectly supported the contribution of ribonucleotide reductase to the phenomenon. Unfortunately, a direct measurement of the enzymatic activity was not feasible: *in vitro* measurement of the enzymatic activity on crude or purified preparation and preliminary assay of a reducing agent, namely DTT or NADPH [16,17] and preliminary assays indicated that azodicarbonamide was rapidly converted to biurea in the presence of these agents even in absence of cellular preparation. When we combined azodicarbonamide and thymidine administration on the cells, we observed as mentioned above a reduced dTTP increase. This could be explained by an effect of azodicarbonamide on the thymidine kinase activity but also to a downregulation of the thymidine kinase 1 due to inhibition of DNA synthesis [18]. To exclude this possibility we tested the direct effect of azodicarbonamide on the activity from growing cells in the standard culture conditions. Azodicarbonamide but not hydroxyurea non-competitively inhibits thymidine phosphorylation. Thymidine kinase is an

enzyme of the pyrimidine nucleotide salvage pathway. There are two thymidine kinases in animal cells, a cytosolic (thymidine kinase 1) and a mitochondrial (thymidine kinase 2) enzyme, with different properties. Thymidine kinase 1 is expressed only in S-phase cells, while thymidine kinase 2 is expressed in all tissues in proportion to the mitochondrial content of the cell type. In many resting cells, such as muscle and nerve cells, thymidine kinase 2 is the only pyrimidine deoxynucleoside phosphorylating enzyme expressed. Previous work on the HeLa cells had demonstrated that the S-phase-specific thymidine kinase accounts for more than 95% of the assayable activity in the cytoplasm (thymidine kinase 1) with the remainder representing mitochondrial thymidine kinase activity (thymidine kinase 2) [19]. Although in our experiments performed in another cell line, the cells were not synchronized, we estimated on the basis of the high activity and the fact that we used a preparation from cells in exponential growth phase that we measured essentially the cytoplasmic enzyme. This is however speculative as the thymidine kinase 1 activity was not specially measured in the present work and we cannot exclude the contribution of the mitochondrial activity. This is an important point that deserves more experiments; indeed, thymidine kinase is involved in phosphorylation of a number of anticancer and antiviral nucleoside analogues. For example, 5-fluoro-dUrd, 3′-azido-2′,3′-dideoxythymidine (AZT, zidovudine), 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T, stavudine) are well recognized by cytosolic thymidine kinase 1 but acyclovir is exclusively recognized by herpetic thymidine kinase and mitochondrial thymidine kinase 2 [20]. It must also be noticed that Prabhakar *et al.* [21] observed that intraperitoneal injection of hydroxyurea decreased both soluble and mitochondrial thymidine kinase activities in regenerating rat liver but had no direct *in vitro* effect on the enzyme.

In conclusion, azodicarbonamide could, at least, *in vitro*, act through several cellular modification, including a decrease in the deoxyribonucleotides pools, a decrease in thymidine phosphorylation, zinc expulsion [4,5] and calcium mobilization [2,3].

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