Physiological cell culture media alter nucleotide metabolism in T lymphocytes and increase their sensitivity to methotrexate

Claudia Cano-Estrada
Universitat Autònoma de Barcelona: Universitat Autonoma de Barcelona
Neus Ontiveros
Universitat Autònoma de Barcelona: Universitat Autonoma de Barcelona
Paula Escudero-Ferruz
IMO: Instituto de Microcirugia Ocular
Veronika Baresova
Charles University: Univerzita Karlova
Marie Zikanova
Charles University: Univerzita Karlova
Daniel Iglesias-Serret
Universitat de Barcelona
José M. López
josemanuel.lopez@uab.cat

Universitat Autònoma de Barcelona: Universitat Autonoma de Barcelona https://orcid.org/0000-0002-4104-6262

Research Article

Keywords: T-lymphocytes, methotrexate, physiological media, nucleotide metabolism, cancer, folic acid

Posted Date: February 4th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8681623/v1

Physiological cell culture media alter nucleotide metabolism in T lymphocytes and increase their sensitivity to methotrexate

Claudia Cano-Estrada [1,2], Neus Ontiveros [1,2], Paula Escudero-Ferruz[3], Veronika Baresova[4], Marie Zikanova[4], Daniel Iglesias-Serret [5], and José M. López [1,2]*

1. Institut de Neurociències, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Barcelona, Spain
2. Departament de Bioquímica i Biologia Molecular, Unitat de Bioquímica, Facultat de Medicina, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Barcelona, Spain
3. Departament de Genètica, IMO Grupo Miranza, Barcelona, Spain
4. Research Unit for Rare Diseases, Department of Paediatrics and Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague, and General University Hospital in Prague, Czech Republic.
5. Departament d'Infermeria Fonamental i Clínica, Facultat d'Infermeria, Universitat de Barcelona, L'Hospitalet de Llobregat, 08907 Barcelona, Spain

* Correspondence: josemanuel.lopez@uab.cat

ORCID ID:

Claudia Cano-Estrada: 0009-0006-9177-9241

Paula Escudero-Ferruz: 0000-0002-8778-392X

Veronika Baresova: 0000-0001-8989-6090

Marie Zikanova: 0000-0002-9375-800X

Daniel Iglesias-Serret: 0000-0001-8656-1308

José M. López: 0000-0002-4104-6262

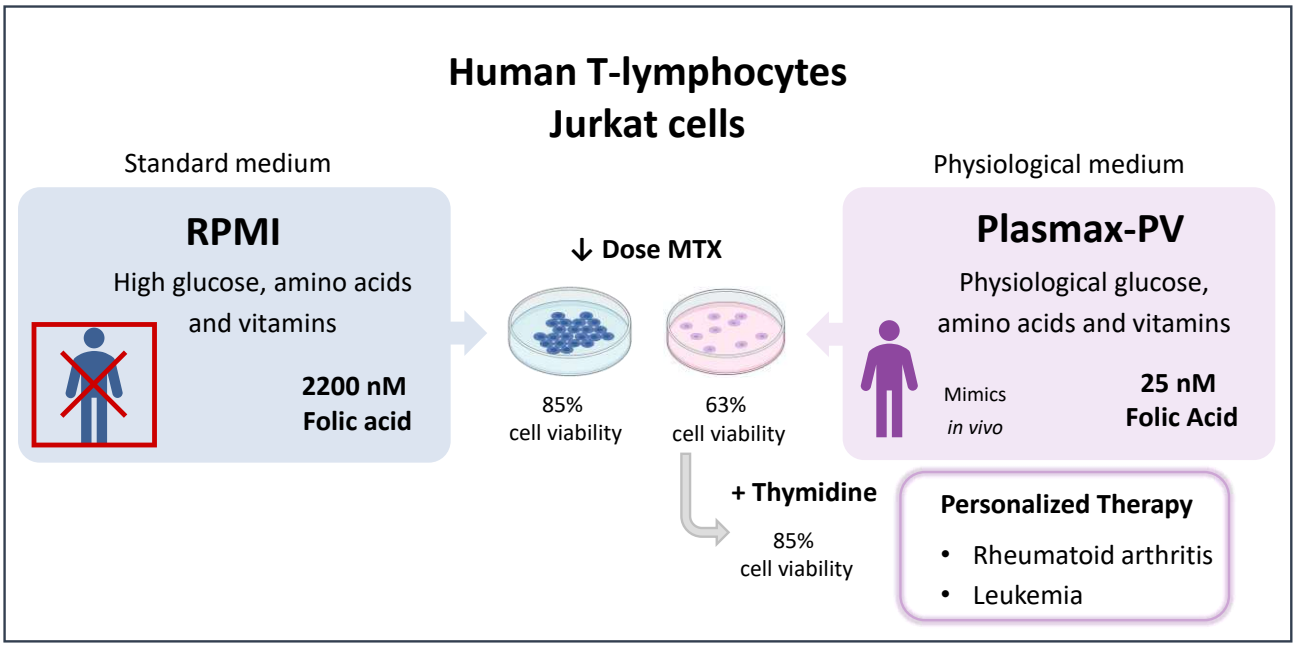
Abstract

Most cancer cells have an increased synthesis of purine nucleotides to fulfil their enhanced division rate. The *de novo* purine synthesis requires folic acid in the form of N10 formyltetrahydrofolate (10-formyl-THF). However, regular cell culture media contain very high non-physiological concentrations of folic acid, which have an impact on cell metabolism. In a previous study, several human cell lines maintained in RPMI containing physiological levels of folic acid (FA) (25 nM) accumulated 5-aminoimidazole-4-carboxamide riboside 5'-monophosphate (ZMP), an intermediary of the de novo purine biosynthetic pathway, but not with the artificially high levels (2200 nM) present in regular media [1]. Here, we investigate how culturing human cell lines in a new medium formulated to mimic physiological levels of all nutrients including vitamins (Plasmax-PV) [2] affects nucleotide content and cell death induced by the antifolate drug methotrexate (MTX). In Plasmax-PV, most of the cell lines do not show any nucleotide alteration, but Jurkat cells show accumulation of ZMP and dUMP, suggesting a disruption of both purine and pyrimidine biosynthetic pathways. Notably, a low dose of MTX induces apoptosis of Jurkat cells cultured in Plasmax-PV more effectively than in standard RPMI medium. Thymidine supplementation of Plasmax-PV completely blocked MTX-induced apoptosis, confirming an impairment of pyrimidine biosynthesis in human lymphocytes. In summary, our findings highlight the critical impact of the culture medium composition on T-cell sensitivity to MTX. These results suggest that physiological culture media can be useful to optimize MTX doses for improving personalized therapy.

Keywords

T-lymphocytes, methotrexate, physiological media, nucleotide metabolism, cancer, folic acid.

Graphical Abstract



1. Introduction

The commercially available media have made cell culture techniques widely accessible to the scientific community. Early formulations were primarily designed to maintain cell viability over extended periods of time [3]. However, these media prioritized nutrient availability and ease of production over physiological accuracy, resulting in media with supraphysiological concentrations of glucose, glutamine, pyruvate, and other components, while lacking many metabolites present in human plasma [4,5]. Despite growing awareness that cellular metabolism is highly responsive to environmental conditions, particularly nutrient availability, traditional media formulations remain widely used.

In recent years, efforts have been made to adjust the concentration of specific nutrients in cell culture media. The emergence of physiological media, such as Human Plasma-Like Medium (HPLM) and Plasmax, marks a significant paradigm shift in the field. These novel formulations aim to recapitulate the composition of human plasma, incorporating not only the essential macronutrients and amino acids but also trace elements, and polar metabolites absent in traditional media [6,7].

The use of HPLM and Plasmax produce significant metabolic changes in cultured cells compared to regular media. For instance, physiological concentrations of uric acid found in HPLM produced a direct inhibition of monophosphate synthase (UMPS), leading to altered sensitivity to 5-fluorouracil, a chemotherapeutic drug [6]. Similarly, Plasmax was found to mitigate artifacts caused by excessive nutrient levels in commercial media, such as pseudohypoxic responses induced by pyruvate and reversed urea cycle reactions caused by high arginine concentrations [7]. Furthermore, breast cancer spheroids grown in Plasmax presented metabolic profiles more closely resembling those of primary mammary tumors, which highlights the potential improvement of *in vitro* models when using physiological media [7].

Despite their advantages, physiological media also present important limitations. One of them is that current commercialized physiological media do not recapitulate *in vivo* vitamin concentrations. For instance, studies performed in standard media such as RPMI revealed that folic acid (FA) concentration profoundly impacts purine metabolism. Using physiological levels of FA (25 nM), we uncovered purine alterations in LND fibroblasts, such as ZMP accumulation (an intermediate in the *de novo* purine synthesis, substrate of the enzyme ATIC, which is FA dependent), and similar alterations may also occur in the brain of LND patients, as suggested by increased urinary levels of AICAr (a ZMP derivative) [8]. Together, these observations support the notion that vitamin concentrations are key determinants of cellular metabolic phenotypes. Recent studies from our group further reinforce this idea: human fibroblasts derived from Lesh Nyhan Disease (LND) cultured in Plasmax-PV (Plasmax with physiological levels of vitamins) presented several metabolic and functional alterations compared to control fibroblasts: ZMP accumulation, higher glycolytic capacity, increased expression of the folate carrier SLC19A1, decreased mitochondrial potential, and reduced cell migration. Notably, most of these alterations were reverted with FA supplementation, suggesting a potential therapeutic approach [2].

Nucleotide biosynthesis is a central metabolic process in cancer cells, where the *de novo* purine and pyrimidine synthesis are frequently upregulated to meet the high nucleotide demand for DNA replication, transcription, and other anabolic processes. Cancer cells reprogram nucleotide metabolism through oncogenic signaling, with key enzymes like PPAT or PAICS often overexpressed in aggressive tumors such as lung adenocarcinomas and bladder cancer [9–11].

Antifolates have been widely used for many years to treat both cancer and inflammatory diseases. Their therapeutic utility relies on the essential role of folate metabolism in the biosynthesis of purines and pyrimidines. Since cancer and activated immune cells have very increased proliferation rate, they are particularly vulnerable to disruptions in the folate metabolism [12]. Among antifolates, methotrexate (MTX) is one of the most widely used and studied drugs. MTX is a structural analog of FA [13]. It inhibits the enzyme dihydrofolate reductase (DHFR), critical to the regeneration of tetrahydrofolate (THF) from dihydrofolate (DHF), necessary for purine and pyrimidine biosynthesis. MTX not only inhibits DHFR, the major MTX target, but also other folate-dependent enzymes such as thymidylate synthase (TS), 5-amino imidazol-4-carboxamide ribonucleotide transformylase (ATIC) and glycinamide ribonucleotide transformylase (GART) [14–16]. Interestingly, MTX also inhibits the

first enzyme in the *de novo* purine biosynthesis, glutamine PRPP amidotransferase (PPAT) [17]. Clinically, MTX is administered in high doses for cancer therapy (5 g/week) [13]. It is used in combined therapy for breast cancer, non-Hodgkin lymphoma, osteosarcoma and choriocarcinoma among others [12]. At lower doses (10 to 25 mg/week), MTX serves as a first-line disease-modifying anti-rheumatic drug (DMARD) for rheumatoid arthritis, psoriasis, systemic lupus erythematosus, and Crohn's disease [13]. The main cause of MTX treatment withdrawal is not the lack of efficacy but toxicity. The most common MTX-related adverse reactions are gastrointestinal manifestations (nausea, vomiting, stomatitis) and hepatotoxicity. Some toxicities such as cytopenia, gastrointestinal intolerance and stomatitis mimic the manifestations of folate deficiency and can be prevented by folic or folinic acid supplementation [18], other toxicities unrelated to folate deficiency include nodulosis, pulmonary fibrosis, lethargy, fatigue and renal insufficiency. Despite all these effects, the mechanisms of MTX toxicity remain unclear. Ongoing research focuses on refining its delivery, reducing side effects, and better understanding its mechanisms of action, so this drug can continue to benefit patients suffering from a wide range of diseases [19].

Most of the cell culture studies performed to address the toxicity of MTX and the mechanisms involved have been done using regular media containing supraphysiological levels of folate. Our previous studies with cancer cells have shown that RPMI containing physiological levels of FA induces ZMP accumulation in certain cell lines. Here, we study the effect of Plasmax-PV, a complete physiological culture media in different human cell lines. Jurkat cells (human T-lymphocytes) cultured in a physiological medium, are markedly more sensitive to low-dose MTX treatment compared to cells grown in standard commercial media. Moreover, MTX-induced apoptosis is completely blocked by thymidine supplementation. These findings highlight the importance of using culture media that better reflect *in vivo* metabolic environments to accurately assess drug efficacy and toxicity. Our results further suggest that incorporating physiological media into preclinical testing or personalized medicine approaches could help define more effective and safer MTX dosing regimens for diseases such as rheumatoid arthritis and leukemia.

2. Methods

2.1. Cell culture

HEK293T (Cultek HCL4517), HeLa (ECACC 93021013), A549 (ECACC 86012804), Jurkat (ECACC 88042803) and EHEB (DSMZ 14916) cells were provided by the culture service of the Inc-UAB. HeLa HPRT-deficient cells obtained by CRISPR-Cas9 gene editing have been described and characterized before [20]. Cells were maintained in RPMI without folic acid (FA) (Sigma-Aldrich, Burlington, MA, USA, R1145) supplemented with 10% fetal bovine serum (FBS) (Sigma- Aldrich, T7524), 2200 nM filtered FA (Sigma- Aldrich, F8758), 1% L-glutamine (Gibco, Waltham, MA, USA, 250381), 1% Pen/Strep (Gibco, 250381) and 0.2% sodium bicarbonate (Sigma-Aldrich, S8761). The day before treatment, 160,000 adherent cells (HEK293T, HeLa, A549) were seeded in 100 mm dishes. Jurkat and EHEB cells were seeded on the first day of the treatment at a concentration of 100,000 cells/mL and 350,000 cells/mL, respectively. Cells were maintained for 7 days in RPMI with 2200 nM or 25 nM FA. In some experiments, hypoxanthine (5 µM) and/or formic acid (33 µM) were added to RPMI. The medium was changed every 2 or 3 days. When the cells were maintained in Plasmax-PV with 2200 or 25 nM FA, the media was changed daily. Plasmax without any vitamin, also known as InVitroNutrition, was prepared as described by its manufacturer (Cell Culture Technologies, Gravesano, Switzerland) and sodium hydrogen carbonate was added to a final concentration of 26.5 mM (Merck, 1.06329). A stock of vitamins 100 × containing biotin (Sigma-Aldrich, B4501), choline chloride (Sigma- Aldrich, C7527), inositol (Sigma-Aldrich, I7508), d-calcium pantothenate (Sigma-Aldrich, 21210), pyridoxal (Sigma-Aldrich, 271748), riboflavin (Sigma-Aldrich, R4500), thiamine HCl (Sigma-Aldrich, T4625), vitamin B12 (Sigma-Aldrich, V2876) was prepared and kept at -20 °C. The specific final concentration of each vitamin is presented in previous work [2]. Plasmax-PV was prepared by supplementing Plasmax with the appropriate amount of FBS (2.5% final concentration), L-glutamine (650 µM), 1% of Pen/Strep, the stock of vitamins 100× to a final 1× concentration, ascorbic acid (62 µM) (Sigma-

Aldrich, A92902) and 2200 nM or 25 nM of filtered FA (Sigma-Aldrich, F8758). Plasmax-PV was stored at 4 °C and protected from the light.

2.2. Cell growth

Cells were counted at 2 days intervals using a Neubauer chamber after trypan blue staining. The population doubling time was estimated by applying the following formula (which considers a linear regression during the exponential growth phase):

$$\text{Doubling time} = \frac{\text{Ln 2 x Time (hours)}}{\text{Ln} \frac{\text{Final cell concentration}}{\text{Initial cell concentration}}}$$

2.3. Purine measurement

For nucleotide extracts, cells were harvested by trypsinization (HEK293T, HeLa and A549) or centrifugation (Jurkat and EHEB), counted in a Neubauer chamber and resuspended in 0.4 N perchloric acid (PCA). Usually, $3x10^6$ cells were resuspended in 150 µL of PCA (a minimum of 10^6 cells in 75 µL of PCA is necessary for HPLC analysis). After 15 min on ice, cells were centrifuged at 12000x g for 5 min at 4°C. Pellet was stored at -20°C for later protein quantification and supernatant was neutralized with 5 M potassium carbonate (Sigma-Aldrich, 20961) and filtered through PVDF micro-spin filters (Thermo Scientific, Waltham, MA, USA, F2517-5) via centrifugation at 10000x g for 10 min at 4°C.

The method for purine determination involved using High-Pressure Liquid Chromatography (HPLC) coupled with a UV detector. Analytes were separated using reverse-phase ion-pair chromatography on an Atlantis T3 column (Waters, 186003729). The optimized method for nucleotides' separation and quantification consists of a sequence of stepped gradients of buffer A (10 mM of ammonium acetate (Sigma-Aldrich, A1542) and 2 mM of tetrabutylammonium phosphate monobasic solution (Sigma-Aldrich, 268100), pH5) and buffer B (10 mM of ammonium phosphate (Sigma-Aldrich, 09709), 2 mM of tetrabutylammonium phosphate, and 25% of acetonitrile (J.T. Baker, 76045) pH7). The gradient sequence includes the following steps: 100% of buffer A for 10 min, a linear gradient of buffer B up to 75% over 10 min, 9 min at 75% buffer B, a linear gradient to 100% buffer B over 3 min, 8 min at 100% buffer B, a linear gradient to 100% A in 1 min, and finally, 10 min maintained at 100% buffer A. The identification of purines was carried out by comparing their retention times to known standards and they were quantified at 254 nm by using standard curves of all the compounds analyzed. Sample analysis was performed using EZ Chrom Elite/ELITE UV-VIS 3.1.7 software.

2.4. Protein quantification

For nucleotide normalization, protein quantification was required. Protein concentration in samples was determined by using a commercial Micro BCA Protein Assay Kit (Pierce, Waltham). Protein extracts were resuspended in 2% SDS and incubated overnight at 37 °C to completely dissolve the protein pellet. 1:100 dilutions were made and incubated with reagent solution at 37 °C for 2 h. After that, absorbance was measured at 625 nm in a PowerWave XS spectrophotometer (Bio-Tek). A standard curve with increasing concentrations of BSA was prepared and used for quantification.

2.5. Cell toxicity treatments

2.5.1. Thioguanine:

HEK293T and HeLa cells were cultured in 6-well plates for 8 days in RPMI media containing 2200 nM FA and 30 µM of 6-thioguanine. On days 0, 3, 6 and 8, cells were fixed for 15 min with 4% paraformaldehyde (PFA) with continuous shaking. Then, cells were washed with PBS and stained with crystal violet solution (0,5%) for 20 min at room temperature, washed with PBS, and air-dried. Images from fixed cells were obtained using EVOS Floid Imaging System, 20x magnification.

2.5.2. Methotrexate:

Cells were treated with different concentrations of MTX (0.05 µM, 0.1 µM, 1 µM, and 5 µM) for 24 h and viability was measured by MTT assay or cell cytometry. In some experiments MTX was added in combination with 10 µM thymidine.

2.6. Studies of cell viability

2.6.1. MTT assay

For MTT assay, 5 x 10^5 cells treated in different conditions were transferred to a 96-multiwell plate and incubated with 0.45 mg/mL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) for 2 h at 37°C. When violet crystals were observed using microscopy, culture medium was removed by centrifugation and cells were lysed in 100 µL DMSO by pipetting up and down. Finally, absorbance at 550 nm and 620 nm was measured (the last one is used as a background activity) in a PowerWave XS spectrophotometer (Bio-Tek).

2.6.2. Annexin V/IP assay

Samples were analyzed by flow cytometry using flux cytometry analyzer Cytoflex (Beckman Coulter). Cytometry results were obtained using CytExpert software (Informer technologies). Annexin V dye was performed following the manufacturer's instruction detailed in the Annexin V kit (Bender Medsystems). Shortly, 3x10^5 cells were washed in 1 mL of annexin buffer, centrifuged at 200 x g for 5 min, and resuspended in 100 µL buffer containing 3 µL annexin FITC. Samples were incubated for 15 min at room temperature in the dark, and then 100 µL of annexin buffer containing 5 µL of PI (20 µg/mL) were added. 10000 cells were analyzed by flow cytometry with 633 nm excitation and a suitable filter for FITC and PI. Viability was quantified as the percentage of PI (-) and Annexin V (-) cells.

2.7. Statistics

All data are presented as mean ± SEM. Statistical analysis was performed using the GraphPad Prism 8.0.1 program. One-way ANOVA, followed by an uncorrected Fisher's LSD test, was used when more than 2 groups were compared. Two-way or three-way ANOVA, followed by an uncorrected Fisher's LSD, was used when comparing more than 2 groups and different conditions within each group. An unpaired two-tailed Student's t-test was used when only 2 groups of data were concerned.

3. Results

3.1. Physiological levels of folic acid induce ZMP and dUMP accumulation in Jurkat cells cultured in Plasmax-PV

Cell media composition can modify cell metabolism, as we have previously described in Jurkat cells cultured in RPMI containing physiological levels of FA (25 nM) [1]. In these conditions, Jurkat cells accumulated ZMP, an intermediary of the *de novo* purine pathway. Although RPMI is a commonly used commercial medium, the concentration of glucose, amino acids and many other metabolites are far from the physiological conditions found in human serum. This can produce important alterations in the cellular metabolism of the cells. To avoid this bias, we cultured the cells with Plasmax-PV, which contain physiological levels of all nutrients, including vitamins, as described by our group in studies with human fibroblasts [2]. As this new medium contains low levels of multiple components, including vitamins, daily cell medium changes are necessary to avoid nutrients exhaustion [21].

Jurkat cells were cultured for 7 days in RPMI or Plasmax-PV containing either 2200 or 25 nM FA and ZMP concentration was determined by HPLC. As shown in **Figure 1A**, ZMP accumulation was dependent on FA content in the cell media since it was observed in RPMI and Plasmax-PV containing 25 nM FA, but not with 2200 nM FA. Interestingly, a marked increase of dUMP was detected in Jurkat cells cultured in Plasmax-PV with 25 nM FA (**Figure 1B**). A representative region of an HPLC chromatogram obtained under this condition highlights that although GMP, ZMP, and dUMP elute with a 1 min difference, the peaks are clearly separated and can be quantified individually (**Figure 1C**).

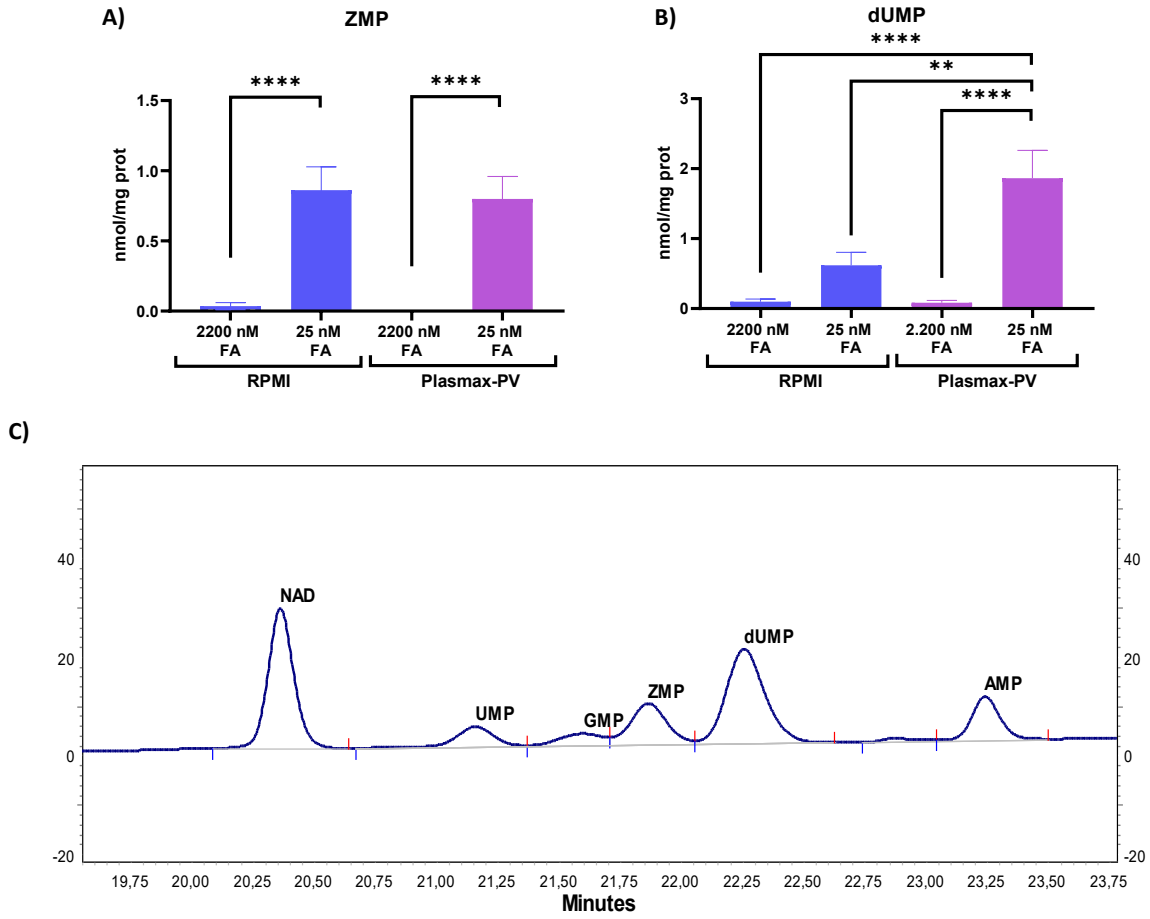


Fig. 1. Physiological levels of folic acid induce ZMP and dUMP accumulation in Jurkat cells cultured in Plasmax-PV. Cells were incubated for 7 days in RPMI medium containing 2200 or 25 nM FA and supplemented with 10% FBS; or in Plasmax-PV containing 2200 or 25 nM FA supplemented with 2.5% FBS. Cell extracts were obtained with 0.4N PCA and ZMP **(A)** and dUMP **(B)** were determined by HPLC. The graphs represent the mean ± SEM of at least 11 independent experiments, expressing the results as nmol/mg protein. One-way ANOVA, uncorrected Fisher's LSD test. ** $p < 0.01$, ****$p < 0.0001$. **(C)** Representative HPLC chromatogram (elution time 19.50 to 23.75) of Jurkat cells maintained in Plasmax-PV with 25 nM FA. Chromatographic conditions are fully described in Methods. mAU: milliabsorbance units.

3.2. Purine nucleotide levels in Jurkat cells cultured in Plasmax-PV

Intracellular levels of the most abundant purine nucleotides were determined by HPLC in Jurkat cells maintained in RPMI or Plasmax-PV containing high (2200 nM) or physiological levels (25 nM) of FA (**Figure 2 A-F**). A significant decrease in ATP levels was observed in RPMI or Plasmax-PV containing 25 nM FA compared to RPMI with 2200 nM FA (**Figure 2A**). Total adenylates and purines also presented significant lower levels in media with 25 nM FA (**Figures 2 G, I**) since ATP is the most abundant nucleotide in cells. For the other purine nucleotides, no significant differences were observed between conditions. ATP>ADP>AMP and GTP>GDP>GMP ratios were well preserved and energy charge, as defined by Atkinson and Walton [22], was similar in the different media conditions (**Figure 2J**).

Cell viability, measured as tetrazolium dye reduction with the MTT assay, increased in Plasmax-PV compared to RPMI, regardless of the FA concentration considered (**Figure 2K**), and there was a tendency to decrease within the medium with low FA concentrations.

Jurkat cells grown in RPMI showed no differences in the doubling time (approximately 30 h) regardless of the FA concentration and was similar to cells grown in Plasmax-PV with 2200 nM FA. However, in Plasmax-PV with physiological levels of FA, the doubling time was significantly increased up to 58 h (**Figure 2L**).

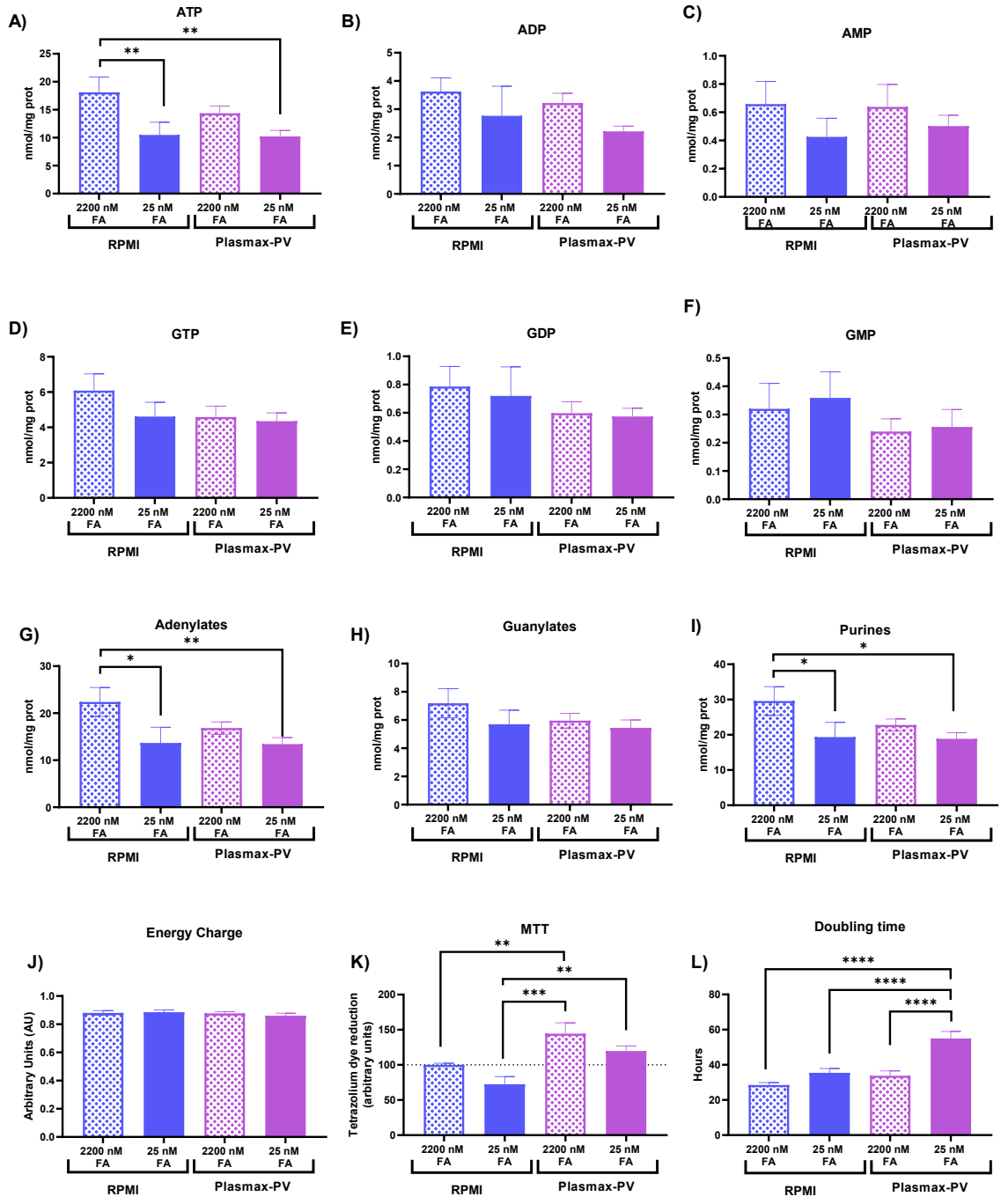


Fig. 2. Purine nucleotide levels of Jurkat cells incubated with different media. Jurkat cells were incubated for 7 days in RPMI medium containing 2200 or 25 nM FA and supplemented with 10% FBS; Plasmax-PV containing 2200 or 25 nM FA supplemented with 2.5% FBS. Cell extracts were obtained with 0.4N PCA and ATP **(A)**, ADP **(B)**, AMP **(C)**, GTP **(D)**, GDP **(E)**, GMP **(F)**, were determined by HPLC. Adenylates **(G)**, guanylates **(H)** and total purine content **(I)** are presented. Results were expressed as nmol/mg protein and represent the mean ± SEM of at least 11 independent experiments. **(J)** Energy charge was calculated as ([ATP] + 1/2 [ADP])/([ATP] + [ADP] [AMP]) and results expressed as arbitrary units (AU). **(K)** MTT assay was performed as described in Methods and the graph represents the mean ± SEM of 4 independent experiments. Results are expressed as arbitrary units (AU) using RPMI 2200 nM FA as a reference condition. **(L)** Jurkat cells doubling time was obtained using the formula described in Methods. Results were expressed in hours and represent the mean ± SEM of at least 11 independent experiments. One-way ANOVA, uncorrected Fisher's LSD test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.3. Hypoxanthine supplementation of RPMI medium induces dUMP accumulation and normalizes ATP levels in Jurkat cells

To assess which factors might be crucial in inducing a marked accumulation of dUMP in Jurkat cells cultured in Plasmax-PV, we added some components only present in this medium to RPMI. We focused on hypoxanthine and formic acid since they are some of the most important metabolites in purine synthesis. Hypoxanthine is an important source of nucleotides in the salvage purine synthesis, while formic acid is a FA precursor, needed for the *de novo* purine synthesis.

Jurkat cells were maintained in RPMI containing either 2200 or 25 nM FA and supplemented with 5 µM hypoxanthine and/or 33 µM formic acid (the concentrations found in Plasmax-PV) for 7 days. As shown in **Figure 3A**, physiological levels of FA (25 nM) induced ZMP accumulation in all conditions assayed. However, dUMP accumulation was only detected in RPMI media containing 25 nM FA plus hypoxanthine (5 µM) and/or formic acid (33 µM) (**Figure 3B**), thus mimicking the dUMP accumulation of cells cultured in Plasmax-PV (**Figure 1B**).

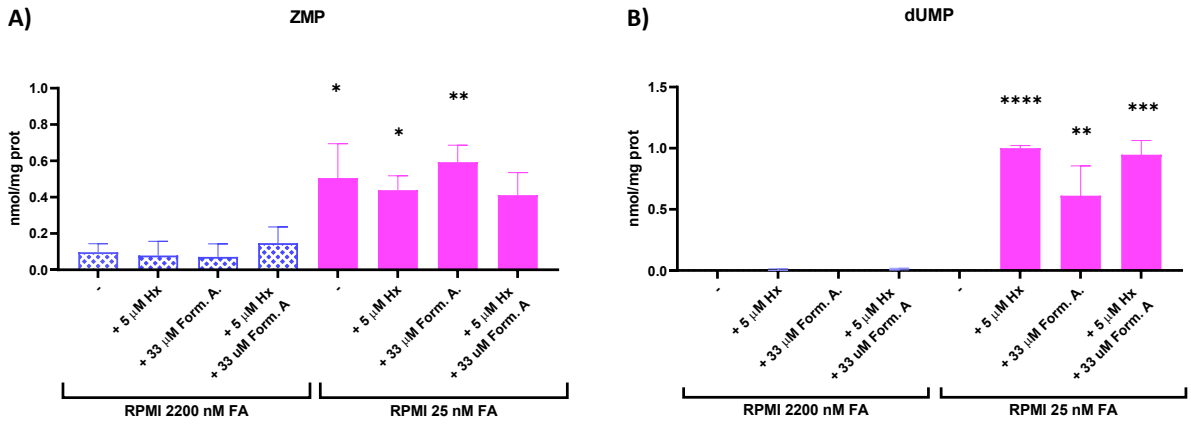


Fig. 3. ZMP and dUMP accumulation in Jurkat cells maintained in RPMI medium supplemented with hypoxanthine or formic acid. Cells were incubated for 7 days in RPMI containing 2200 or 25 nM FA with or without 5 µM hypoxanthine (Hx) and/or 33 µM formic acid (Form. A), plus 10% FBS. Cell extracts were obtained with 0.4N PCA and ZMP **(A)** or dUMP **(B)** levels determined by HPLC. The graphs represent the mean ± SEM of at least, 3 independent experiments, expressing the results as nmol/mg protein. One-way ANOVA, uncorrected Fisher's LSD test comparing each condition with 2200 nM FA to the corresponding one with 25 nM FA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

As shown in **Table 1**, Jurkat cells cultured in RPMI containing high levels of FA (2200 nM) with or without 5 µM hypoxanthine and/or 33 µM formic acid had similar levels of purine nucleotides, but at physiological levels of FA there was a clear decrease of ATP, ADP, and AMP. Importantly, the addition of hypoxanthine (but not formic acid) reverted the depletion of all adenylates, achieving concentrations closer to those observed in cells cultured with high levels of FA (**Table 1**).

3.4. Several cell lines maintained in Plasmax-PV do not accumulate ZMP or dUMP

It has been reported that human cell lines maintained in RPMI with physiological levels of FA present differential ZMP accumulation [1, 22]**.** HEK293T, Jurkat, and A549 accumulate ZMP, while HeLa and EHEB do not [1]. To further explore whether these alterations persist in a more complex and physiologically balanced culture environment, we maintained these cell lines in Plasmax-PV and intracellular purine nucleotides were analyzed by HPLC (**Table 2**). ATP and GTP concentrations varied depending on the cell line. A549 and HeLa presented the lowest levels, but the differences were not statistically significant. Importantly, Jurkat showed the highest levels of ZMP and dUMP among the cell lines analyzed, and dUMP levels were significantly different in Jurkat compared to HEK293T, A549 and HeLa, but not EHEB, which presented intermediate values.

Table 1. Purine nucleotide levels of Jurkat cells maintained in different RPMI media. Results expressed as nmol/mg protein.

		ATP	ADP	AMP	GTP	GDP	GMP
		Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
RPMI 2200 nM FA	-	11.78 ± 2.7	3.03 ± 0.82	0.57 ± 0.21	3.06 ± 0.62	0.62 ± 0.15	0.26 ± 0.09
	+ 5 µM Hx	13.14 ± 4.59	3.08 ± 0.55	0.55 ± 0.22	2.74 ± 0.57	0.62 ± 0.11	0.25 ± 0.10
	+ 33 µM Form. Acid	11.55 ± 2.49	2.97 ± 1.27	0.65 ± 0.46	2.90 ± 0.17	0.58 ± 0.17	0.24 ± 0.12
	+ 5 µM Hx + 33 µM Form. Acid	12.07 ± 2.31	3.39 ± 1.45	0.70 ± 0.39	2.78 ± 0.77	0.73 ± 0.36	0.29 ± 0.15
RPMI 25 nM FA	-	5.66 ± 0.56 ##	0.64 ± 0.31 ##	0.16 ± 0.18 #	2.19 ± 0.73	0.45 ± 0.26	0.42 ± 0.19
	+ 5 µM Hx	8.97 ± 1.65 *	2.22 ± 1.09 *	0.47 ± 0.36	2.74 ± 0.65	0.58 ± 0.24	0.22 ± 0.18
	+ 33 µM Form. Acid	6.55 ± 1.90 #	1.07 ± 0.21 #	0.20 ± 0.13	2.68 ± 0.88	0.42 ± 0.06	0.12 ± 0.09 *
	+ 5 µM Hx + 33 µM Form. Acid	9.26 ± 2.90 *	2.19 ± 1.03 *	0.44 ± 0.31	2.85 ± 1.08	0.55 ± 0.24	0.20 ± 0.15

Jurkat cells were incubated for 7 days in RPMI containing 2200 or 25 nM folic acid (FA) and supplemented with 5µM hypoxanthine (Hx) and/or 33 µM formic acid (Form. Acid), plus 10% FBS. Purine nucleotides were determined by HPLC as mean ± SEM of at least 3 independent experiments. ATP > ADP > AMP and GTP > GDP > GMP ratios were well preserved in each condition. To analyze folic acid effect, a t-test was performed for each condition comparing 2200 vs 25 nM FA. # $p < 0.05$, ## $p < 0.01$. To analyze the effect of supplements, a one-way ANOVA, uncorrected Fisher's LSD test, was performed comparing each treatment with the control condition (-) in RPMI 25 nM FA and RPMI 2200 FA independently. * $p < 0.05$.

Table 2. Purine nucleotide levels of different cell lines maintained in Plasmax-PV. Results expressed as nmol/mg protein.

	ZMP	dUMP	ATP	ADP	AMP	GTP	GDP	GMP
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
Jurkat	0.79 ± 0.16	1.86 ± 0.39 *	10.22 ± 1.09	2.21 ± 0,19	0.50 ± 0.07	4.35 ± 0.47	0.57 ± 0.06	0.26 ± 0.06
HEK293T	0.17 ± 0.08	0.01 ± 0.01	17.27 ± 2.72	2.42 ± 0.71	0.42 ± 0.22	4.33 ± 0.63	0.39 ± 0.14	0.08 ± 0.04
A549	0.13 ± 0.07	0.03 ± 0.01	6.14 ± 1.89	0.38 ± 0.03	0.10 ± 0.03	2.40 ± 0.42	0.07 ± 0.04	0.16 ± 0.07
HeLa	0.11 ± 0.014	0.036 ± 0.01	7.70 ± 0.63	0.90 ± 0.20	0.13 ± 0.01	2.54 ± 0.17	0.19 ± 0.04	0.07 ± 0.01
EHEB	0.34 ± 0.09	0.47 ± 0.24	17.09 ± 2.18	2.68 ± 0.38	0.64 ± 0.10	3.81 ± 0.65	0.40 ± 0.23	0.41 ± 0.06

Cells were incubated for 7 days in Plasmax-PV containing 25 nM folic acid (FA) plus 2,5% FBS and nucleotides determined by HPLC. Results are the mean ± SEM of at least 3 independent experiments. ATP > ADP > AMP and GTP > GDP > GMP ratios were well preserved in all cell lines. One-way ANOVA, uncorrected Fisher's LSD test comparing each nucleotide concentration between the different cell lines studied. * $p < 0.05$.

These results indicate that ZMP and dUMP accumulation is not a general feature of cells maintained in media with physiological levels of folate, but rather a cell-type–specific characteristic, which seems particularly pronounced in lymphocytic cell lines (Jurkat and EHEB). In contrast to standard RPMI with 25 nM FA, the more complete nutrient composition of Plasmax-PV likely provides additional nucleotide precursors (hypoxanthine and formic acid) to prevent ZMP and dUMP accumulation in other cell lines, further emphasizing the relevance of media composition in modulating cellular metabolic phenotypes.

3.5. Effect of methotrexate (MTX) in cell viability

In previous works, we have proposed that HEK293T and Jurkat cell lines, compared to other cell lines, rely more on the *de novo* than on the salvage pathway for purine nucleotide biosynthesis [1]. If this is the case, HEK293T and Jurkat cells should be more sensitive to MTX toxicity. To test this hypothesis in a more physiological culture medium, cells grown in Plasmax-PV with 25 nM FA were exposed to increasing concentrations of MTX (0.1-5 μM) for 24 h, and viability was assessed by MTT. Jurkat and HEK293T cells were more sensitive to MTX-induced toxicity than A549 and HeLa cells (**Figure 4A, B, D, E**). EHEB, a cell line with a very high doubling time (178h) compared to the other cell lines (24-30h) also showed a progressive decrease in cell viability with increasing concentrations of MTX (**Figure 4C**). It is remarkable that the effect of MTX on Jurkat and HEK293T cells was dose independent. Viability decreased to 70% with 0.1 μM MTX and remained similar at higher doses (1 and 5 μM). Therefore, these cells appear to be very sensitive to low doses of MTX.

The resistance to MTX-induced toxicity of HeLa cells was dependent on the salvage purine synthesis. As shown in **Figure 4F**, 0.1 μM MTX induced a significant 24% decrease in viability of HPRT-deficient cells, whereas wild type cells were resistant up to 5 μM MTX (**Figure 4E**). This result confirms that HeLa cells preferentially rely on the salvage pathway for purine biosynthesis, in contrast to Jurkat and HEK293T cell lines.

Although the relative fluxes of the purine biosynthetic pathways were not directly measured, HEK293T, HeLa, and HeLa HPRT-deficient cells were cultured in the presence of 6-thioguanine (6-TG), a guanine analogue converted to the toxic metabolite thioguanine monophosphate (TGMP) by HPRT. Cells with reduced or absent HPRT activity are therefore resistant to 6-TG toxicity [24,25]. As shown in **Figure 4G**, HeLa wild-type cells were highly sensitive to 6-TG, whereas HPRT-deficient cells remained viable for up to 8 days. HEK293T cells displayed intermediate resistance, supporting the idea that HeLa cells rely more on the salvage pathway for purine nucleotide synthesis than HEK293T cells.

3.6. Effects of a low dose of MTX on Jurkat cells cultured in different media

After characterizing the effects of MTX on different cell lines, we then compared the impact of a low dose of MTX (0.05 μM for 24 h) on the viability of Jurkat cells maintained for 7 days in RPMI or Plasmax PV containing 2200 or 25 nM FA. For a more accurate detection of cell death and apoptosis, viability was measured as the percentage of annexin and propidium iodide negative cells (see Methods). Physiological levels of folate induced a significant reduction of cell viability in the cells maintained in RPMI, but not in those maintained in Plasmax-PV (**Figure 5C**). MTX treatment induced a slight decrease in viability in cells maintained in RPMI with 2200 nM FA (90 to 84%), which was only statistically significant when analyzing the results with a T-test ($p = 0.016$), but not with ANOVA (**Figure 5C**). MTX had no effect on cells grown in RPMI with 25 nM FA, where cell viability was already reduced compared to the standard condition containing high levels of FA. Importantly, MTX markedly reduced viability (86 to 63%) of Jurkat cells grown in Plasmax-PV with 25 nM FA ($p < 0.0001$, ANOVA) (**Figure 5 A-C**).

To gain insight into the mechanism of MTX-induced apoptosis, thymidine was added to the culture medium. Jurkat cells maintained in Plasmax-PV with 25 nM FA were treated with 0.05 μM MTX for 24 h in the presence or absence of 10 μM thymidine. As shown in **Figure 5D**, thymidine slightly increased cell viability in untreated cells (from 80 to 85%) and completely blocked MTX-induced apoptosis. Thus, low doses of MTX induce apoptosis in Jurkat cells through pyrimidine deprivation.

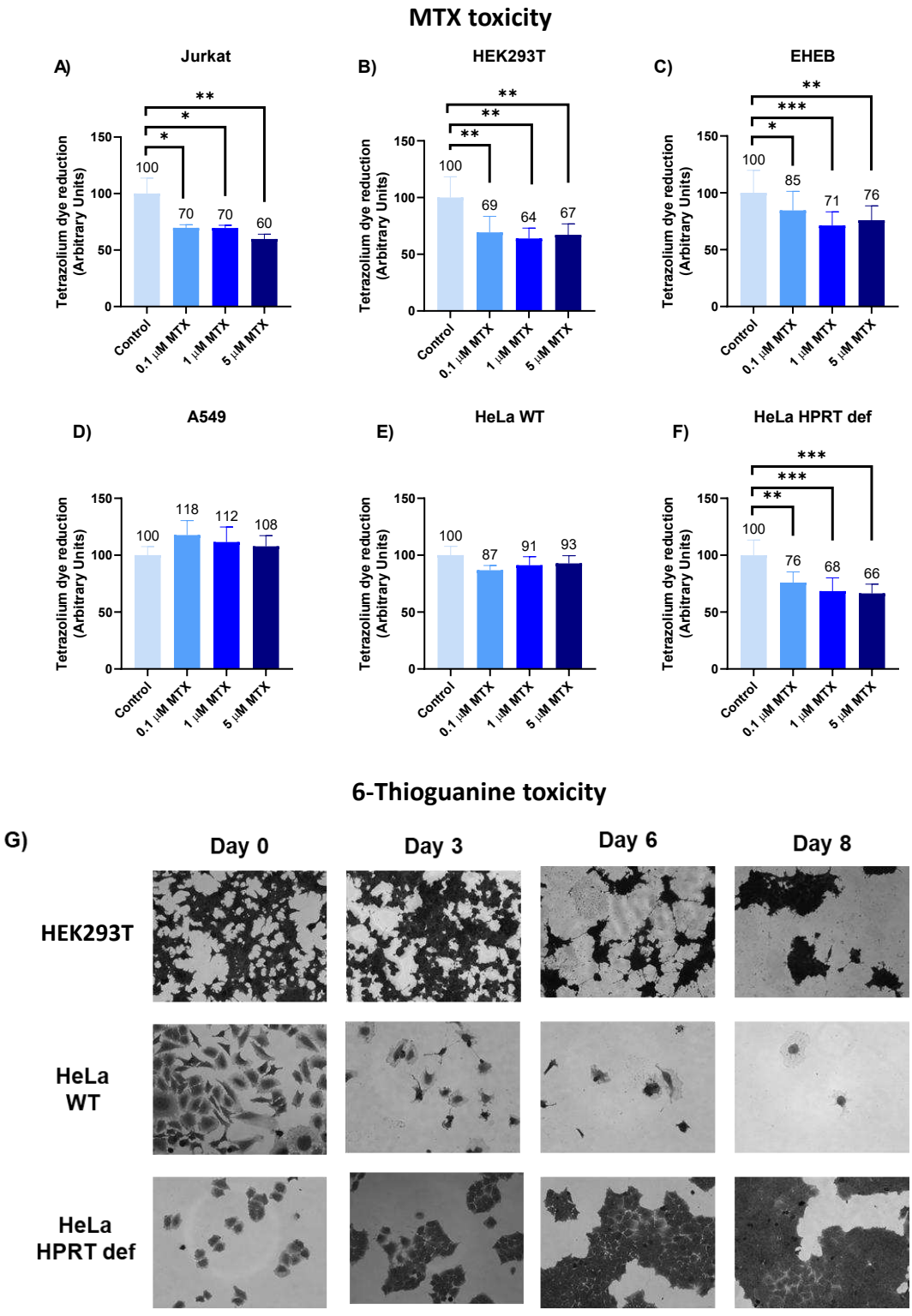


Fig. 4. MTX-induced toxicity in different cell lines**. (A-F)** Jurkat, HEK293T, EHEB, A549, HeLa WT and HeLa HPRT-deficient cell lines were cultured for 7 days in Plasmax-PV with 25 nM FA. Cells were treated for 24 h with increasing doses of MTX (0.1, 1 and 5 µM). MTT assay was performed as detailed in Methods and results were expressed as tetrazolium dye reduction arbitrary units, using the mean of untreated cells as reference condition. Graphics represent the mean ± SEM of at least 3 independent experiments. One-way ANOVA, uncorrected Fisher's LSD test comparing MTX treatments with the control condition. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(G)** HEK293T, Hela WT and HeLa HPRT-deficient cells cultured in Plasmax-PV were treated with 30 µM 6-thioguanine for 8 days. After cell fixation and crystal violet staining, representative pictures were taken every 2-3 days. EVOS Floid Imaging System, 20x magnification.

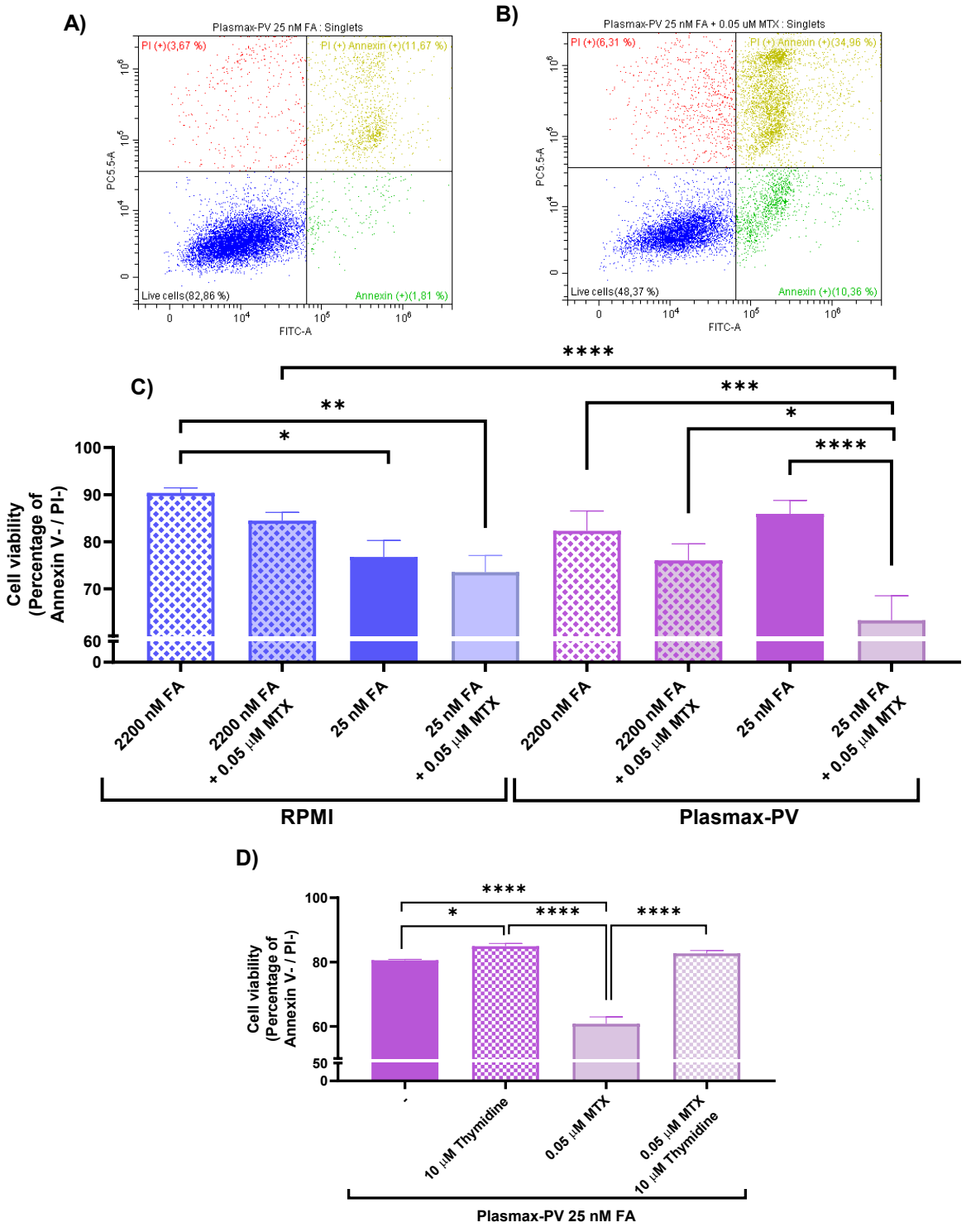


Fig. 5. MTX-induced apoptosis of Jurkat cells grown in Plasmax-PV is blocked with thymidine supplementation. Jurkat cells were cultured for 7 days in RPMI with 2200 or 25 nM FA plus 10% FBS, or in Plasmax-PV with 2200 or 25 nM FA plus 2.5% FBS and treated with 0.05 µM MTX for 24 h. Annexin V and propidium iodide were determined by flow cytometry as described in Methods. (**A, B**) Representative cytometry of Jurkat cells cultured in Plasmax-PV with 25 nM FA untreated (A) or treated with 0.05 µM MTX for 24 h (B). **(C)** Percentage of live cells (annexin V -, propidium iodide -) grown in different media for 7 days and treated with or without 0.05 µM MTX for 24 h and expressed as mean ± SEM of at least 4 independent experiments. Three-way ANOVA uncorrected Fisher LSD test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. **(D)** Cells maintained for 7 days in Plasmax-PV with 25 nM FA were treated with or without 0.05 µM MTX in the presence or absence of 10 µM thymidine. Percentage of live cells (annexin V -, propidium iodide -) expressed as mean ± SEM of 4 independent experiments. One-way ANOVA uncorrected Fisher LSD test. * $p < 0.05$, **** $p < 0.0001$.

4.Discussion

4.1. Influence of Plasmax-PV on nucleotide metabolism: Role of hypoxanthine and formic acid

The culture medium supports cell survival and proliferation and it's composition directly affects various cellular functions [26]. For this reason, some authors have put the focus on this topic, because non-physiological culture media can have a significant impact on studies of cell metabolism [4,5]. We previously demonstrated that reducing folic acid in RPMI to physiological levels (25 nM) can reveal purine nucleotide alterations in human fibroblasts from patients with Lesch-Nyhan disease [8]. Furthermore, culturing these fibroblasts in a complete physiological media, like Plasmax-PV, uncovers hidden metabolic and functional alterations [2]. We have also described that physiological levels of folate in RPMI produces differential accumulation of ZMP in several human cell lines [1].

Here we show that Jurkat cells cultured in Plasmax-PV have some alterations in purine and pyrimidine metabolism compared to cells cultured in RPMI. Notably, at physiological levels of FA (25 nM), there is a marked accumulation of ZMP and dUMP in Plamax-PV and only high levels of ZMP in RPMI (**Figure 1**). Importantly, these nucleotides are not observed with high non-physiological levels of FA (2200 nM). Moreover, ATP levels are significantly reduced in Jurkat cells maintained in media with 25 nM FA, either RPMI or Plasmax-PV (**Figure 2A**), since folate is necessary for the *de novo* synthesis of purine nucleotides. Importantly, Jurkat cells maintained in RPMI with 25 nM FA show a significant decrease in viability (**Figure 5C**). It has been reported that low levels of folic acid (12 nM) in RPMI decrease cell proliferation and induces apoptosis in PHA-activated human T lymphocytes [27]. Interestingly, despite the cells cultured in Plasmax-PV exhibit an increased doubling time **(Figure 2L**), the viability assessed by MTT assay or by annexinV/IP is higher in Plasmax-PV than in RPMI (**Figures 2K and 5C**). Although Plasmax-PV contains lower concentrations of several key nutrients compared to RPMI, it can be considered a richer medium in metabolic terms. This is because it includes a broader diversity of metabolites that contribute to essential cellular pathways, such as Fe, Se, Zn, Cu in the form of salts. These elements bound to organic small molecules and proteins such as transferrin, selenoproteins, ceruloplasmin and albumin and their availability is important for metabolic catalysts [5]. Conventional media like RPMI often overlook low-molecular-weight compounds [2].

When RPMI medium, containing physiological levels of FA, is further enriched with hypoxanthine and formic acid, Jurkat cells accumulate ZMP and dUMP, like in Plasmax-PV (**Figure 3**). This suggests that both hypoxanthine and formic acid, which are present in Plasmax-PV but absent in standard RPMI, are critical for nucleotide biosynthesis. Hypoxanthine can enhance purine salvage synthesis and formic acid can support 1C metabolism. Moreover, the addition of hypoxanthine, and to a lesser extend formic acid, allows Jurkat cells to recover from ATP depletion induced by physiological folate (**Table 1**).

4.2. Differential impact of physiological medium in human cell lines and their response to MTX

As we reported previously, different cell lines (HEK293T, Jurkat, A549, HeLa and EHEB) maintained in RPMI exhibit different metabolic profiles, along with variable energy and cellular structural biosynthesis demands, which are reflected in their total purine content [1]. When these cell lines are maintained in RPMI with physiological folate they present differential ZMP accumulation [1]. Here we show, that when these cell lines are cultured in Plasmax-PV differences in total purine content are also evident (**Table 1**), but the accumulation of ZMP occurred only in Jurkat cells, and to a lesser extent in EHEB cells, both lymphocytic cell lines. This suggests that other compounds present in Plasmax-PV can activate compensatory pathways. Hypoxanthine may enhance purine salvage through the activity of the HPRT enzyme. Meanwhile, formic acid and other small molecules might support 1C metabolism or improve mitochondrial function, thereby facilitating folate utilization and preventing a bottleneck in the *de novo* purine biosynthesis pathway [28]. Although enzymes such as ATIC require 10-formyl-THF, the conversion of FA from the medium into this active form depends on the cell's redox and energy status [29]. Therefore, the lack of ZMP accumulation in many cell lines may indicate that Plasmax-PV is a more suitable culture medium for cell lines such as HEK293T, A549, HeLa and EHEB.

The question is why Jurkat cells behave differently from other cell lines in Plasmax-PV, showing accumulation of ZMP and dUMP. EHEB cells (B-lymphocytes) also show a slight increase in these metabolites (**Table 2**). It has been previously described that hypoxanthine can inhibit the *de novo* purine synthesis in bone marrow mononuclear cells. In bone marrow, where hypoxanthine concentrations are relatively high, cells rely more on salvage pathways than on the *de novo* synthesis for purine nucleotides [30]. It has also been reported that activated human lymphocytes are very sensitive to purine bases restriction and folic acid changes in the medium [31]. It seems that the concentration of hypoxanthine present in Plasmax-PV (5 μM) is not sufficient to completely inhibit the *de novo* purine biosynthesis, since ZMP accumulates in Jurkat cells. The decreased proliferation of Jurkat cells in Plasmax-PV with limited FA availability (**Figure 2L**) is reflected in the accumulation of ZMP and dUMP (**Figure 1A, B**). This indicates that, although both purine and pyrimidine synthesis pathways are active, they are not sufficient to support maximum proliferation.

dUMP accumulation in Jurkat cells is consistent with prior findings showing that human T-lymphocytes have increased levels of dTTP compared with other deoxyribonucleotides [32]. It seems that, in Plasmax-PV with 25 nM FA, thymidylate synthase activity is compromised, leading to dUMP accumulation. It is possible that Jurkat cells maintained in Plasmax-PV have increased purine synthesis via the salvage pathway, which in turn would upregulate pyrimidine synthesis and thus, under FA limitation, induce dUMP accumulation. This aligns with our findings in Jurkat cells maintained in RPMI with physiological levels of FA and supplemented with hypoxanthine, showing increased ATP levels, as well as dUMP accumulation, a phenomenon not seen in standard RPMI (**Figure 3** and **Table 1**). Furthermore, T lymphocytes are particularly vulnerable to disruptions in nucleotide metabolism. T cells often exhibit reduced activity of enzymes involved in deoxyribonucleotide degradation, potentially contributing to imbalances such as dUMP accumulation under specific nutrient conditions [32].

Due to the differential metabolic behavior among cell lines, we also observed distinct responses to MTX treatment (**Figure 4**). Cell lines such as Jurkat, HEK293T and EHEB grown in Plasmax-PV with 25 nM FA show reduced viability (measured as MTT assay) when treated with different doses of MTX (0.1-5 μM). Interestingly, this reduction in viability is dose independent, which may be explained by saturation of the target enzyme [33] or the activation of compensatory mechanisms. As a result, cell viability appears to reach a lower threshold of approximately 60% (**Figure 4**). Very recently it has been described that MTX induces salvage purine biosynthesis through activation of the lysosomal pathway [34]. This could be one of the compensatory mechanisms. In contrast, high doses of MTX do not reduce viability of A549 or HeLa cells. This supports our previous results showing that differences among cell lines arise from the differential use of the *de novo* pathway versus the salvage pathway [1]. HEK293T and Jurkat cells, which rely more on the *de novo* purine synthesis (FA-dependent), are more sensitive to MTX. In contrast, A549 and HeLa cells, which are likely more dependent on the salvage pathway, are less affected by MTX treatment. The clearest evidence comes from HeLa cells. When these cells are genetically modified using CRISPR-Cas9 to be HPRT-deficient, they exhibit a markedly increased sensitivity to MTX toxicity (**Figure 4 E, F**).

4.3. Physiological culture media increase the sensitivity of Jurkat cells to MTX-induced apoptosis: Insights for better personalized therapy

MTX is commonly used as an anticancer therapy in leukemia and in anti-inflammatory diseases such as rheumatoid arthritis [12]. This supports our findings, showing that highly proliferative lymphocytes (like Jurkat cells) are particularly vulnerable to this drug. While the anti-proliferative effects of MTX in cancer cells are well understood, its anti-inflammatory mechanisms in autoimmune diseases remain less clear. Recent evidence suggests that MTX can modulate inflammatory processes altering different signaling pathways such as JAK/STAT, NF-κB or even p38MAPK. It can also modulate the T lymphocytes CD4 + subsets and regulate immune balance by reducing the proportion of Th1 and Th17 cells while promoting Th2 and Treg cells [33, 34]. Despite all these effects, the mechanisms of MTX-induced toxicity remain unclear. As we show here, thymidine addition in Plasmax-PV completely blocked MTX-induced apoptosis (**Figure 5D**). Therefore, the decreased viability caused by MTX in Plasmax-PV appears to be primarily due to disruption in pyrimidine synthesis. This is in agreement with a previous report showing that MTX-induced apoptosis of *in vitro* activated T cells from human peripheral blood is completely abrogated by thymidine addition [37].

Different results have been reported for Jurkat cells maintained with regular RPMI and treated with low doses of MTX. It has been described that 0.1 µM MTX induces apoptosis in Jurkat cells [38] or has no effect [39]. These conflicting results could be due to intrinsic variability among the cells used in different laboratories or to other methodological issues. Here we show that 0.05 µM MTX induces a slight decrease in viability in cells maintained with standard RPMI, but a marked reduction in the same cells grown in Plasmax-PV (**Figure 5C)**. Clearly, Jurkat cells under physiological conditions are much more sensitive to MTX-induced apoptosis. These findings suggest that therapeutic doses of MTX in patients with rheumatoid arthritis or leukemia could potentially be reduced while still maintaining treatment efficacy [37,38]. The main cause of MTX treatment withdrawal is not the lack of efficacy but toxicity [18]. Our results suggest that the use of physiological media in cells obtained from the patients could help to establish an effective dose on treatment for rheumatoid arthritis or leukemia, thus avoiding the overtreatment of patients, and potentially reducing toxicity effects (**Figure 6**). In conclusion, here we show how the use of a complete physiological medium like Plasmax-PV modifies drug efficacy and cellular responses to MTX. Extending the use of physiological culture media in research laboratories could improve clinical translation and optimize patients' outcomes by guiding drug dosage and delivery strategies more effectively.

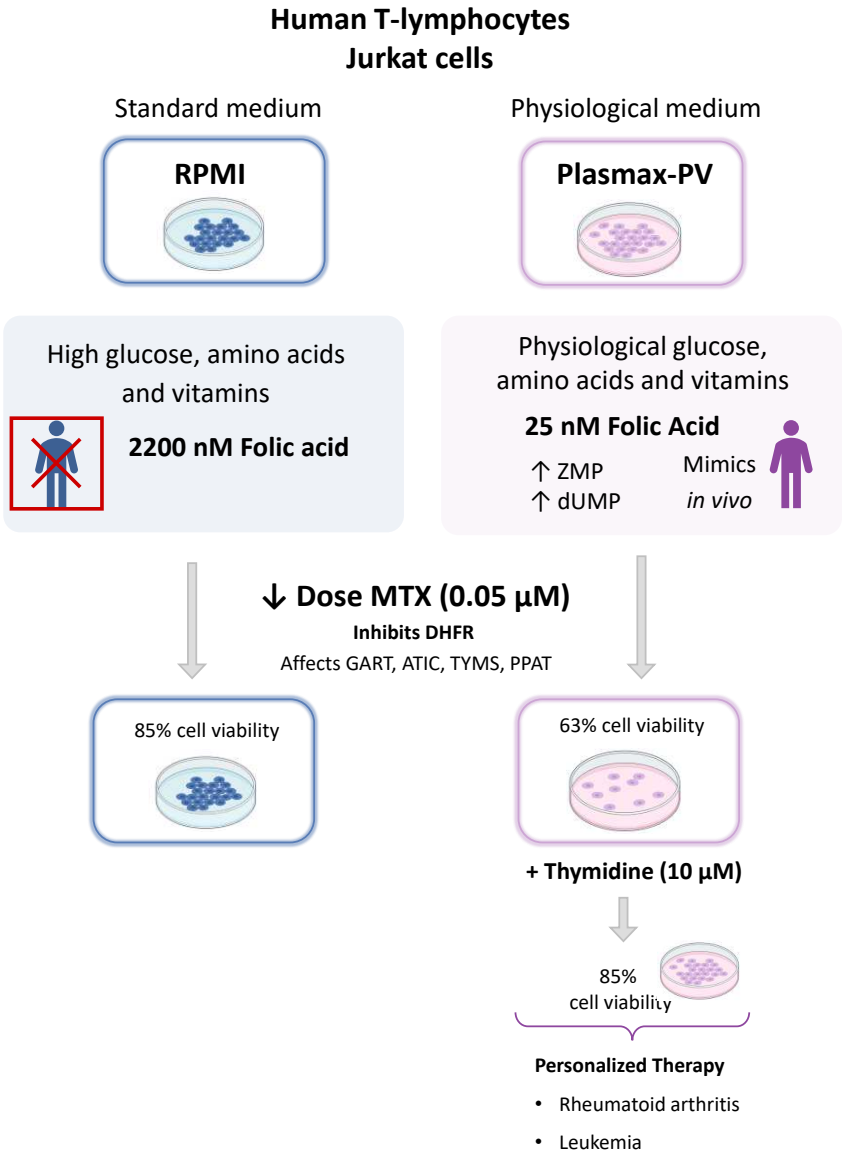


Fig. 6. A complete physiological cell culture medium (Plasmax-PV) alters nucleotide metabolism in T lymphocytes and increases their sensitivity to methotrexate (MTX). Jurkat cells maintained in Plasmax-PV have ZMP and dUMP accumulation and decreased viability in response to 0.05 µM MTX compared to standard RPMI. Thymidine supplementation completely blocked MTX-induced apoptosis. DHFR: dihydrofolate reductase, GART: glycinamide ribonucleotide transformylase, ATIC: AICAR transformylase, TYMS: thymidylate synthetase, PPAT: glutamine PRPP amidotransferase.

Statements and Declarations

Funding

This research was supported by Fundation La Marató de TV3, grant 776/C/2020, and grant PID2020-113124RB-C21 funded by MCIN/AEI/https://doi.org/10.13039/501100011033. Obtaining HPRT-deficient HeLa cells was supported by the Ministry of Health of the Czech Republic and AZV (grant NU23-01-00500) and by the MULTIOMICS_CZ project, (Program Johannes Amos Comenius, Ministry of Education, Youth and Sports of the Czech Republic; project ID: CZ.02.01.01/00/23_020/0008540), which is co-funded by the European Union.

Contributions

CCE and JML conceptualized the study and wrote the manuscript; CCE, NO and PEF performed the research; VB and MZ provided the HPRT-deficient HeLa cells; CCE, DIS and JML reviewed the results; JML administered and supervised the project and acquired funding. All autors read the article and approved the submitted version.

Consent for publication

All the authors have approved and agreed to publish this manuscript.

Competing interests

The authors declare that they have no competing interests.

Data availability

The datasets supporting the findings of this study can be obtained by contacting the corresponding author upon reasonable request.

Corresponding author

Correspondence to José M. López.

Bibliography:

[1] Cano-Estrada C, de Benito-Gómez L, Escudero-Ferruz P, Ontiveros N, Iglesias-Serret D, López JM. Purine Nucleotide Alterations in Tumoral Cell Lines Maintained with Physiological Levels of Folic Acid. Int J Mol Sci 2023;24. https://doi.org/10.3390/ijms241612573.

[2] Escudero-Ferruz P, Ontiveros N, Cano-Estrada C, Sutcliffe DJ, Jinnah HA, Torres RJ, et al. A new physiological medium uncovers biochemical and cellular alterations in Lesch-Nyhan disease fibroblasts. Mol Med 2024;30. https://doi.org/10.1186/s10020-023-00774-8.

[3] Vis MAM, Ito K, Hofmann S. Impact of Culture Medium on Cellular Interactions in in vitro Co-culture Systems. Front Bioeng Biotechnol 2020;8:911. https://doi.org/10.3389/FBIOE.2020.00911.

[4] Cantor JR. The Rise of Physiologic Media. Trends Cell Biol 2019;29:854–61. https://doi.org/10.1016/J.TCB.2019.08.009.

[5] Ackermann T, Tardito S. Cell Culture Medium Formulation and Its Implications in Cancer Metabolism. Trends in Cancer 2019;5:329–32. https://doi.org/10.1016/j.trecan.2019.05.004.

[6] Cantor JR, Abu-Remaileh M, Kanarek N, Freinkman E, Gao X, Louissaint A, et al. Physiologic Medium Rewires Cellular Metabolism and Reveals Uric Acid as an Endogenous Inhibitor of UMP Synthase. Cell 2017;169:258-272.e17. https://doi.org/10.1016/j.cell.2017.03.023.

[7] Voorde J Vande, Ackermann T, Pfetzer N, Sumpton D, Mackay G, Kalna G, et al. Improving the metabolic fidelity of cancer models with a physiological cell culture medium. Sci Adv 2019;5. https://doi.org/10.1126/SCIADV.AAU7314.

[8] López JM, Outtrim EL, Fu R, Sutcliffe DJ, Torres RJ, Jinnah HA. Physiological levels of folic acid reveal purine alterations in Lesch-Nyhan disease. Proc Natl Acad Sci 2020;117:12071–9. https://doi.org/10.1073/pnas.2003475117/-/DCSupplemental.

[9] Goswami MT, Chen G, Chakravarthi BVSK, Pathi SS, Anand SK, Carskadon SL, et al. Role and regulation of coordinately expressed de novo purine biosynthetic enzymes PPAT and PAICS in lung cancer. Oncotarget 2015;6:23445–61. https://doi.org/10.18632/ONCOTARGET.4352.

[10] Villa E, Ali ES, Sahu U, Ben-Sahra I. Cancer Cells Tune the Signaling Pathways to Empower de Novo Synthesis of Nucleotides. Cancers 2019, Vol 11, Page 688 2019;11:688. https://doi.org/10.3390/CANCERS11050688.

[11] Chakravarthi BVSK, Rodriguez Pena MDC, Agarwal S, Chandrashekar DS, Hodigere Balasubramanya SA, Jabboure FJ, et al. A Role for De Novo Purine Metabolic Enzyme PAICS in Bladder Cancer Progression. Neoplasia 2018;20:894–904. https://doi.org/10.1016/J.NEO.2018.07.006.

[12] Gonen N, Assaraf YG. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. Drug Resist Updat 2012;15:183–210. https://doi.org/10.1016/J.DRUP.2012.07.002.

[13] Bedoui Y, Guillot X, Sélambarom J, Guiraud P, Giry C, Jaffar-Bandjee MC, et al. Methotrexate an old drug with new tricks. Int J Mol Sci 2019;20. https://doi.org/10.3390/ijms20205023.

[14] Allegra CJ, Chabner BA, Drake JC, Lutz R, Rodbard D, Jolivet J. Enhanced inhibition of thymidylate synthase by methotrexate polyglutamates. J Biol Chem 1985;260:9720–6.

[15] Allegra CJ, Drake JC, Jolivet J, Chabner BA. Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. Proc Natl Acad Sci U S A 1985;82:4881–5. https://doi.org/10.1073/PNAS.82.15.4881,.

[16] Chabner BA, Allegra CJ, Curt GA, Clendeninn NJ, Baram J, Koizumi S, et al. Polyglutamation of methotrexate: Is Methotrexate a prodrug? J Clin Invest 1985;76:907–12. https://doi.org/10.1172/JCI112088,.

[17] Fairbanks LD, Rückemann K, Qiu Y, Hawrylowicz CM, Richards DF, Swaminathan R, et al. Methotrexate inhibits the first committed step of purine biosynthesis in mitogen-stimulated human T-lymphocytes: A metabolic basis for efficacy in rheumatoid arthritis? Biochem J 1999;342:143–52. https://doi.org/10.1042/0264-6021:3420143.

[18] Morgan SL, Oster RA, Lee JY, Alarcón GS, Baggott JE. The effect of folic acid and folinic acid supplements on purine metabolism in methotrexate-treated rheumatoid arthritis. Arthritis Rheum 2004;50:3104–11. https://doi.org/10.1002/ART.20516,.

[19] Wang W, Zhou H, Liu L. Side effects of methotrexate therapy for rheumatoid arthritis: A systematic review. Eur J Med Chem 2018;158:502–16. https://doi.org/10.1016/J.EJMECH.2018.09.027.

[20] Baresova V, Skopova V, Souckova O, Krijt M, Kmoch S, Zikanova M. Study of purinosome assembly in cell-based model systems with de novo purine synthesis and salvage pathway deficiencies. PLoS One 2018;13:1–13. https://doi.org/10.1371/journal.pone.0201432.

[21] Gardner GL, Moradi F, Moffatt C, Cliche M, Garlisi B, Gratton J, et al. Rapid nutrient depletion to below the physiological range by cancer cells cultured in Plasmax. Am J Physiol Cell Physiol 2022;323:C823–34. https://doi.org/10.1152/AJPCELL.00403.2021.

[22] Atkinson DE, Walton GM. Adenosine Triphosphate Conservation in Metabolic Regulation. J Biol Chem 1967;242:3239–41. https://doi.org/10.1016/s0021-9258(18)95956-9.

[23] Lee WD, Pirona AC, Sarvin B, Stern A, Nevo-Dinur K, Besser E, et al. Tumor Reliance on Cytosolic versus Mitochondrial One-Carbon Flux Depends on Folate Availability. Cell Metab 2021;33:190-198.e6. https://doi.org/10.1016/J.CMET.2020.12.002.

[24] Nelson JA, Carpenter JW, Rose LM, Adamson DJ. Mechanisms of action of 6-thioguanine, 6-mercaptopurine, and 8-azaguanine. Cancer Res 1975;35:2872–8.

[25] Munshi PN, Lubin M, Bertino JR. 6-Thioguanine: A Drug With Unrealized Potential for Cancer Therapy. Oncologist 2014;19:760–5. https://doi.org/10.1634/THEONCOLOGIST.2014-0178.

[26] Yao T, Asayama Y. Animal-cell culture media: History, characteristics, and current issues. Reprod Med Biol 2017;16:99–117. https://doi.org/10.1002/RMB2.12024.

[27] Courtemanche C, Elson-Schwab I, Mashiyama ST, Kerry N, Ames BN. Folate Deficiency Inhibits the Proliferation of Primary Human CD8+ T Lymphocytes In Vitro. J Immunol 2004;173:3186–92. https://doi.org/10.4049/jimmunol.173.5.3186.

[28] Pietzke M, Meiser J, Vazquez A. Formate metabolism in health and disease. Mol Metab 2020;33:23–37. https://doi.org/10.1016/j.molmet.2019.05.012.

[29] Ducker GS, Rabinowitz JD. One-Carbon Metabolism in Health and Disease. Cell Metab 2017;25:27–42. https://doi.org/10.1016/j.cmet.2016.08.009.

[30] King ME, Honeysett JM, Howell SB. Regulation of de novo purine synthesis in human bone marrow mononuclear cells by hypoxanthine. J Clin Invest 1983;72:965–70. https://doi.org/10.1172/JCI111068.

[31] Van Der Weyden MB, Rose IS, Newitt P. Folate-deficient human lymphoblasts: changes in de novo purine and pyrimidine synthesis and phosphoribosylpyrophosphate. Eur J Haematol 1991;47:213–8. https://doi.org/10.1111/j.1600-0609.1991.tb01557.x.

[32] Cohen A, Barankiewicz J, Lederman HM, Gelfand EW. Purine and pyrimidine metabolism in human T lymphocytes. Regulation of deoxyribonucleotide metabolism. J Biol Chem 1983;258:12334–40. https://doi.org/10.1016/s0021-9258(17)44179-2.

[33] Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The Pharmacology and Clinical Use of Methotrexate. N Engl J Med 1983;309:1094–104. https://doi.org/10.1056/NEJM198311033091805,.

[34] Liu Y, Pareek V, Bhowmik D, Zhang X, Benkovic SJ. Purinosomes and lysosomes interact to maintain the purine pools. Int J Biochem Cell Biol 2025;186. https://doi.org/10.1016/j.biocel.2025.106830.

[35] Alqarni AM, Zeidler MP. How does methotrexate work? Biochem Soc Trans 2020;48:559–67. https://doi.org/10.1042/BST20190803.

[36] Zhao Z, Hua Z, Luo X, Li Y, Yu L, Li M, et al. Application and pharmacological mechanism of methotrexate in rheumatoid arthritis. Biomed Pharmacother 2022;150:113074. https://doi.org/10.1016/j.biopha.2022.113074.

[37] Genestier L, Paillot R, Fournel S, Ferraro C, Miossec P, Revillard JP. Immunosuppressive properties of methotrexate: Apoptosis and clonal deletion of activated peripheral T cells. J Clin Invest 1998;102:322–8. https://doi.org/10.1172/JCI2676.

[38] Herman S, Zurgil N, Deutsch M. Low dose methotrexate induces apoptosis with reactive oxygen species involvement in T lymphocytic cell lines to a greater extent than in monocytic lines. Inflamm Res 2005;54:273–80. https://doi.org/10.1007/s00011-005-1355-8.

[39] Spurlock CF 3rd, Aune ZT, Tossberg JT, Collins PL, Aune JP, Huston JW 3rd, Crooke PS, Olsen NJ AT. Increased sensitivity to apoptosis induced by methotrexate is mediated by JNK. Arthritis Rheum 2011;63:2606–16. https://doi.org/10.1002/art.30457.