

Low-dose methotrexate enhances cycling of highly anaplastic cancer cells.

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Keywords:

methotrexate, cell cycle, folates, glycine, serine

ABSTRACT

We previously showed that cellular RedOx state governs the G₁-S transition of AH130 hepatoma, a tumor spontaneously reprogrammed to the embryonic stem cell stage. This transition is impaired when the mitochondrial electron transport system is blocked by specific inhibitors (antimycin A) or the respiratory chain is saturated by adding to the cells high concentrations of pyruvate. The antimycin A or pyruvate block is removed by the addition of adequate concentrations of folate (F). This suggests that the G₁-S transition of AH130 cells depends on a respiration-linked step of DNA synthesis related to folate metabolism. In the study reported here, we characterized the effects of methotrexate (MTX), an inhibitor of dihydrofolate-reductase, on the G₁-S transition of hepatoma cells, in the absence or the presence of exogenously added F, dihydrofolate (FH₂) or tetrahydrofolate (FH₄). MTX, at 1 μM or higher concentrations, inhibited G₁-S transition. This inhibition was completely removed by exogenous folates. Surprisingly, 10 nM MTX stimulated G₁-S transition. The addition of F, but not FH₂ or FH₄, significantly increased this effect. Furthermore, 10 nM MTX removed the block of the G₁-S transition operated by antimycin A or pyruvate, an effect which was enhanced in the presence of F. Finally, the stimulatory effect of 10 nM MTX was inhibited in the presence of serine. Our findings indicated that, under certain conditions, MTX may stimulate, rather than inhibiting, the cycling of cancer cells exhibiting a stem cell-like phenotype, such as AH130 cells. This may impact the therapeutic use of MTX and of folates as supportive care.

INTRODUCTION

We previously showed that metabolic networks which are active in cancer stem cells and can be target of a radical antineoplastic therapy are susceptible to inhibition by substrates playing a physiological role, such as pyruvate, glutamine or folates.^{1,2} These metabolic networks are also targeted by antineoplastic drugs such as methotrexate (MTX). MTX is an inhibitor of dihydrofolate-reductase (DHFR), the enzyme that catalyzes the NADPH-dependent reduction of dihydrofolate (FH₂) to tetrahydrofolate (FH₄), crucial step of the synthesis of purine ring and thymidylate.³ The study reported here was directed to deepen the effects of the combination of MTX, a widely used antineoplastic drug, with folates, nutritional compounds often included in supportive cancer care protocols, in the regulation of mitotic cycle in cancer cells.

The Yoshida's AH130 ascites hepatoma was chosen for this work because this is a highly homogeneous cell population characterized, under determined experimental conditions (see below), by an embryonic-like stem cell phenotype, as witnessed by the expression of CD133 as well as Embryonic Transcription Factors (ETF), such as *Nanog*, *Klf4* and *c-Myc*.¹ AH130 cells are maintained *in vivo* in the rat peritoneal cavity. At the time of harvesting from donor animals, cells are cell cycle arrested due to the shortage of oxygen and nutrients⁴ and exhibit, in vast majority, the stem cell phenotype. The combination of these two features represents a feasible example of cells sustaining minimal residual disease (MRD). Studies of cycle progression of AH130 cells are facilitated by the fact that these cells, when transferred *in vitro* in the presence of oxygen and nutrients, can be induced to G₁-S transition in a synchronized fashion. Thus, the use of AH130 cells enabled to study the effects of antineoplastic drugs on G₁-S transition of cancer stem cells.

The results obtained revealed unexpected effects of relatively low MTX concentrations (10 nM), such as the stimulation of G₁-S transition and the removal of the inhibition of this transition by physiological substrates such as pyruvate.^{1,2,4} Furthermore, these effects of low MTX concentrations were enhanced by the addition of folate (F) but not FH₄. Taken together, these results suggest that the use of MTX and the related supportive care with folates should be considered with caution and deepened further, in view of a potential detrimental effect due to the risk of stimulating cancer growth.

MATERIALS AND METHODS

Cells and animals. The Yoshida's AH130 ascites hepatoma was obtained by treating Wistar rats with the carcinogen *o*-aminoazotoluene,⁵ which induced genomic instability via the binding to and suppression of the p53 protein,¹ promoting oncogenesis and neoplastic progression. AH130 cells display unlimited self-renewal and a totally undifferentiated phenotype, with a high nucleus/cytoplasm ratio and few mitochondria. AH130 cells were maintained via serial transplantations in the peritoneal cavity of male Wistar rats (Harlan-Nossan S.R.L., San Pietro al Natisone, UD, Italy) weighing 150-200g and given water and food *ad libitum*. Cells are fed by the ascites fluid exuded from the rat peritoneal vessels. Animal manipulation was performed according to the European Union guidelines for animal care (DL 116/92, application of the European Communities Council Directive 86/609/EEC). Any effort was made to minimize animal suffering and to use the minimal number of animals sufficient to produce reliable scientific data.

Tumor transplantation and cell transfer to culture. The animals used for experiments were inoculated intraperitoneally with 3×10^7 AH130 cells rescued from an animal that had received transplantation 7 days previously. The experiments were carried out 11 days after transplantation, when peritoneal tumor cell population reaches the plateau of its growth, after a period of intense proliferation. At this stage, oxygen and glucose in ascites fluid are zeroed and most cells are blocked in G₁. A single tumor-bearing animal with haemorrhage-free ascites was sacrificed and tumor cells were harvested from the peritoneal cavity under sterile conditions by aspiration with a syringe. A small aliquot of ascites fluid was saved as it was (2-3 ml) and the remainder (70-80) ml was centrifuged at 1000 g for 10 min in order to separate cells from ascites plasma, which was recovered and used for the preparation of incubation medium. Incubation medium was prepared by mixing 4 volumes of ascites plasma and 6 volumes of a saline containing 133 mM NaCl, 3.8 mM KCl, 0.58 mM MgSO₄, 0.88 mM CaCl₂, 0.24 mM Na₂HPO₄, 0.32 mM KH₂PO₄, 0.04 mM phenol red and antibiotics (100 U/ml of penicillin and 100µg/ml of streptomycin). This solution was buffered with HEPES (Eurobio, Paris) at a final 20 mM concentration and subsequently adjusted to pH 7.4 with NaOH at room temperature. To the medium (ascitic plasma plus saline) glucose was added to a final concentration of 15mM. About 1 ml of tumor cell suspension in ascites fluid was diluted with incubation medium to obtain a final concentration of $3-3.5 \times 10^5$ viable cells/ml and placed into a Warburg flask. Cell viability was assessed by the trypan blue exclusion test, diluting 1:1 the cell suspension with a 1% trypan solution. Cells were incubated in air at 38°C in a Warburg apparatus (B. Braun, model V85, 80

oscillations/min). By the exclusive effects of the oxygen and glucose supply, the whole AH130 cell population is synchronously recruited to S phase.⁴

Cell labeling and radioactivity measurement. The overall estimate of DNA labeling was carried out using 2-¹⁴C-thymidine with a specific activity of 61 mCi/mmol (Amersham International PLC, Little Chalfont, U.K.). Cells were pulse-labeled for 90 minutes with 0.6 μ Ci/flask at different times of incubation. At the end of labeling time, the content of flask was centrifuged and cell pellet resuspended in 10 ml of trichloroacetic acid (TCA) 10% solution in water. The TCA-precipitated material was washed 3 times with 10 ml of TCA 10% solution in water, dissolved in 0.5 ml of NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, IL, U.S.A.) and mixed with scintillation medium (4.0 g of 2,4-diphenyloxazolone and 0.05 g of 2,2'-p-phenyloxazolone in 1 l of toluene (Sigma-Aldrich, St. Louis, MO, U.S.A.). Radioactivity was measured with a TriCarb 460CD β -counter (Packard Instrument Co., Fallbrook, CA, U.S.A.) to determine the rate of 2-¹⁴C-thymidine incorporation into DNA (R).

Autoradiography. The percentage of cells synthesizing DNA was estimated by autoradiography, using ³H-thymidine with a specific activity of 2 Ci/mmol (Amersham). Cells were pulse-labeled for 90 minutes with 1.0 μ Ci/flask at different times of incubation. At the end of labeling time, the content of flask was centrifuged at 1000 g for 10 min. Cell pellet was first washed with 1.0 ml of cold culture medium and then fixed by resuspension in 10 ml of acetic acid:methanol (1:3) mixture. Fixed cells were then washed three times with the same mixture and centrifuged as above and finally spread onto glass slides pre-treated with a solution containing 0.05% KCr(SO₄) and 0.5% gelatine. After drying, slides were dipped into NTB-2 emulsion for autoradiography (Kodak Corp., Rochester, NY, U.S.A.) and kept in the dark at 4°C for 20-30 days. At the end, the slides were developed in D-19 developer (Kodak) for 5 minutes at room temperature, fixed for 15 min and stained with Giemsa. One thousand cells were observed for each slide, counting cells separately on the basis of the number of granules per cell: (a) none; (b) 1 to 5; (c) 6 to 15; (d) more than 15; (c) and (d) cells were considered positive.

Drugs and chemicals. MTX, F, FH₂ and FH₄, pyruvate, antimycin A and aminoacids were all purchased from Sigma Aldrich and added at time zero of incubation at the concentrations indicated in the Legends to Figures. Aminoacids were neutralized to pH 7.4 before addition to cultures.

Statistical Analysis. The significance of differences was calculated by the Student's *t* test for paired samples; * = $p < 0.05$; ** = $p < 0.02$; *** = $p < 0.005$.

RESULTS

The transfer of AH130 cells blocked in G_1 *in vivo* into the culture system described in Materials and Methods determines the recruitment of most cells to enter the S phase during the first 18 hours after plating *in vitro*. As in this experimental system the number of cells synthesizing DNA is directly proportional to rate of ^{14}C -thymidine incorporation into DNA (R), the G_1 -S transition of cell population can be easily monitored by comparing R values at time 0 with those at 18 hours of incubation.⁴ The concentration/response effect of MTX on G_1 -S transition, as determined by measuring R at 18 hours of incubation, is reported in **Figure 1A**. At 100 nM and higher concentrations, MTX inhibited G_1 -S transition, indicating that this transition requires F reduction by DHFR. Surprisingly, a significant stimulation of G_1 -S transition in comparison to the untreated control was obtained with 10 nM MTX, while lower MTX concentrations were neither inhibitory, nor stimulatory. **Figure 1B** shows that, as expected, the addition of reduced folates to the system antagonized completely the inhibitory effect of 1 μM MTX on G_1 -S transition. However, F was found equally capable of antagonizing the effect of 1 μM MTX, as previously shown.⁶ Surprisingly, F also enhanced the stimulatory effect of 10 nM MTX, whereas the reduced folates were ineffective in this respect. On the other hand, F was ineffective in the absence of MTX (data not shown), a clear indication that the endogenous F metabolism is sufficient to sustain the G_1 -S transition of AH130 cells at its maximum extent. **Figure 1C** shows the results obtained by autoradiography after pulse-labeling (90 minutes) with ^3H -thymidine, immediately after plating or from 16.5 to 18 hours of incubation, in the presence or not of the 10 nM MTX/F combination. Incubation for 18 hours determined a marked increase of the percentage of nucleus-labeled cells in untreated controls; this increase was further enhanced in MTX/F-treated cultures, reaching values around 50% higher than in untreated controls.

Previous work from our laboratory revealed that cellular RedOx state governs the G_1 -S transition of AH130 cells.^{2,4} This transition is indeed impaired when the mitochondrial electron transport system is blocked by specific inhibitors (antimycin A) or the respiratory chain is saturated by adding to the cells high concentrations of pyruvate or other oxidizable substrates. The inhibitory effects of antimycin A or pyruvate are removed by the addition of purines, but not pyrimidines, indicating that the G_1 -S transition of AH130 cells depends on respiration-linked steps of purine synthesis.⁷ Since a complex series of NADP-dependent interconversions of F derivatives is necessary for purine synthesis,⁸ we hypothesized that some respiration-linked reaction involved in F metabolism represent the limiting step of G_1 -S transition.² On this basis, in the study reported here, we tested the effects of 10 nM MTX

and/or F on G₁-S transition of AH130 cells, as determined by R measuring, at 18 hours of incubation in the presence or the absence of pyruvate or antimycin A (**Figure 2**). The addition of MTX or F, or their combination, removed completely the inhibition of G₁-S transition produced by pyruvate or antimycin A.

On the basis of the results of Figure 2, we tested the effects of aminoacids involved in folate metabolism, such as serine, glycine, aspartic acid or glutamic acid, on G₁-S transition of AH130 cells, as determined by R measuring (**Figure 3A**). While glutamic and aspartic acids were ineffective, glycine and serine strongly inhibited G₁-S transition. The concentration/response relationship of the inhibitory effect of serine on G₁-S transition is reported in **Figure 3B**. The inhibition became significant at 0.5 mM (-42%), to reach its maximal value at 5 mM (-95%). The effects of the combination of 2.5 mM serine with MTX are shown in **Figure 3C**. Serine alone induced a 74% inhibition of G₁-S transition with respect to the untreated control. In the presence of serine, the stimulatory effect of low-concentration MTX was abolished, while the inhibitory effect of high-concentration MTX was unchanged.

DISCUSSION

The results obtained revealed unexpected effects of low MTX concentrations (10 nM), such as the stimulation of G₁-S transition and the removal of the inhibition of this transition operated by the block of respiratory chain (antimycin A) or its saturation with physiological substrates (pyruvate). Furthermore, these effects of low MTX were enhanced by F addition (Figures 1 and 2). Serine, on the contrary, inhibited the stimulatory effect of low-concentration MTX.

MTX is a potent inhibitor of DHFR, the main function of which is the regeneration of FH₄ from FH₂ (Figure 4). As a consequence of DHFR inhibition, the intracellular levels of FH₄-based coenzymes are decreased, resulting in the inhibition of pyrimidine and purine biosyntheses.³ The pivotal substrate of these syntheses is N⁵N¹⁰methylenFH₄. This substrate, indeed, on one hand enables thymidilate synthesis generating FH₂ as a by-product, on the other is converted to N⁵N¹⁰methenylFH₄, which sustains purine synthesis via N⁵formylFH₄ (folinic acid) and then N¹⁰formylFH₄. In this scenario, it has been shown that N⁵N¹⁰methylenFH₄ decreases under high MTX concentration, as expected, while it increases under low-concentration MTX, an apparent paradox.⁹ However, it is worth pointing out that DHFR reduces FH₂ to FH₄ operating at very low levels of saturation, so that only 5% of DHFR activity is sufficient to cope with the cell needs of FH₄-based coenzymes.¹⁰ On this basis, low concentrations of MTX (which reduce but do not block completely DHFR activity) were found not only ineffective in reducing, but actually capable to increase, N⁵N¹⁰methylenFH₄.⁹ Thus, the stimulatory effect of low-concentration MTX can be due to the fact that the enhanced N⁵N¹⁰methylenFH₄ availability fosters purine and thymidilate syntheses, thereby maintaining DNA synthesis.

Purine and thymidilate syntheses are also controlled by the NADP/NADPH ratio, the decrease of which inhibits DNA synthesis.² An increase of this ratio can explain our data that the addition of preformed F, but not FH₄ or FH₂, markedly enhanced the stimulatory effect of low-concentration MTX. Added F can be converted to FH₂ (not shown in Figure 4) and then to FH₄ under the reductive action of DHFR.¹¹ Thus, as DHFR is not blocked by low-concentration MTX (see above), this action may result in the generation of NADP. FH₄ or FH₂ addition, on the contrary, cannot determine such an increase, as they, being already in a reduced state, do not enhance NADPH to NADP conversion. Actually, the addition of FH₄ alone to cultures produced a cytostatic effect similar to that of MTX (data not shown), as previously demonstrated for other cancers such as melanoma.² It is worth pointing out here that, in several different cancer (stem) cell populations, the purine pools are sensitive to the inhibition/saturation of respiratory chain (antimycin A/pyruvate), while

the pyrimidine pools are usually sufficient to ensure cell cycling.^{7,12,13} This implies that purines but not pyrimidines are critical to DNA synthesis. Purine synthesis requires the NADP-dependent oxidation of N^5N^{10} methylenFH₄ to N^5N^{10} methenylFH₄, which is paralleled by NADPH production. Antimycin A or pyruvate block cell recruitment to S because they inhibit purine synthesis via the block of mitochondrial respiratory chain and of respiration-dependent NADPH reoxidation to NADP². When low MTX concentrations (with or without F) determine an increase⁹ of N^5N^{10} methylenFH₄ and the consequent enhancement of MTHFD activity, the excess NADPH produced can be reoxidized to NADP in the cytosol by DHFR (insensitive to low-concentration MTX, as explained above). This may well explain the complete removal by low-concentration MTX of the effect of antimycin A or pyruvate (Figure 2) and the maintenance of purine synthesis independently of mitochondrial NADPH reoxidation.

A reduction of NADP/NADPH ratio may also explain the effects of serine or glycine (Figure 3), aminoacids strictly related to the conversion of FH₄ to N^5N^{10} methylenFH₄ synthesis, which occurs via the SOG pathway (serine synthesis, one-carbon cycle, glycine cleavage).¹⁴ An excess of serine determines the production of an excess of NADPH and consequently the reduction of NADP/NADPH ratio in cells with low mitochondrial activity, such as AH130 cells (see below).^{1,12} Serine may also determine NADPH production by forcing the conversion of N^5N^{10} methylenFH₄ to N^5N^{10} methenylFH₄. As far as the observed effects of the serine/MTX combination are concerned, one can conclude that serine counteracts the stimulatory effects of low-concentration MTX via the production of an excess of NADPH. On the other hand, in the presence of high concentrations of MTX alone, it is the complete block of DHFR which accounts for the accumulation of NADPH, an effect which is further enhanced by the addition of serine.

A more general question is whether the paradoxical effect of low-concentration MTX we observed is linked to the fact that the AH130 cell population is homogeneously endowed with a stem cell profile. It is a fact that AH130 is a highly anaplastic cancer cell population which expresses a number of stem cell markers. Furthermore, AH130 cells are endowed with a very scanty mitochondrial apparatus,¹ another feature which typically distinguishes stem from progenitor cells.¹⁵ Thus, the mitochondrial activity of stem cells is easily saturable by the reducing equivalents coming from the oxidation of physiological substrates, such as pyruvate, at concentrations which are instead easily catabolized by progenitor cells characterized by a high proliferation rate.¹² This implies that in stem cells NADPH cannot be easily oxidized to NADP. Accordingly, the paradoxical effect of low-concentration MTX observed for AH130 cells could not be obtained with a number of more

differentiated cancer cell populations such as the WEHI3B human myelomonocytic leukemia or the Friend's murine erythroleukemia cells (data not shown).

Our findings may impact the therapeutic use of MTX and of folates as supportive care. The low MTX concentrations we found to stimulate G₁-S transition of AH130 cells match the plasmatic levels reached at the end of treatment cycles or during the necessary therapeutic intervals. This may represent a stimulus to the maintenance of slow-cycling cancer stem cells which are best suited to survive treatment.¹⁶ On the other hand, the possibility has been also taken into consideration that folates themselves may support tumor growth. Indeed, although the majority of clinical studies points to a relatively high folate intake as a factor protecting patients from the adverse effects of anti-folate therapy,^{17,18} recent evidences are against folate treatment of patients with prostate cancer and suggest that F may accelerate the progression of colorectal cancer.^{19,20} The detrimental effects –enhancement instead of inhibition of tumor growth- of MTX and/or folate treatment observed under some conditions can be framed within the more general issue that cancer therapy may sometimes promote cancer.²¹ The best known example in this sense is the failure of anti-angiogenetic therapy,²² due to the selection of hypoxia-resistant cancer stem cells. This scenario suits very well our experimental system, where low MTX concentrations enhance cycling under hypoxia-mimicking conditions (treatment with antimycin A or pyruvate) of cells endowed with a cancer stem cell profile.

ACKNOWLEDGEMENTS

This paper is dedicated to the memory of Dr. Manuela Balzi, Director of the Radiobiology Unit of Università degli Studi di Firenze.

Research was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC; grants #IG5220 and #IG13466), Istituto Toscano Tumori (ITT), Ministero della Salute (grant #RF-TOS-2008-1163728) and Regione Toscana – Programma per la Ricerca in Materia di Salute and Ente Cassa di Risparmio di Firenze.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Effects of MTX on AH130 cell recruitment to S. (A) Effects of different concentrations of MTX added at time zero on ^{14}C -thymidine incorporation (R) in the 90-minute interval between 16.5 and 18 hours of incubation; R values (DPM incorporated per 10^6 viable cells in 90 minutes), representing a measure of DNA synthesis, are means \pm SEM of 3 independent experiments; dashed line: untreated control. (B) Effects of the treatment or not (ctr/light grey) with $1\mu\text{M}$ (1000 nM) or 10nM MTX on R (determined under the above conditions) in the presence of (ctr/hatched) MTX alone or (dark grey) MTX plus $50\mu\text{M}$ folate (F), dihydrofolate (FH_2) or tetrahydrofolate (FH_4); values are means \pm SEM of 10 independent experiments. (C) Effects of the treatment or not (ctr) with 10nM MTX plus $50\mu\text{M}$ F on the percentage of labeled cells (autoradiography) following a 90 minute incubation in the presence of ^3H -thymidine from 16.5 to 18 hours of incubation; the time zero value in the absence of treatment is also shown (incubation with ^3H -thymidine from time 0 to 90 minutes); values are means \pm SEM of 3 independent experiments. Significance of differences was evaluated by the Student's *t* test for paired samples (** $p < 0.02$; *** $p < 0.001$).

Figure 2. Effects of MTX and/or F on the inhibition of AH130 cell recruitment to S by pyruvate or antimycin A. R values (DPM ^{14}C -thymidine) incorporated per 10^6 viable cells in the interval 16.5-18 h of incubation were determined in the absence (dashed line) or the presence of 10 mM pyruvate (light grey) or $6\mu\text{M}$ antimycin A (dark grey), without (ctr) or with 10 nM MTX and/or $50\mu\text{M}$ F, and are means \pm SEM of 5 independent experiments. Significance of differences was evaluated by the Student's *t* test for paired samples (* $p < 0.05$; ** $p < 0.02$).

Figure 3. Effects of aminoacids involved in folate metabolism on AH130 cell recruitment to S. R values (DPM ^{14}C -thymidine) incorporated per 10^6 viable cells in the interval 16.5-18 h of incubation were determined in the presence or the absence of aminoacids, and/or MTX added at time 0. (A) Effects of 5mM serine, glycine, aspartic acid or glutamic acid (ctr: untreated control). (B) Effects of different serine concentrations; values are expressed as $\Delta\%$ of untreated control (0). (C) Effects of 2.5mM serine (light grey), MTX (hatched) or serine plus MTX (dark grey); untreated control: dashed line. Values are means \pm SEM of 3 independent experiments. Significance of differences was evaluated by the Student's *t* test for paired samples (* $p < 0.05$; ** $p < 0.02$).

Figure 4. Pathways linking folate metabolism, MTX action and serine/glycine interconversions. See text for explanation. Notes: (a) the level of action of the most important drugs interfering with folate metabolism is shown; (b) the enzymatic activity of MTHFD the acronym refers to (methylenetetrahydrofolate-dehydrogenase) is NADP-dependent; (c) MS exhibits 5-methylTHF:homocysteine-methyltransferase activity; (d) pathways 1 are cytosolic, 2 cytosolic or mitochondrial, 3 mitochondrial; (e) arrows point to the product(s) of enzymatic reaction; (f) ⊥ inhibitory action.

Figure 1

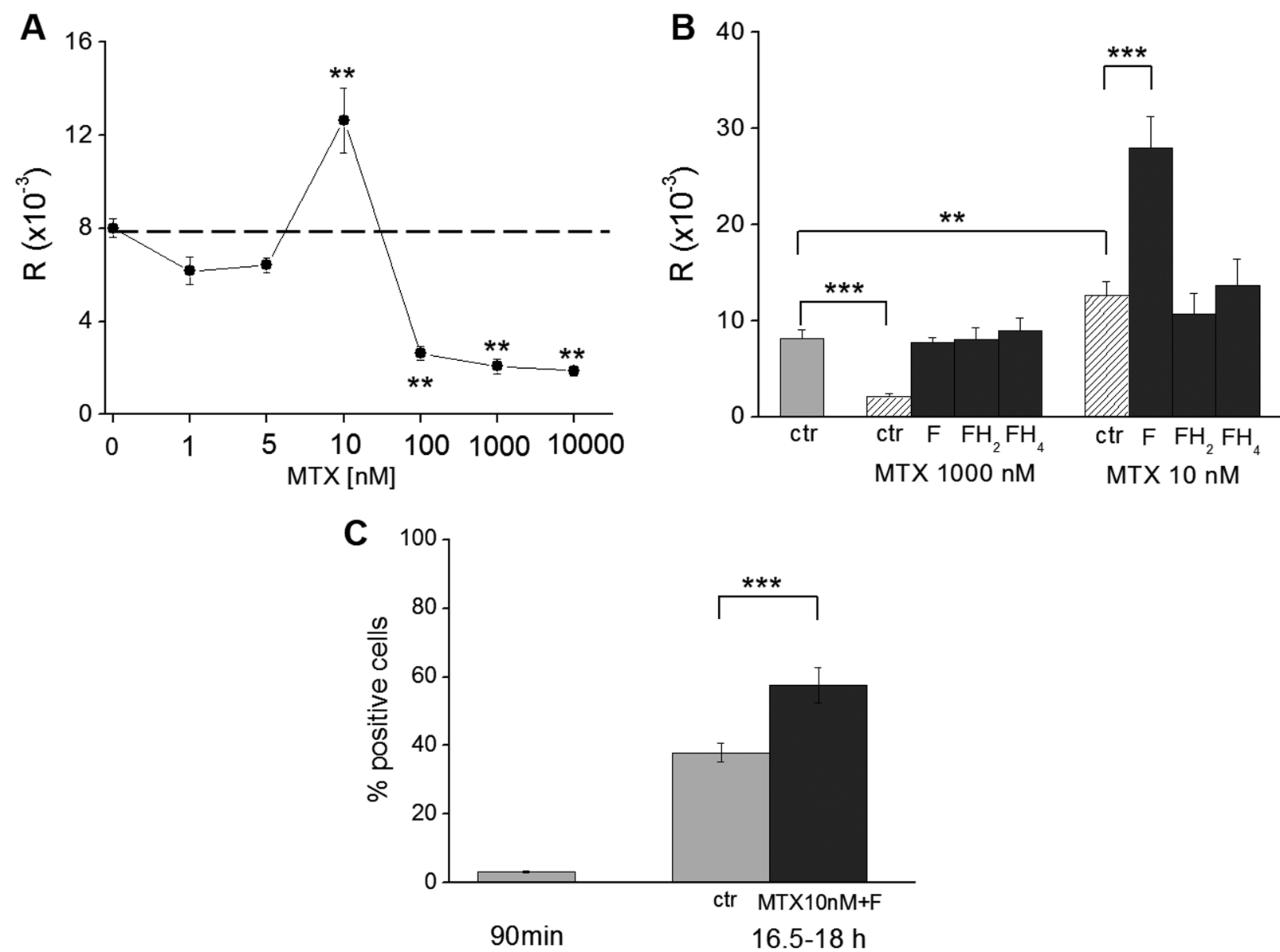


Figure 2

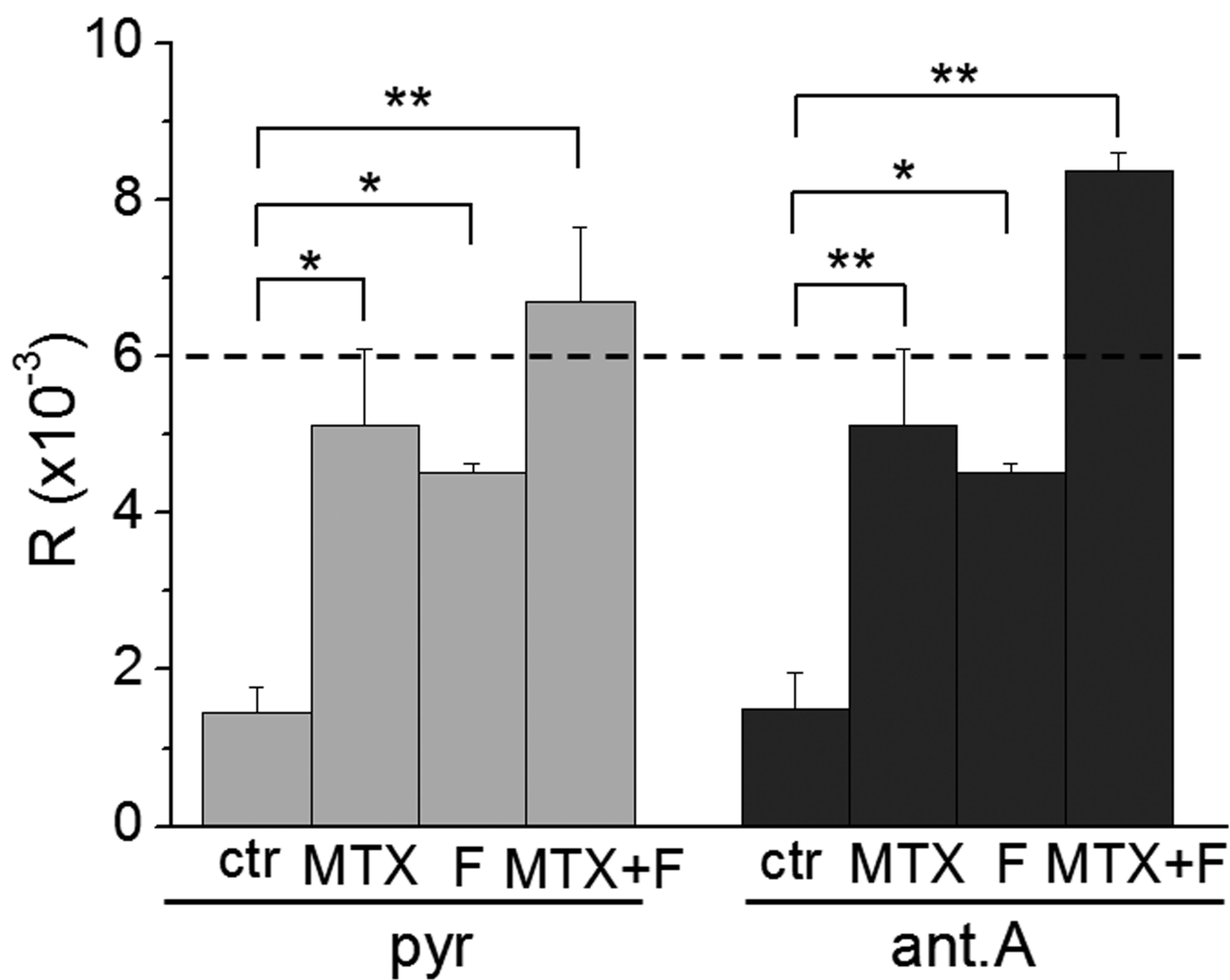


Figure 3

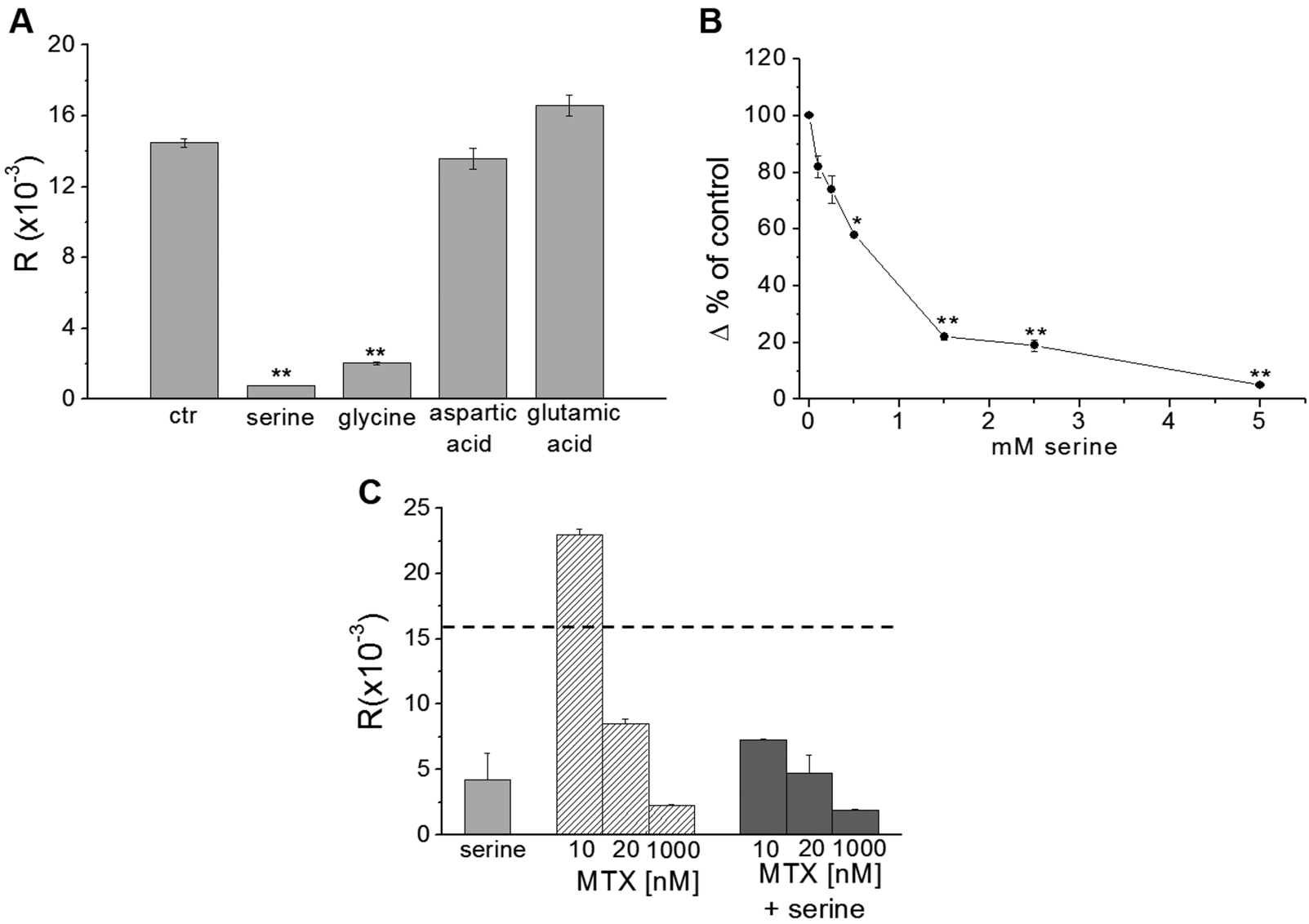


Figure 4

