Regulations of expressions of rat/human sulfotransferases by anticancer drug, nolatrexed, and micronutrients

Smarajit Maiti[a,b], Sangita MaitiDutta[c] and Guangping Chen[d]

Cancer is related to the cellular proliferative state. Increase in cell-cycle regulatory function augments cellular folate pool. This pathway is therapeutically targeted. A number of drugs influences this metabolism, that is, folic acid, folinic acid, nolatrexed, and methotrexate. Our previous study showed methotrexate influences on rat/human sulfotransferases. Present study explains the effect of nolatrexed (widely used in different cancers) and some micronutrients on the expressions of rat/human sulfotransferases. Female Sprague-Dawley rats were treated with nolatrexed (01–100 mg/kg) and rats of both sexes were treated to folic acid (100, 200, or 400 mg/kg) for 2-weeks and their aryl sulfotransferase-IV (AST-IV; β-napthol sulfation) and sulfotransferase (STa; DHEA sulfation) activities, protein expression (western blot) and mRNA expression (RT-PCR) were tested. In human-cultured hepatocarcinoma (HepG2) cells nolatrexed (1 nM–1.2 mM) or folinic acid (10 nM–10 μM) were applied for 10 days. Folic acid (0–10 μM) was treated to HepG2 cells. PPST (phenol catalyzing), MPST (dopamine and monoamine), DHEAST (dehydroepiandrosterone and DHEA), and EST (estradiol sulfating) protein expressions (western-blot) were tested in HepG2 cells. Present results suggest that nolatrexed significantly increased sulfotransferases expressions in rat (protein, STa, $F=4.87$, $P<0.05$/ mRNA, AST-IV, $F=6.702$, $P<0.014$; Student's *t* test, $P<0.01$–0.05) and HepG2 cells. Folic acid increased sulfotransferases activity/protein in gender-dependant manner. Both folic and folinic acid increased several human sulfotransferases isoforms with varied level of significance (least or no increase at highest dose) in HepG2 cells pointing its dose-dependent multiphasic responses. The clinical importance of this study may be furthered in the verification of sulfation metabolism of several exogenous/endogenous molecules, drug–drug interaction and their influences on cancer pathophysiological processes. Further studies are necessary. *Anti-Cancer Drugs* 33: e525–e533

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Introduction

Phase-II drug metabolizing enzymes are important for their role in the modification of endogenous and exogenous compounds. Sulfotransferases are one group of enzymes that catalyzes sulfation-mediated polarization of drugs that increase solubility hence, the circulation rate and bioavailability of that compound [1–3]. At the same time, excretion of that compound is also increased. During this process, biotransformation and bioactivation of some drug may occur. These steps may generate carcinogenic potentials in those drugs. Through the phase-II metabolic pathway, a drug may transform from pro- to proximate and then to ultimate carcinogen [4]. The process of tumorigenesis and carcinogenesis are driven by some dysregulation steps at cellular and metabolic levels. The best example is estradiol (E_2) [4,5]. Reports reveal that postmenopausal women may develop tumor due to the higher level of E_2 with low level of estrogen sulfotransferase (EST; SULT1E1) expression [6,7]. Low SULT1E1 fails to catalyze E_2–E_2S (estrogen sulfate), so high E2 participates in cellular malfunctioning. Some bioamines or polyphenolic compounds are also associated with sulfation–desulfation-related carcinogenic manifestation [6]. Drugs used in cancer therapy are of great interest to study their potentials in the expression of sulfotransferases. And whether the sulfation metabolism of these drugs or their potentiality for sulfotransferases induction has some influence on cancer pathogenesis may be a subject of concern. Reports from our and some other laboratories revealed that tamoxifen; an antiestrogenic drug could regulate sulfotransferases expression. Further, alterations of sulfotransferases expressions by therapeutic materials/prescription drugs may also influence the sulfation of important endogenous molecules. A large number of prescription drugs are used in cancer therapeutics. And sometimes these drugs are applied for years to decades. Long-term consumption of these drugs may have effects on sulfation metabolism

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of endogenous molecules. So, studies on cancer drugs or vitamins or micronutrients (those have direct cell cycle regulatory potentials) are important. Our previous studies showed that cancer drug tamoxifen and methotrexate could alter sulfotransferases expression [1,8]. Metabolism of 4OH-tamoxifen by sulfation has also been demonstrated. Some other report showed that cancer drug may alter sulfotransferases expression. Anticancer and apoptosis-inducing drug rhein and emodin may also alter sulfotransferases expression [9].

In this background, our present study is intended to elucidate the role of folic acid, folinic acid, nolatrexed on the expressions of human and rat sulfotransferases. This study will help to reveal the role of prescription drugs on sulfotransferases expression. Moreover, how the alterations of sulfotransferases expression may influence the sulfation metabolism of endogenous and exogenous drugs that can be elucidated. The physiological and pathological consequences sulfation metabolism and drug–drug interactions may also be explored.

Materials and methods

β-Naphthol, [14C]b-naphthol (4.7 mCi/mmol), ρ-nitro-phenyl sulfate (PNPS), 3′-phosphoadenosine-5′-phosphosulfate (PAPS), and [1,2,6,7-3H(N)] dehydroepiandrosterone ([3H]DHEA, 60 Ci/mmol) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). SDS-PAGE reagents were obtained from Bio-Rad (Hercules, California, USA). Western blot chemiluminescence reagent kits (Super Signal West Pico Stable Peroxide and Super Signal West Pico Luminol/Enhancer solutions) were purchased from Pierce Chemical (Rockford, Illinois, USA). Nitrocellulose membrane (Immobilon-P; Millipore Corporation, Bedford, Massachusetts, USA) used during western blot procedure was purchased from Fisher Scientific (Fair Lawn, New Jersey, USA). Total RNA extraction kit (RNeasy mini protection kit) was supplied by Qiagen (Valenica, California, USA). Antibodies against aryl sulfotransferase-IV (AST)-IV [10] and hydroxysteroid sulfotransferase (STa) [11] were provided by Dr. Michael W. Duffel (Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, Iowa, USA). Protein assay reagent was purchased from Bio-Rad. All other reagents and chemicals were of the highest analytical grade available.

Study design

In the current study, female rats were used to test the dose-dependent nolatrexed effects on hepatic sulfotransferases expression. Rats of either sex were used to test the dose-dependent folic acid effects on hepatic sulfotransferases expression. Nolatrexed, folic acid, and folinic acid were used in dose-dependent manner to test their effects on the expressions on several sulfotransferase isoforms in human cultured hepatocarcinoma cell lines (HepG2). In all cases, protein expressions were studied by western blot technique. In nolatrexed experiment with rat, sulfotransferases mRNA expressions and enzymes activities were tested. Densitometry analyses were conducted for all blot and mRNA data.

Experimental procedure

Animals and drug treatment

Sprague-Dawley rats of both gender (Harlan, Indianapolis, Indiana, USA) 10-week-old to 11-week-old and 200–300 g body weight were used in this investigation.

For all animal experiments, proper permissions were obtained from the concerned Institutional (Department of Physiological Sciences, Oklahoma State University, USA and Oriental Institute of Science and Technology, India) Internal Review Board. We followed all ethical norms and maintained requisite regulatory affairs. All the procedures were done in accordance with the Helsinki Declaration (2000) and the National Institutes of Health guidelines. This is to confirm that all experimental protocols were approved by the institutional ethical committee. Rats were housed in a temperature and humidity-controlled room and supplied with rodent chow and water for at least 1 week before use. Rats were divided into four groups with three in each. The nolatrexed was suspended in corn oil by gavages with 1, 10, 100 mg/kg/day for 2 weeks to three separate groups of female rats. Control group received only the corn oil. Folic acid was administered by gavages with 100, 200, or 400 mg/kg/day for 2 weeks to three separate groups of both male and female rats. The animals were sacrificed 24 h after the final drug treatment. Livers were collected, washed with sterile, ice-cold NaCl (0.9%, w/v) solution, and kept in dry ice bath. Samples were stored at –80°C until use.

Cell culture and drug treatment

HepG2 cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA). The cells were grown and maintained in Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham (Sigma-Aldrich) supplemented with L-glutamine and 15 mM HEPES, and 10% fetal bovine serum (FBS, Sigma-Aldrich). The cultures were incubated at 37°C in a humidified incubator containing 5% CO_2, 95% air [9]. After seeding at 0 days, on day 1, folic acid (FA) (0.01, 0.1, 1, and 10 μM final) was added to the medium in properly marked plates. A group of plates were added with folinic acid (1,10,100, and 1 μM) and a suitable control was made. Similarly, for the nolatrexed experiment, nolatrexed was added (1 ηM–1.2 mM) to the properly marked plates with HepG2 cells. Control plates are added with the vehicle. The medium was refreshed every 3 days with the new addition of corresponding drug. On day 10, the cells were harvested. Cytosols are prepared from the cells by suitable methods as explained [9].

Cytosolic sample preparation

Liver homogenates were prepared with 50 mM Tris buffer containing 0.25 M sucrose, pH 7.5. Homogenates were centrifuged at 100 000 g for 1 h at 4°C cytosol aliquots were collected and preserved at 80°C for enzymatic assay and western blot.

Sulfotransferases assay

Two different enzyme assay methods were used.

ρ-Nitro-phenyl sulfate assay method

The β-naphthol sulfation activity was determined from the liver cytosols as previously described [10–12]. This assay determines phenol sulfation activities of different isoforms of phenol sulfating sulfotransferases. Briefly, sulfation activity was determined in a reaction mixture containing 50 mM Tris buffer, pH 6.2, 5 mM PNPS, 20 µM PAPS, and 0.1 mM β-naphthol. Rat liver cytosols (50 µg protein) were used as the enzyme source in a total reaction volume of 250 µL. After 30 min incubation at 37°C in a shaking water bath, the reaction was stopped by adding 250 µL of 0.25 M Tris, pH 8.7. The reaction mixtures were read at 401 nm in a spectrophotometer. Specific activity was expressed as nmole/min/mg of protein. The data shown in the figures are the average of three independent datasets collected from three different animals.

Radioactive assay method

The β-naphthol sulfation activity in HepG2 cell cytosols and DHEA sulfation activities in liver cytosol was determined by the radioactive assay method as described previously [10]. The ingredients and reaction conditions were same as the PNPS assay method mentioned above. For rat liver β-naphthol sulfation activity, [14C]β-naphthol (4.7 mCi/mmol; 0.1 mM final concentration) was used as the substrate. To determine DHEA sulfation activity in liver cytosol, [3H] DHEA (diluted to 0.4 Cµ/mmol; 2 µM final concentration) was used as the substrate. For all the assays, 20 µM PAPS was used. Liver cytosol protein (50 µg) was used as enzyme source in a total reaction volume of 250 µL. After 30 min incubation at 37°C in a shaking water bath, the reaction was stopped by adding 250 µL of 0.25 M Tris, pH 8.7. Extraction of the final reaction mixture was performed twice by the addition of 0.5 mL of water-saturated chloroform. After the final extraction, 100 µL of aqueous phase was used for scintillation counting. The data shown in the figures are the average of three independent datasets collected from three different animals. PAPS was eliminated from the controls of both assay methods. Assays were run in duplicate and the average of the results was used for enzyme activity calculations.

Western blot analysis for the detection of sulfotransferases

Cytosol protein from rat liver (10 µg) was used in a 10% PAGE in an electrophoresis system (Novex, San Diego, California, USA) to run the western blot experiment. After running the gel at 200 V, the protein bands were transferred overnight at 40 V onto a nitrocellulose membrane. For rat liver cytosols, membranes were incubated with either rabbit anti-rat AST-IV or rabbit anti-rat STa (1:5000) in TBST [50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% (v/v) Tween 20] containing 5% (w/v) dried milk for 2 h on a shaker at room temperature. For HepG2 cell cytosols, membranes were incubated with rabbit anti-hPPST or hMPST or hDHEAST or hEST (1:5000 to 1:2000) antibodies. After incubation, all membranes were washed with TBST for 4 × 15 min and incubated in the secondary antibody (horseradish peroxidase-conjugated Immuno-Pure goat anti-rabbit IgG; H+ L) at 1:5000 dilutions in the same buffer for 2 h. The membranes were washed with TBST for 4–15 min and then with PBS 3 × 5 min. The chemiluminescent bands were developed with 1 mL of substrate containing the same volume of each Super Signal West Pico Luminol Enhancer solution and Super Signal West Pico Stable Peroxidase solution at room temperature for 5 min. The X-ray films were exposed to the membrane and then developed. Films were scanned and the densitometry analysis was performed in a Gel Documentation and Analysis System from Advanced American Biotechnology and with AAB software (Fullerton, California, USA).

Extraction of total RNA and RT-PCR for sulfotransferases mRNA expression study

Total RNA was extracted from rat liver using RNeasy mini protection kit from Qiagen according to supplier's guidelines. The concentration and purity of the extracted RNA were checked spectrophotometrically by measuring 260/280 absorption ratios. The primer pair for AST-IV was designed in our laboratory using the Gene Fisher primer designing and Multalin alignment software. Using the forward primer 5′-GTGTCCTATGGGTCGTGGTA-3′ and reverse primer 5′-TTCTGGGCTACAGTGAAGGTA-3′ (GenBank Accession No.: X52883), the 299-bp AST-IV cDNA was synthesized [12]. The 264-bp STa cDNA was synthesized using the primer pair forward primer 5′-TCCTCAAAGGATATGTTCCG-3′ and reverse primer 5′-CAGTTCCTTCTCCATGAGAT-3′ (GenBank Accession No.: M33329) [12]. The nucleotide sequences X52883 and M33329 (GenBank Accession No.) were used as the reference sequence for the synthesis of AST-IV and STa cDNA, respectively. For control, a 500-bp cDNA of rat β-actin was synthesized from the same amount of RNA. The primer pair (forward primer 5′-GATGTACGTAGCCATCCA-3′ and reverse primer 5′-GTGCCAACCAGACAGCA-3′) for the synthesis of rat β-actin cDNA was designed in our laboratory using the same software mentioned earlier [8].

The specificity of all primers was tested using the BLAST of the National Centre for Biotechnology Information Open Reading Frame software. The synthesis of cDNA from 1 µg of liver total RNA was performed in a 50-µL of

reaction mixture. The concentrations of different ingredients were used following supplier's protocol.

Statistical analysis

Student's *t*-test was performed to calculate the statistical significance of the difference s between means of control and drug-treated rats or cultured cells. Data presented in the figures denote means ± SEM of the results collected separately from five individual experiments. Multicomparison analysis of variance was performed to test the significances in the changes in the band densities of several sulfotransferases proteins and mRNA in response to drug treatment.

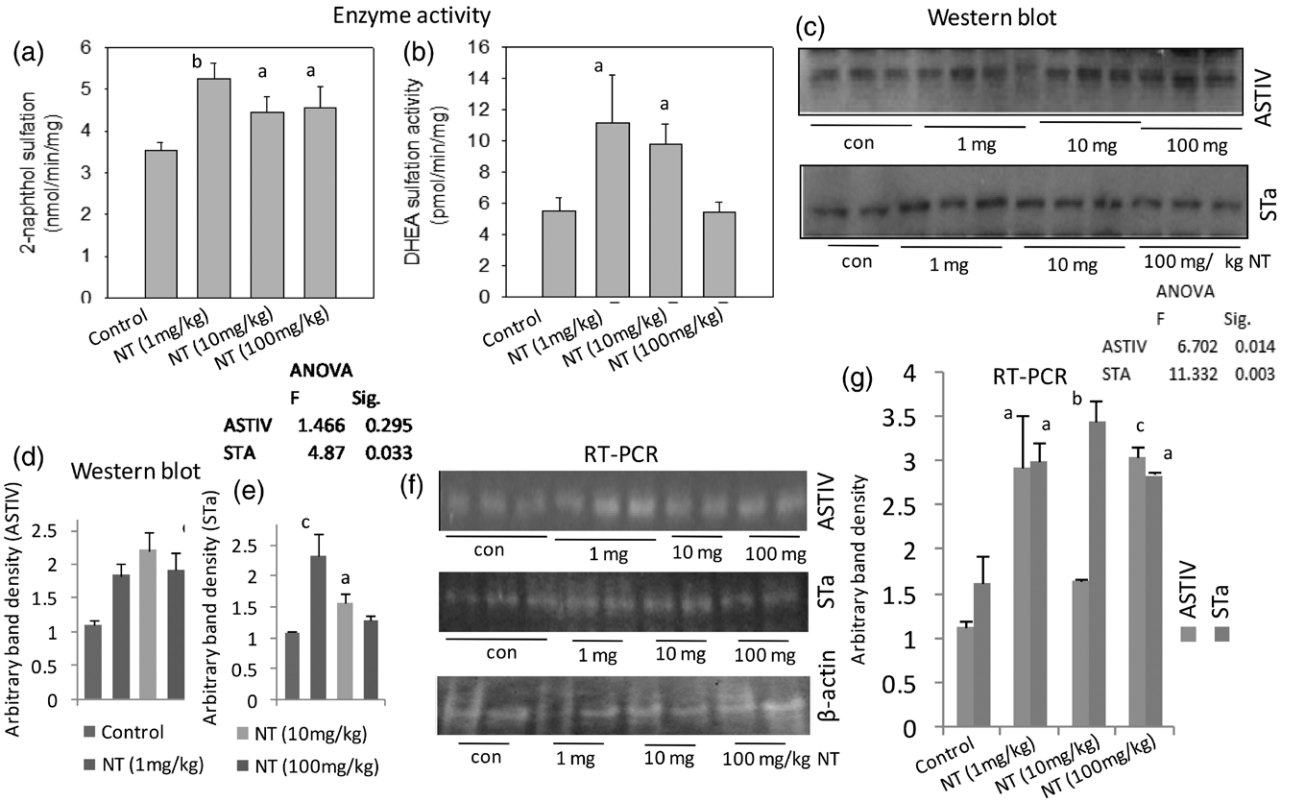
Results and discussion

Present results suggests that nolatrexed significantly increased β-napthol sulfation (SULT1A1 or AST-IV, $P<0.01$ and $P<0.05$) and DHEA sulfation (SULT2A1 or STa, $P<0.01$ and $P<0.05$) activities in rat liver with all drug doses except the highest dose in case of SULT2A1 activity (Fig. 1). The densitometry analysis data and their statistical study suggest that nolatrexed dose-dependent changes in protein and mRNA expressions of AST-IV and STa is significant. Initially, the protein expression increased but at higher doses (higher than the therapeutic dose) the expression decreased. Drug-induced multiphasic gene/protein expression has been reported [13]. Nonresponsive nature at higher dose is possibly due to the moderate antimetabolic effects of nolatrexed. Chemotherapeutic materials are usually strong agents which perform drastic action against cancerous cells. Nevertheless, these drugs exert adverse effects in normal cells also. The toxicity of antifolates is regarded to be sporadic in nature [14]. In the current situation, possibly enzymes became non/less responsive due to some modulation in the internal cytosolic environment. The drug nolatrexed has been widely used as an anticancer drug in last decade against a number of cancers. Here, we report for the first time that nolatrexed can induce sulfotransferases expressions and alter their activities in human and rat cells/tissues. This work has notable importance in relation to the pathological and pharmacological sciences. Antimetabolites are active chemotherapeutic agents for many solid tumor and hematologic malignancies [14]. Folate antagonists, purine analogs, and pyrimidine analogs are the three main categories of antimetabolites [15]. Methotrexate, the most studied folate antagonist (inhibitor of dihydrofolate reductase), is effective in many malignancies [16]. However, resistance to methotrexate develops by decreased folate carrier-mediated membrane transport. Our previous study suggested that methotrexate can induce different sulfotransferases in rat liver [1]. Supplementation with folic acid and vitamin B12 has been shown to reduce the toxicity of some antifolate anticancer drug-like pemetrexed [14]. Similarly, this is indicative that methotrexate-related sulfation metabolism and possible cytotoxic events may be counteracted by the folate supplementation in the presence of B12 for more effective nucleotide metabolism.

Regulation of expressions of phase-II enzymes especially, sulfotransferases by prescription drugs are important to learn the influences of different sulfation reactions in pathophysiological conditions. Nolatrexed dihydrochloride, a thymidylate synthase inhibitor was tested to determine the most tolerable dose for phase II studies in a number of patients [17], but its role on sulfation metabolism has not been verified earlier. In the current study, it has been shown that nolatrexed may induce rat and human sulfotransferases isoforms at protein and RNA level (Fig. 1). Two randomized trials (one in the USA and other in Europe) demonstrated a brief comparison between methotrexate and nolatrexed in patients with certain type of cancer. This study demonstrated similar pattern of response, prognosis and overall survival period for both the drugs [18]. In our previous studies, we have clearly demonstrated changes in sulfotransferases expressions by methotrexate in rat tissues and in human cultured cells [1,11]. In some trial, nolatrexed was administered with as high as the dose of 725 mg/m^2/day to hepatocellular carcinoma patients without any toxicity symptom. The present experimental doses were far lower than the clinical doses. Neither the current dose (data not shown) nor the clinical doses developed any cumulative toxicity [19]. No systemic investigation has been shown nolatrexed effect on sulfotransferases expression. Both the drugs nolatrexed and methotrexate have been shown to be associated to folic acid and folinic acid with their structural/functional analogy (Fig. *2*).

Xenobiotic induction of sulfotransferases is not well known. Our enzyme assay, western blot, RT-PCR results demonstrated that protein and mRNA expressions of AST-IV and STa were induced in liver of male and female rats following methotrexate [1]. Further, we report that folic acid treatment inhibited methotrexate induction of AST-IV in female rat and STa in male rat. This is important for the understanding of the clinical mechanisms of methotrexate [10]. In the current study, in addition to the sulfotransferase induction in rat by nolatrexed, this drug inconsistently induced DHEAST in human HepG2 cell. But the same treatment moderately inhibited the EST expression, but this needs further verification with varied dose and duration studies. The multiphasic action of nolatrexed is noticed at a low (ηM) to high (mM) doses in the current study (Fig. 3). This should be taken into account at the time of clinical use of this drug for a longer period. Sex dimorphic increase of sulfotransferases expressions are noticed in rat liver in response to folic acid treatment. The basal level of AST-IV and STa are found to be lower in female and male rats, respectively (Fig. 3), but the drug-induced increase in these enzymes are found to be in reverse order ($P<05$ to $P<0.001$). This trend also has been observed in our previous drug-induction paper like methotrexate and tamoxifen [1,8]. It can be suggested

Fig. 1



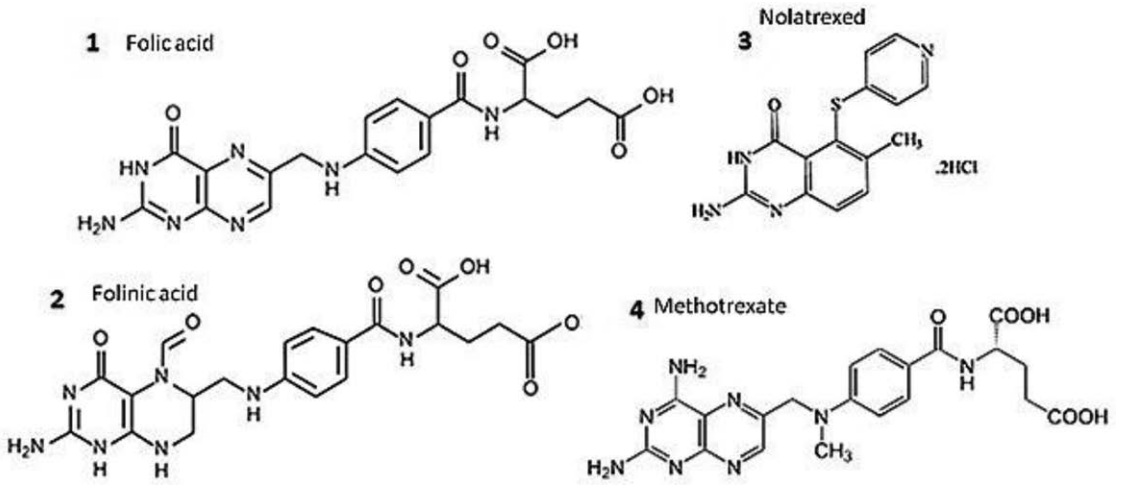
Studies of femae rat experimental model demonstrate AST-IV and STa activity and in liver tissue increased by the nolatrexed treatment for 2 weeks (a, b). AST-IV and STa protein expressions results from Western blot analysis are presented (c) and their densitometry analysis are drawn as bar diagram (d, e). Western blot study on this protein expression and their densitometry analysis study basically agree with the enzymatic activity data. Lane distribution: AST-IV: 1–3=control, 4–6=nolatrexed 1 mg/kg, 7–9=10 mg/kg, 10–13=100 mg/kg. STa: 1–2=control, 3–5=nolatrexed 1 mg/kg, 6–8=10 mg/kg, 9–11=100 mg/kg. RT-PCR data of AST-IV and STa are presented in Fig. 1e. Though there are some inconsistencies this basically supports the western blot results. Lane distribution: AST-IV: 1–3=control, 4–6=nolatrexed 1 mg/kg, 7,8=10 mg/kg, 9,10=100 mg/kg. STa: 1–2=control, 3–5=nolatrexed 1 mg/kg, 6,7=10 mg/kg, 8,9=100 mg/kg. Results in bar diagram represent mean ± SE of five independent sample/experiment. Level of significance is represented [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$. AST-IV, aryl sulfotransferase-IV; EST, estrogen sulfotransferase; HepG2, human cultured hepatocarcinoma cell lines; DHEAST, dehydroepiandrosterone; STa, sulfotransferase.

that either gene is expressible in both male and female. It is also hypothesized that in normal physiological condition, AST-IV is high in male and STa is high in female suggesting their specific metabolism pattern in male and female. But in some specialized condition, exogenous/endogenous inducer or drug-dependant AST-IV induction in female and STa induction in male may be evident as adaptive mechanism. This sex-dimorphic gene expressions and regulations may have some implications in adaptive drug metabolism process and in different pathological condition [20]. Folic acid was noticed to be inducing the phenol catalyzing sulfotransferase (PPST), monoamine catalyzing sulfotransferase (MPST), and EST expressions in human carcinoma HepG2 cells. This suggests that FA or its analog or related drugs have similar pattern on influence on sulfotransferases expressions and activities. The overall changes on sulfotransferases activities might have some influence on the sulfation metabolism of several biomolecules and endobiotic or xenobiotic.

In the current study, methotrexate and tamoxifen have not been tested, but several laboratory published reports on this issue. In brief, it has been shown that methotrexate can induce EST in human HepG 2 cells but not in Caco-2 cells [21]. Methotrexate has also been shown to induce other steroid metabolizing enzyme like DHEAST [21]. Report reveals that tamoxifen can induce EST in in-vivo and in-vitro experimental condition [22]. From the results of the current study, it is suggested that both folic acid and folinic acid can induce EST in human HepG2 cells and in case of nolatrexed, EST expression showed multiphasic pattern, initially decreased but at higher doses it restored up to the control but at very high dose the expression decreased (Fig. 4). Drug-induced multiphasic gene/protein expression has been reported [13]. Further explorations are required in this regard.

Not only for adult cancers, had the use of nolatrexed in cancer of children reported to be well tolerated. The therapeutic activity is correlated to its antiproliferative toxicity

Fig. 2



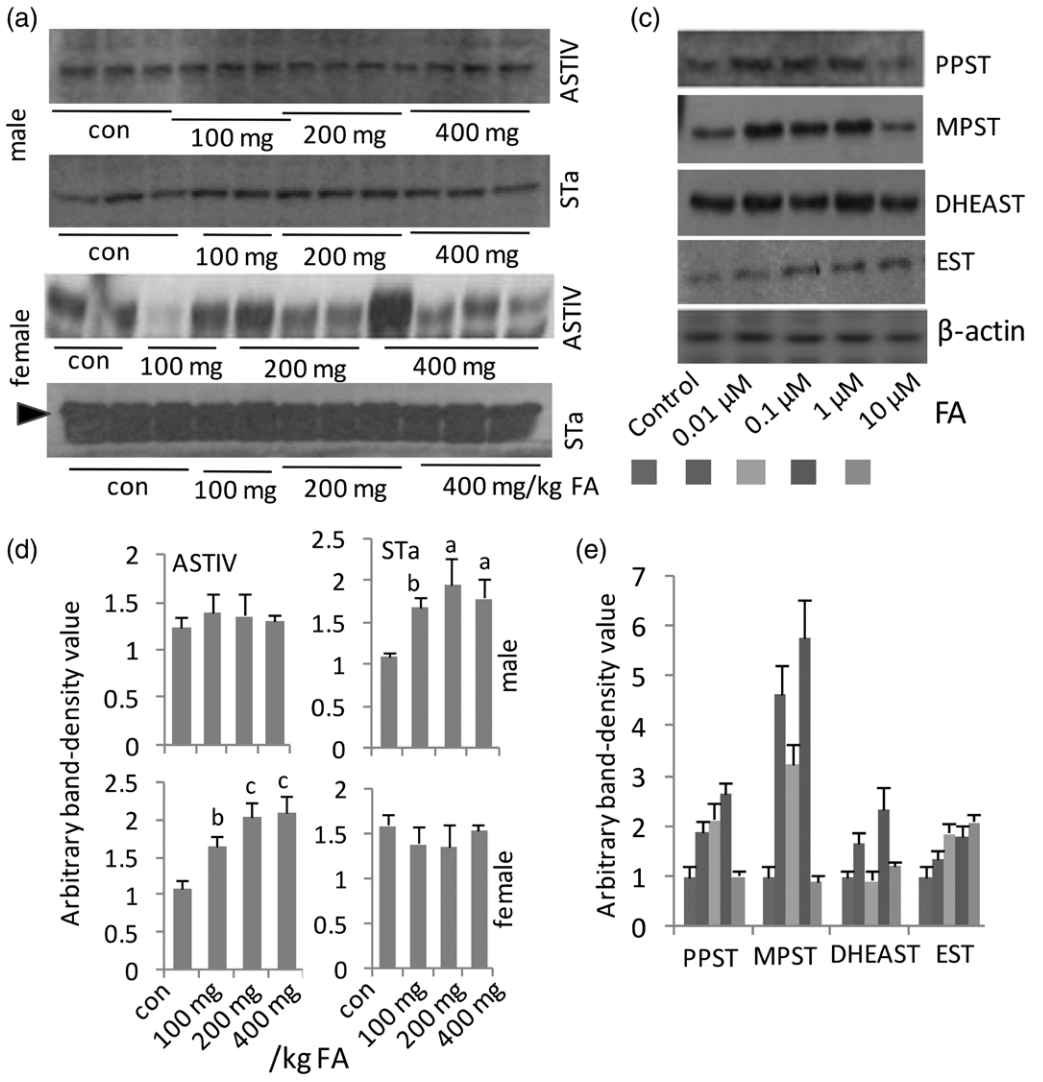
Chemical structures of anticancer drugs and micronutrient, folic acid.

[23]. For metastatic hepatocellular carcinoma, treated with nolatrexed or doxorubicin showed minimal activity in this phase III trial [24]. The pathophysiological outcome of nolatrexed in relation to different cancer has not been enlightened with its role on phase I and phase II drug-metabolizing enzymes. We want to draw the attention that what is explained as 'safe use' may be extrapolated as the most effective and optimized use of this drug. And for this sulfation metabolism, study by nolatrexed is important.

With reference to our previous discussion, it is notable that cancer chemotherapy is the application of multiple drug regimens where drug–drug interaction is possible. And this interaction may influence the therapeutic mechanism of that drug. As for example, cantharidin and nolatrexed were used to inhibit PP and thymidylate synthase activity, respectively. Synergistic manner of response of these drugs are more effective than only nolatrexed use [25]. Further studies are necessary to explore whether this effect or possible toxicities are imposed by drug-induced sulfation-metabolism or not. Similarly pharmacokinetics of nolatrexed is more therapeutically favorable when it is administered sequentially with paclitaxel [26]. These schedules of uses necessitate the study of its impact on sulfotransferases expressions to evaluate the possible sulfation metabolism of other drugs and further possible events of drug–drug interactions. Metabolic reprogramming of tumor cells toward serine catabolism is now recognized as a hallmark of cancer [27]. These observations provide insights into the mechanism of action of antifolate drugs and that may help to more rational drug designing [28].

Though methotrexate has not been investigated in this study, a comparative analysis with the nolatrexed effects and some previous reports may help in the better understanding on the current issue. Earlier report suggests that four isoforms of human sulfotransferases were variably induced by methotrexate in human HepG2 cells but only MPST and DHEAST were induced in Caco-2 cells. The inductions of these enzymatic activities were shown to be associated with their corresponding protein and mRNA expressions [21]. Further, high dose of folic acid was found to inhibit these inductions which suggest the possible role of folate receptor in the methotrexate induction mechanism [21]. Continuation of that study suggests that constitutive androstane receptor (hCAR) and vitamin D receptor (hVDR) are involved in methotrexate induction of DHEAST [(human dehydroepiandrosteronesulfotransferase (hSULT2A1)] in both HepG2 and Caco-2 cells [29]. The 5′-flanking region of hSULT2A1 in the pGL3-Basic luciferase reporter vector assay suggests that hCAR and human retinoid X receptor alpha (hRXRalpha) are involved in the transcriptional upregulation of hSULT2A1 [30]. With comparison to our current study, it can be summarized that both the anticancer drugs methotrexate and nolatrexed can induce human and rat sulfotransferase isoforms. But the degree or extent of inductions may not be ideally compared because the drugs were used with different dose, duration and with other variable conditions. Interestingly, the anticancer drug tamoxifen which is not an anti-folate was also shown to induce rat phenol-catalyzing AST-IV in female and steroid-catalyzing STa in male [8], the same pattern that is observed in case of methotrexate and nolatrexed studies. This may suggests that sulfotransferases inductions by these drugs may be partially if not completely influenced by the folate regulations. Further studies are required to gain a better understanding of the relevance of certain sulfotransferases' expression in cancer pathogenesis.

Fig. 3

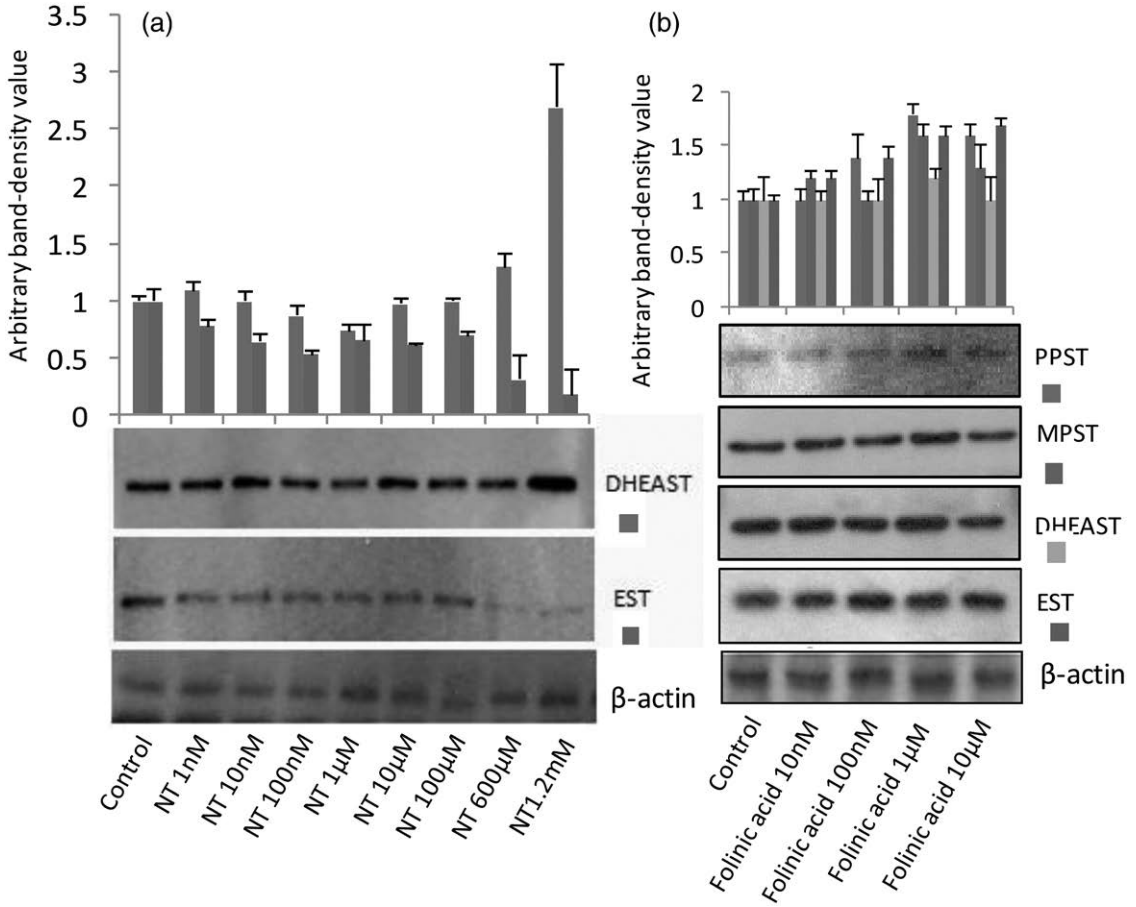


Sex dimorphic protein expressions (AST-IV and STa) were noticed after folic acid exposure to experimental rat. Folic acid – dose (100, 200, or 400 mg/kg/day) dependent increase in STa in male and AST-IV in female rat liver were noticed (a). Treatment of folic acid to Hep-G2 cells suggests consistent increases of PPST, MPST, and gradual increase in EST expression. At highest 10 μM of folic acid-dose PPST, MPST, and DHEAST did not respond significantly (b). Results in bar diagram represent densitometry data of AST-IV and STa (c). Normalized densitometry values of the sulfotransferases protein bands are plotted in the figure (d). Bar diagrams represent mean ± SE of five independent experiments. Level of significance is represented $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$. AST-IV, aryl sulfotransferase-IV; EST, estrogen sulfotransferase; DHEAST, dehydroepiandrosterone; FA, folic acid; MPST, monoamine catalyzing sulfotransferase; STa, sulfotransferase.

Drug resistance is the main barrier to more effective treatment of cancers with antifolates; therefore, mechanisms of antifolate resistance are the topic of worth for investigation [31]. As because, thymidylate synthase is a nucleotide and cell cycle regulator so thymidylate synthase-targeted chemotherapy may impact on pharmacogenetics which is considered in selection of 5-fluorouracil therapy for colorectal cancer [32]. Similarly pharmacogenomic influence by nolatrexed also should be verified. Both folate and methotrexate transport were inhibited by classical antifolates but not by nonclassical antifolates or biopterin [33]. Not only the drug-metabolizing enzyme genes but also the role of tumor suppressor genes (i.e. p53) has been important to alter the sensitivity to thymidylate synthase inhibitors which accounts for the outcome of chemotherapy [34]. In further search on the mechanism of methotrexate inductions of sulfotransferases expression our previous studies explained in involvement of several nuclear receptors like CAR and PXR [29,30]. And these and other report justifies molecular impact of antifolate drugs is occurring at the transcriptional signaling level.

Tissue specific activation of sulfotransferase expression may help in treating hormone-dependent breast or

Fig. 4



Dose responsive increases of DHEAST in HepG2 cells to very low level of nolatrexed. At highest 1.2 mM nolatrexed concentration, two and half fold increase of DHEAST was noticed. Gradual decrease of EST was noticed in these cells after same dose of nolatrexed application (a). Folinic acid increased PPST, DHEAST and EST in dose responsive manner. The fold of increase was found to be less at the highest concentration of folinic acid. HepG2, human cultured hepatocarcinoma cell lines; EST, estrogen sulfotransferase; DHEAST, dehydroepiandrosterone; EST, estrogen sulfotransferase.

endometrial cancer. Estrogen regulating enzymes sulfatases STS and EST are shown to be involved in the metabolism of estrogens in human prostate cancers. So, drug-induced modification of estrogen level may be beneficial in prostate cancer patients. Association between cancer therapeutic drugs and sulfotransferases regulations has been studied scanty in number. Report suggests that SULT1A1-mediated biotransformation of 4-OHT is crucial for its effects in breast tumors. The potential role for 4-OHT sulfated product in tamoxifen therapy has been noted. The metabolism like 4-OHT sulfation has been demonstrated earlier [35,36].

In parity with our previous finding of the role of VDR in methotrexate induction of human SULT2A1, report suggested the role of this receptor in the induction of SULT2B1 which upon inactivation promoted the proliferation of DHEA stimulated prostate cancer cells [37]. This suggests that abnormal steroid regulations may correlate human cancer and that steroid metabolizing sulfotransferases may restrict the disease. Whether, the pharmacogenomic role of chemotherapeutic drugs on sulfotransferases modulations could be their one of the mechanistic action needs further exploration. Single Nucleotide Polymorphism SNPs are found in several sulfotransferases (mainly in SULT1A1) which vary by ethnicity. This factor is a strong determinant of the pharmacogenetics of sulfotransferase-associated cancer risk. And some of these SNPs are associated with higher risk of breast carcinogenesis [4,38].

In conclusion, here for the first time we demonstrate the role of nolatrexed on sulfotransferases expression and induction of their increased activities [39]. In our earlier studies sulfotransferases inductions by methotrexate and tamoxifen have been shown. All are anticancer drug. This induction may have some unidentified impact on sulfation metabolism of different molecules, drug–drug interaction with physiological and pathological consequences. Further studies are necessary in this regard.

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Conflict of interests

There are no conflicts of interest.

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