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Modulation of the cytotoxic mechanism of 6-thioguanine by 4-amino-5-imidazolecarboxamide

Jonathan Maybaum[1], Catherine W. Morgans[1], Paul Ting[1], and Clare E. Rogers[2]

Departments of [1]Pharmacology and [2] Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109-0504, USA

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Summary. Previous evidence has indicated that either purine starvation or incorporation into DNA may be the dominant biochemical effect of the antileukemic agent 6-thioguanine (TG), depending on exposure conditions. Furthermore, it has been suggested that the paradoxical decrease in TG-induced cytotoxicity at high drug concentrations may be due to an antagonistic interaction between these two mechanisms, in which purine starvation inhibits DNA synthesis and, therefore, incorporation of TG into DNA. In this report we test the hypothesis that by concurrent treatment of L1210 cells with TG and the purine precursor 4-amino-5-imidazolecarboxamide (AIC) it is possible to alleviate DNA synthesis inhibition caused by high concentrations of TG, thus enhancing TG incorporation into DNA and TG-induced cell kill. Both the cytotoxic and cytokinetic results presented support this hypothesis. However, gross incorporation of TG into DNA was not increased by AIC under conditions in which a significant enhancement of cytotoxicity (i.e., 1 log) was observed. These findings suggest that the potentiating effect of AIC may be most prominent on the subpopulation of cells that are resistant to treatment with TG alone, and they demonstrate that the cytotoxic effects of TG treatments are more accurately reflected by observing specific cytokinetic changes (delayed late S/G2 arrest) than by measuring the average extent of TG incorporation into DNA within a given population. Finally, we propose that it may be possible to select conditions for administration of TG that favor one or the other cytotoxic mechanism, depending on whether the clinical objective is induction of remission (where rapid cell lysis due to purine starvation would be desired) or eradication of subclinical disease during remission (where proliferation-dependent cytotoxicity due to DNA incorporation should be more effective).

Offprint requests to: J. Maybaum

Introduction

Like most antimetabolites, the thiopurines 6-thioguanine (TG) and 6-mercaptopurine (MP) can produce a number of biochemical disturbances that have the potential to kill cells. The most prominent of these are incorporation of the analog into DNA and inhibition of de novo purine biosynthesis [13], although incorporation into RNA can also disrupt cellular functions [1]. Although DNA incorporation has frequently been invoked as being responsible for thiopurine cytotoxicity in vitro, the dominant cytotoxic mechanism can vary, depending on a number of factors including the cell line and thiopurine used, as well as being concentration-dependent within a single cell line (summarized in [12]). Although it is presently unknown whether DNA incorporation or purine starvation is more important in producing cytotoxicity in human leukemic cells in vivo, it seems likely that DNA incorporation would more selectively target a leukemic cell population because it is dependent upon continued proliferation, whereas extended purine starvation would be toxic to both proliferating and nonproliferating cells. We have recently demonstrated that cytokinetic disturbances characteristic of either mechanism can be induced by TG in L1210 cells in vivo in a dose-dependent manner [12], suggesting that it may be possible to manipulate the predominant cytotoxic mechanism in vivo by altering the schedule of administration used.

In addition to using drug concentration as a means of favoring a particular cytotoxic mechanism, we also proposed that combined treatment with TG and a carefully selected concentration of 4-amino-5-imidazolecarboxamide (AIC) can decrease the relative importance of purine starvation and increase that of DNA incorporation, with a net increase in total cytotoxicity.

Although we have previously shown that nontoxic concentrations of AIC can indeed potentiate the toxicity of TG in CHO cells [9], we have not tested the specific hypothesis that this modulation is indeed due to a shift in cytotoxic mechanism. In the present report we address this hypothesis in experiments using L1210 cells in vitro.

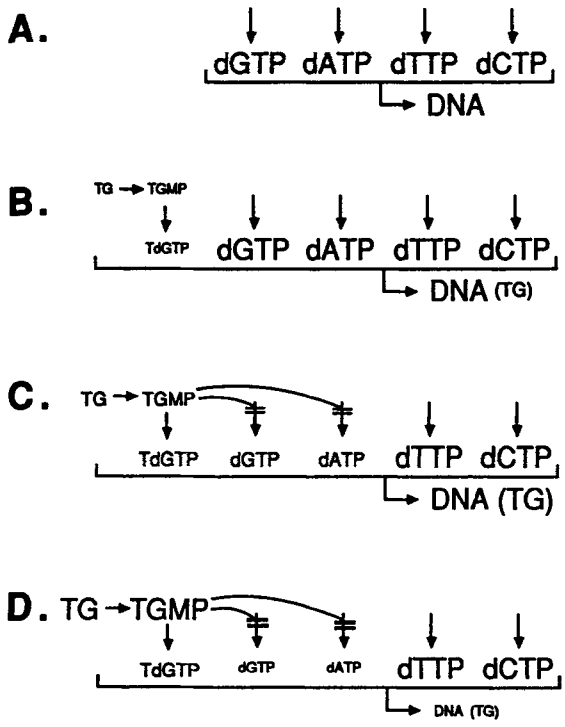

Fig. 1 A–D. Model for the interaction of biochemical effects due to TG treatment. **A** In the absence of TG, synthesis and utilization of DNA precursors proceeds normally. **B** At low TG concentrations, there is a modest amount of analog incorporation into DNA but little inhibition of purine nucleotide synthesis. **C** Moderate levels of TG result in more DNA incorporation and some purine synthesis inhibition. **D** High TG concentrations deplete dGTP and dATP to the point that overall DNA synthesis (and therefore analog incorporation) is inhibited

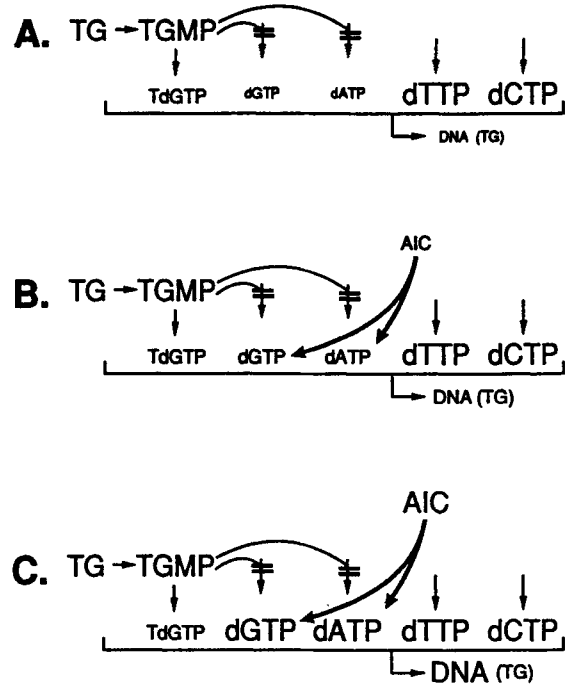

Fig. 2 A–C. Model for interaction of AIC with TG. **A** Picking up from the last frame of Fig. 1, high TG levels in the absence of AIC result in substantial inhibition of purine and DNA synthesis. **B** Addition of a moderate concentration of AIC partially repletes purine pools, thus enabling DNA synthesis to proceed. The ratio of thio-dGTP to dGTP remains sufficiently high to result in significant analog incorporation. **C** Very high levels of AIC produce so much dATP and dGTP that both purine synthesis inhibition and analog incorporation are antagonized

Materials and methods

L1210 cells were grown in RPMI 1640 medium supplemented with 10% horse serum at 37°C in a humidified incubator with an atmosphere containing 5% CO_2. All enzymes and reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise indicated. Stock drug solutions were prepared in 0.1 *N* NaOH at a concentration at least 1,000-fold higher than the final concentration in medium. Cytotoxicity of various treatments was assessed by exposure to drugs for 12 h, after which cells were washed twice with drug-free medium and then cloned in soft agar [12]. For cytokinetic measurements cells were also exposed to drugs for 12 h and washed twice with medium, at which time a portion of the population was fixed with ethanol, whereas the remaining cells were resuspended in fresh, drug-free medium and incubated for an additional 24 or 48 h before being fixed. Cells were then processed as before [12] by staining with propidium iodide/RNase and analyzed on a Coulter EPICS C flow cytometer.

Incorporation of TG into DNA was measured using a modification of earlier procedures [6, 16]. DNA was first isolated by lysis of cells with an SDS-TRIS buffer, followed by sequential treatment with proteinase K and RNase [11]. After ethanol precipitation the DNA was dried, dissolved in 10 m*M* TRIS (pH 8.0) and 10 m*M* $MgCl_2$, and hydrolyzed to nucleosides by treatment with 0.5 mg DNase I, 1 mg snake-venom phosphodiesterase, and 18 units of alkaline phosphatase in a total volume of 250 µl at 37°C for 1 h. The reaction was stopped by extraction with chloroform and each sample was then split into two equal portions.

The first portion was analyzed for thiodeoxyguanosine (TdGuo) by quantitative oxidation with $KMnO_4$ (to produce the fluorescent 6-sulfonate derivative [4]), followed by reverse-phase HPLC chromatography (Regis C-18 column, 25 cm × 4.6 mm) using an ion-pairing mobile phase (5 m*M* NaH_2PO_4, 5 m*M* tetrabutyl ammonium hydroxide, 8% MeOH, pH 7.0) with fluorescence detection (Perkin-Elmer LS-5 spectrofluorimeter, excitation at 330 nm, emission at 410 nm). The second portion of hydrolyzed DNA was analyzed for dAdo (as an index of total DNA content) by chromatography using a similar system, differing only in that the tetrabutyl ammonium hydroxide was omitted from the mobile phase and that UV absorbance at 254 nm was the mode of detection. In each case an external calibration curve was used for quantitation, and the extent of TG incorporation was calculated as pmol TdGuo/100 pmol dAdo.

Results and Discussion

Cytotoxicity

In a previous study we determined that in L1210 cells there is an unusual relationship between TG concentration and loss of clonogenicity, such that intermediate drug concentrations (~0.2 µ*M*) were more lethal than high drug concentrations (≥ 1 µ*M*) when a 12-h exposure period was used (Table 1 [12]). Flow cytometric data obtained in the same study showed that relatively rapid inhibition of progression through S-phase occurred at drug levels of ≥ 1 µ*M*, indicative (in this context) of purine starvation. At 0.2 µ*M* we observed no cytokinetic disturbances until 24–48 h after the drug was removed, at which time G2 arrest was seen. In earlier work we provided evidence that this delayed G2 arrest is a consequence of unilateral chro-

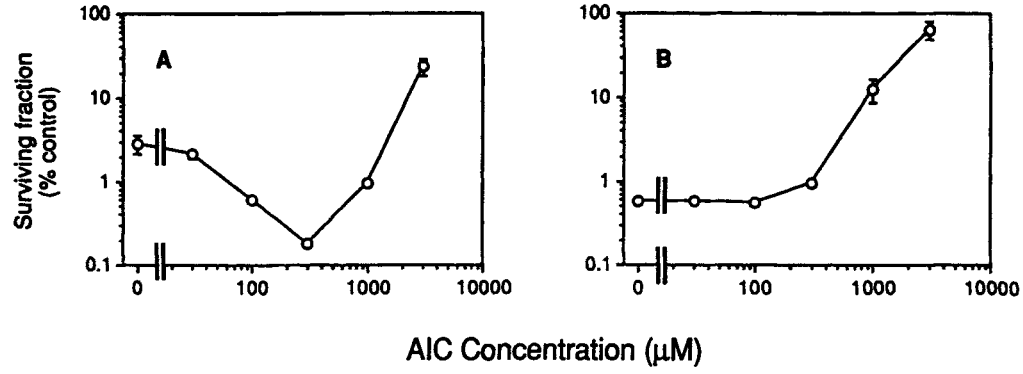

Fig. 3 A, B. Effects of AIC on TG cytotoxicity. L1210 cells were exposed for 12 h to a range of AIC concentrations plus either A 5 μ*M* TG or B 0.2 μ*M* TG, then washed and cloned in soft agar. Symbols denote the mean values (± SE) obtained from three separate experiments

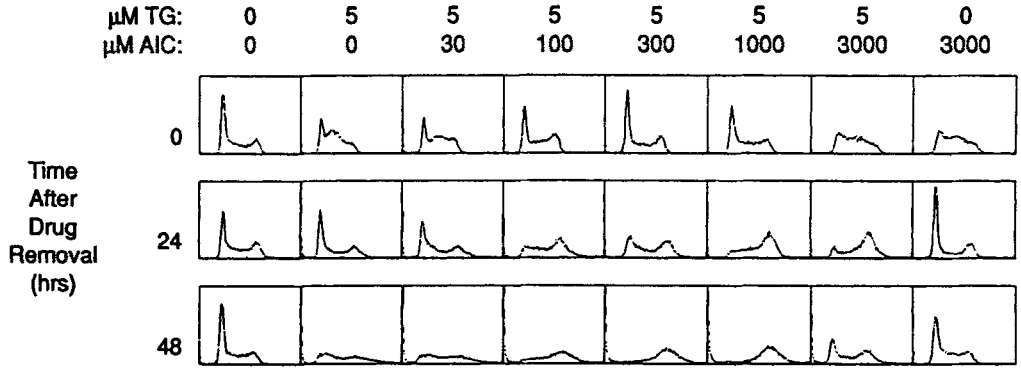

Fig. 4. Effects of TG/AIC combinations on cytokinetics of L1210 cells. Exponentially growing cells were exposed to the indicated drug combinations for 12 h, then washed free of drug. Samples were fixed in ethanol and stained with propidium iodide/RNase for single-parameter flow cytometric analysis of DNA content (cell cycle distribution)

matid damage resulting from TG incorporation into DNA [3, 7–9]. These results are therefore consistent with the hypothesis that although intermediate levels of TG killed L1210 cells as a result of analog incorporation into DNA, purine depletion at higher TG concentrations inhibited S-phase progression and TG incorporation into DNA, as illustrated in Fig. 1.

One prediction of this model is that alleviation of purine starvation, e.g., by addition of an exogenous source of purines, should increase the cytotoxicity of high TG concentrations by enabling a greater fraction of cells to traverse S-phase and thereby incorporate TG into their DNA, provided that the level of exogenous purine source is not so high as to produce excessive intracellular dGTP, which would then compete with thio-dGTP for incorporation (see Fig. 2). Furthermore, we would expect that with a TG concentration at which DNA incorporation is dominant and purine depletion is not a significant factor (in this system, 0.2 μ*M*), addition of exogenous purines should not enhance cytotoxicity. To test these predictions, L1210 cells were simultaneously exposed to either 5 μ*M* or 0.2 μ*M* TG plus a range of concentrations of AIC, which has previously been shown to circumvent purine synthesis inhibition induced by thiopurines [5, 14].

As illustrated in Fig. 3 A, the dose dependence of the effects of AIC on cytotoxicity induced by 5 μ*M* TG in L1210 cells was biphasic, generating a curve similar to that seen after treatment with a range of concentrations of TG alone [12]. Potentiation of cytotoxicity was significant when AIC concentrations from 100 to 1,000 μ*M* were used, with maximal effect observed at 300 μ*M* AIC. Under these conditions, the fraction of clonogenic cells was reduced by >1 log unit compared with the value resulting from treatment with 5 μ*M* TG only. The observation that potentiation of TG cytotoxicity reached a maximum at 300 μ*M* AIC, with protection of cells as the dose of AIC was further increased, suggests that these higher concentrations of AIC may raise intracellular levels of purine nucleotides to such a degree that they competitively inhibit the production or utilization of TG nucleotide metabolites. When the same range of AIC doses was combined with 0.2 μ*M* TG, no potentiation of cytotoxicity was detected. As in the previous case, AIC levels of 1,000 and 3,000 μ*M* protected cells against this concentration of drug. Exposure of cells to AIC as an individual agent at concentrations up to 3,000 μ*M* caused no significant loss of clonogenicity (data not shown). We conclude from these data that, with respect to cytotoxicity, the aforementioned model accurately predicted the effects of AIC on TG action in L1210 cells.

Cytokinetics

We also had specific expectations as to the consequences of AIC/TG combinations on cytokinetic profiles of L1210 cell populations. These predictions were tested by exposing cells for 12 h to TG/AIC combinations, washing out the agents, and then examining the cell cycle distributions of the treated populations at various times thereafter. As illustrated in Fig. 4, cells given 5 μ*M* TG alone experienced an S-phase arrest that was detected immediately at the end of drug treatment. At 24 h post-washout these cells had a distribution like that of a control population, whereas at 48 h post-washout a broad histogram was observed, indicative of arrest throughout the cell cycle. Addition of

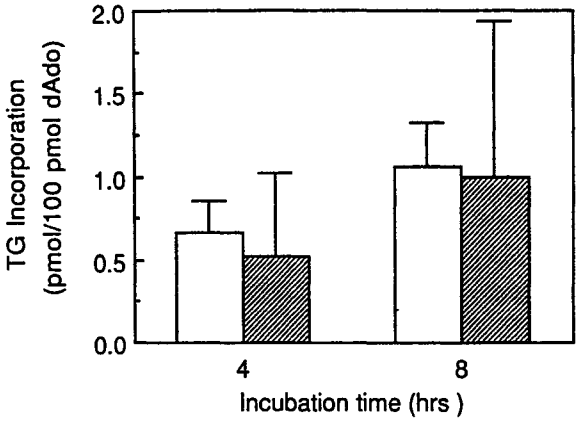

Fig. 5. Incorporation of TG into DNA. Exponentially growing L1210 cells were treated with 5 μ*M* TG with (□) or without (□) 300 μ*M* AIC for 4 or 8 h, at which time cells were lysed and their DNA was extracted. The extent of TG incorporation was determined by HPLC analysis of the nucleosides derived from these DNA samples by enzymatic hydrolysis. Data represent the mean (± SE) of four experiments

30 μ*M* AIC did not substantially alter these responses. Combination with 100 μ*M* AIC produced distinct changes at both the early time point, attenuating the appearance of a broad hump corresponding to cells arrested in mid-S-phase, as well as at later time points, when the G2 peak became prominent. Similar changes were seen at AIC concentrations of 300 and 1,000 μ*M*. The data at 3,000 μ*M* AIC were somewhat more difficult to interpret since this concentration of AIC caused cell cycle perturbations by itself. Exposure of cells to lower concentrations of AIC alone did not alter DNA histograms (not shown). These results are also compatible with the model, in that the range of AIC concentrations over which early S-phase arrest was prevented is the same as that in which delayed G2 arrest was most pronounced and in which cytotoxicity was enhanced over that seen with TG only.

DNA incorporation

Since the addition of 300 μ*M* AIC to a treatment with 5 μ*M* TG enhanced cytotoxicity and delayed G2 arrest, we initially predicted that such a combination should result in an increased extent of TG incorporation into DNA, compared with that obtained with 5 μ*M* TG only. As shown in Fig. 5, no significant change in TG incorporation was observed during the initial 8 h of drug exposure as a result of combining 300 μ*M* AIC with 5 μ*M* TG. Although this finding is in apparent contradiction to the model in Fig. 2, it is consistent with our previous result obtained using CHO cells [9].

A number of interpretations of these data are possible. The first interpretation we considered is that the model in Fig. 2 is incorrect and that the modulation of TG action by AIC proceeds through a mechanism other than DNA incorporation. Although this is certainly possible, adoption of this explanation would require that some other cytotoxic mechanism be invoked to account for the observed increase in cytotoxicity upon addition of AIC. This other

Table 1. Biphasic dose-response curve for loss of clonogenicity of L1210 cells treated with TG. Cells were exposed for 12 h, washed free of drug, then plated in soft agar. These data have been presented elsewhere in graphical form [12]

TG concentration (μ*M*)	Surviving fraction
0	1.00
0.008	0.951
0.04	0.148
0.2	0.0026
1.0	0.0156
5.0	0.0180
25.0	0.0283

mechanism could not be purine starvation, since AIC acts to replete purine pools. Another possibility is that TG incorporation into RNA might be the cause of enhanced cytotoxicity [1]. Although we did not measure RNA incorporation in the present study, our previous experiments showed that AIC significantly decreased the total pool of acid-soluble TG-containing species (TG nucleotides) that would be the precursors for RNA incorporation, making it unlikely that this pathway would be potentiated by AIC. More importantly, the delayed nature of the cell cycle arrest caused by TG and accentuated by AIC are not characteristic of an RNA-related lesion.

An alternative interpretation that would be consistent with all of the present data is that the applicability of the scheme in Fig. 2 varies among the members of the treated cell population. For example, in the absence of AIC, the maximal cell kill produced by a 12-h TG treatment corresponds to a reduction in cloning efficiency by about 99%, or 2 log units [12]. Although potentiation of this effect by addition of AIC is significant from a therapeutic standpoint (i.e., increased cell kill from 2 logs to 3 logs), one would not expect increased DNA incorporation in 1% of the population to have a detectable impact on the extent of incorporation measured as an average of the total cell population.

Still another possibility is that AIC could alter the pattern of TG incorporation into DNA. We have recently demonstrated that incorporated TG can disrupt sequence-specific DNA/protein interactions [10]. It is therefore possible that the biological impact of TG incorporation is dependent on the location and frequency with which such incorporation occurs. Although we have not explored the patterns of TG incorporation in cellular DNA, it has been reported that bromodeoxyuridine can be incorporated in a nonrandom manner [2, 15]. If TG incorporation is also nonrandom, one could speculate that AIC-induced changes in the distribution of TG in DNA could produce changes in biological effects without necessarily altering the total extent of analog incorporation.

Therapeutic implications

At present it is unknown whether purine starvation or DNA incorporation is the major cause of cell kill in humans

being treated with thiopurines. Our previous results using mice bearing L1210 leukemia cells provide cytokinetic evidence that the identity of the dominant mechanism may be concentration-dependent in vivo, as it was in vitro [12]. Because of the different properties of these two mechanisms, it might be desirable to accentuate one or the other, depending on the circumstances at hand. For example, induction of remission in a patient experiencing blast crisis calls for treatment that rapidly lowers the density of leukemic cells. In this case, purine starvation would be the mechanism of choice since it causes cell lysis with a much more rapid time course than that induced by DNA incorporation [9]. In contrast, long-term maintenance of remission would probably be better served by conditions favoring DNA incorporation, since this mechanism would be more likely to affect cells undergoing multiple rounds of replication (such as regenerating leukemic cells) and less likely to be toxic to mature, normal leukocytes. If the present results are representative of the response of human leukemic cells in vivo, this would suggest that manipulation of the cytotoxic mechanism of TG may be possible through coadministration of AIC.

Conclusion

Most of the present data support the idea that the combination of AIC with TG in L1210 cells acts to shift the relative significance of purine starvation and DNA incorporation as cytotoxic mechanisms, in favor of the latter. Although the gross extent of TG incorporation into DNA did not increase upon addition of AIC to the treatment regimen, the enhanced cytotoxic effect could be due to an effect on a relatively small subpopulation of cells (i.e., the 1% of cells surviving treatment with TG alone), which would not be detected against the background of the majority of the population.

We propose that a rational approach to the use of TG in treating human leukemias would be to use conditions favoring purine starvation (and therefore rapid cell lysis), such as short exposures to high TG concentrations, for induction of remission while using combinations of TG+AIC given by extended infusion (to favor DNA incorporation) for maintenance of remission.

Regardless of the validity of the present model, these data demonstrate that quantitation of the average extent of TG incorporation does not necessarily reflect the cytotoxicity of TG treatment and that such cytotoxicity is more consistently correlated with the extent of delayed late S/G2/M arrest exhibited by the subject population.

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References

1. Armstrong RD, Vera R, Snyder P, Cadman E (1982) Enhancement of 6-thioguanine cytotoxic activity with methotrexate. Biochem Biophys Res Commun 109: 595
2. Cohn SM, Lieberman MW (1984) The distribution of DNA excision-repair sites in human diploid fibroblasts following ultraviolet irradiation. J Biol Chem 259: 12463
3. Fairchild CR, Maybaum J, Kennedy KA (1986) Concurrent unilateral chromatid damage and DNA strand breakage in response to 6-thioguanine treatment. Biochem Pharmacol 35: 3533
4. Finkel JM (1975) Fluorometric assay of thioguanine. J Pharm Sci 64: 121
5. Hakala MT, Nichol CA (1964) Prevention of the growth-inhibitory effect of 6-mercaptopurine by 4-aminoimidazole-5-carboxamide. Biochim Biophys Acta 80: 665
6. Herbert BH, Drake S, Nelson JA (1982) A dual-column HPLC method for the simultaneous measurement of 6-thioguanine and adenine in RNA or DNA. J Liquid Chromatogr 5: 2095
7. Maybaum J, Mandel HG (1981) Differential chromatid damage induced by 6-thioguanine in CHO cells. Exp Cell Res 135: 465
8. Maybaum J, Mandel HG (1983) Unilateral chromatid damage: a new basis for 6-thioguanine cytotoxicity. Cancer Res 43: 3852
9. Maybaum J, Hink LA, Roethel WM, Mandel HG (1985) Dissimilar actions of 6-mercaptopurine and 6-thioguanine in Chinese hamster ovary cells. Biochem Pharmacol 34: 3677
10. Maybaum J, Bainnson AN, Roethel WM, Ajmera S, Iwaniec LM, TerBush DR, Kroll JJ (1987) Effects of incorporation of 6-thioguanine into SV40 DNA. Mol Pharmacol 32: 606
11. Maybaum J, Kott MG, Johnson NJ, Ensminger WD, Stetson PL (1987) Analysis of bromodeoxyuridine incorporation into DNA: comparison of gas chromatographic/mass spectrometric, CsCl gradient sedimentation, and specific radioactivity methods. Anal Biochem 161: 164
12. Maybaum J, Morgans CW, Hink LA (1987) Comparison of in vivo and in vitro effects of continuous exposure of L1210 cells to 6-thioguanine. Cancer Res 47: 3083
13. Paterson ARP, Tidd DM (1975) 6-Thiopurines. In: Sartorelli AC, Johns DG (eds) Handbook of experimental pharmacology. Springer, Berlin, pp 383–403
14. Sartorelli AC, Booth BA (1965) The effect of 4-amino-5-imidazolecarboxamide on the synergistic antineoplastic activity of 6-chloropurine and azaserine. Experientia 21: 457
15. Schwartz SA, Horio D, Kirsten WH (1974) Non-random incorporation of 5-bromodeoxyuridine in rat cell DNA. Biochem Biophys Res Commun 61: 927
16. Tidd DM, Dedhar S (1978) Specific and sensitive combined high-performance liquid chromatographic-flow fluorometric assay for intracellular 6-thioguanine nucleotide metabolites of 6-mercaptopurine and 6-thioguanine. J Chromatogr 145: 237