Biochemical Pharmacology, Vol 31, No 6, pp. 979–982, 1982.
Printed in Great Britain.

0006-2952/82/060979–04 $03.00/0

POTENTIATION OF THE GROWTH INHIBITORY EFFECTS OF ADENOSINE 3′,5′-MONOPHOSPHATE ANALOGUES BY HOMOCYSTEINE

MICHAEL J. TISDALE

Department of Pharmacy, University of Aston in Birmingham, Gosta Green, Birmingham B4 7ET, U.K.

(*Received* 10 *June* 1981; *accepted* 23 *September* 1981)

Abstract—The growth inhibitory effects of N^6-monobutyryl adenosine 3′,5′ monophosphate (mbcAMP) and $N^6,O^{2'}$-dibutyryl adenosine 3′,5′ monophosphate (dbcAMP) towards Walker carcinoma *in vitro* are significantly potentiated by the addition of L-homocysteine to the culture medium. This effect is not seen with L-cysteine or when exogenous cAMP or prostaglandin E_2(PGE_2) replace the butyrylated cyclic nucleotide. Combinations of mbcAMP or dbcAMP and L-homocysteine significantly inhibit nucleic acid methylations. Both the butyrylated cyclic nucleotides cause an elevation of the intracellular level of *S*-adenosyl-L-homocysteine (SAH), a potent inhibitor of *S*-adenosyl-L-methionine (SAM) dependent methyltransferases, and this is significantly enhanced in combination with L-homocysteine. The increase in SAH level produced by such combinations is proportional to the inhibition of methyl group incorporation into 5-methyl cytosine and 7-methyl guanine. These results suggest that L-homocysteine potentiates accumulation of SAH in the presence of mbcAMP and dbcAMP and that the resultant inhibition of methylation accounts for the enhanced growth inhibition.

S-Adenosyl-L-methionine (SAM) is known to participate as a methyl donor in many reactions of physiological importance [1]. A general feature of most SAM-dependent methyltransferases is the inhibition produced by the demethylated product *S*-adenosyl-L-homocysteine (SAH) [2]. SAH can be formed by the condensation of adenosine and L-homocysteine by the enzyme *S*-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1). This reaction has an extremely low K_{eq} in the hydrolytic direction suggesting that under certain conditions of adenosine excess SAH may accumulate with significant cytotoxic effect. Kredich and Martin [3] have shown that L-homocysteine markedly potentiates adenosine toxicity in mouse T lymphoma cells due to the formation of SAH. In addition L-homocysteine has also been shown to potentiate the cellular elevation of adenosine 3′,5′ monophosphate (cAMP) caused by adenosine, but not that produced by prostaglandin E_1(PGE_1) [4]. In addition lymphocytes preloaded with high levels of SAH exhibit a markedly enhanced cAMP response to PGE_1, adenosine, 2-chloroadenosine, isoproterenol and cholera toxin. The enhancement of cAMP response by intracellular SAH was attributed both to the amplification of the activity of adenylate cyclase and to inhibition of cAMP phosphodiesterase [5].

The present report describes the synergistic effect of homocysteine on growth inhibition produced by cAMP and its butyrylated analogues and suggests that the effect may be due to inhibition of transmethylation reactions by an accumulation of SAH.

MATERIALS AND METHODS

Materials. L-[Methyl-^{3}H]methionine (sp. act. 12 Ci/mmole), *S*-adenosyl-L-[methyl-^{3}H]methionine (sp. act. 12 Ci/mmole) and [^{14}C]deoxycytidine (sp. act. 462 mCi/mmole) were purchased from the Radiochemical Centre (Amersham, U.K.). L-Homo-cysteine thiolactone hydrochloride, cAMP, N^6 monobutyryl cAMP (mbcAMP), and $N^6,O^{2'}$ dibutyryl cAMP (dbcAMP) were from Sigma Chemical Co. (London, U.K.).

Cell culture. Cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (Gibco) under an atmosphere of 10% CO_2 in air. For growth experiments cells were grown in duplicate wells (3.5 ml) of a 24-well plastic plate (Flow Laboratories, Irvine, U.K.). Cell number was enumerated daily using a Coulter counter model F_B. Growth curves were constructed for each experiment and growth inhibition was calculated from the linear portion of the growth curve. The cyclic nucleotides were dissolved in growth medium. L-Homocysteine was generated from the thiolactone by treatment with 1N KOH, followed by neutralisation with 1N HCl and used immediately.

Effects on methylation of specific bases. Walker cells were incubated in Dulbecco's modified Eagle's medium together with 20 mM sodium formate, 20 μM adenosine and 20 μM guanosine to minimize incorporation of carbon units through the "one carbon pool"; 50 μCi, L-[methyl-^{3}H]methionine (sp. act. 46 Ci/mmole), 2.5 μCi [^{14}C]deoxycytidine (sp. act. 462 mCi/mmole), and the concentrations of cyclic nucleotides and homocysteine as indicated in Table 1. After 24 hr the medium was removed and the cell pellet was washed with 0.9% NaCl and homogenized together with freezing and thawing in 1 ml 0.01 M Tris–HCl, pH 7.5. The homogenate was made 1% with respect to sodium dodecyl sulphate and extracted three times with phenol. After chloroform extract the solution was supple-

Table 1. Effect of cyclic nucleotides and L-homocysteine on base methylation in nucleic acids after 24 hr of treatment

Culture conditions	Ratio $^3H/^{14}C$ 1-Methyl adenosine	N^6-Methyl adenine	5-Methyl cytosine	7-Methyl guanine
No addition	4.8	1.0	1.7	7.6
L-Homocysteine (Hcy) 0.1 mM	4.6	0.9	1.1	4.3
L-Homocysteine (Hcy) 0.4 mM	4.4	0.6	2.3	4.4
mbcAMP 0.24 mM	2.2	0.6	0.4	2.2
mbcAMP 0.24 mM + L-Hcy 0.1 mM	0.9	0.5	0.2	1.4
mbcAMP 0.24 mM + L-Hcy 0.4 mM	0.4	0.4	0.2	0.7
dbcAMP 0.2 mM	0.8	1.4	0.8	5.1
dbcAMP 0.2 mM + L-Hcy 0.1 mM	0.7	1.2	0.6	2.6
dbcAMP 0.2 mM + L-Hcy 0.4 mM	0.3	1.0	0.3	1.9

mented with NaCl to 0.2 M. The nucleic acids were precipitated with 2.5 vol. of 95% ethanol and left overnight at −20°. The collected nucleic acids were subjected to hydrolysis in 0.5 ml 88% formic acid at 180° for 2 hr as described [6]. The hydrolysates were evaporated under nitrogen and the residue stored at −20° for later chromatographic processing. For chromatography the residues were dissolved in 30 μl of formic acid containing 3–5 μg of each of the standard methylated bases and 10 μl of this solution was applied to the corner of 20 × 20 cm cellulose plates (Merck, Darmstadt, West Germany), which were then developed in pre-equilibrated tanks containing ethyl acetate–methanol–water–88% formic acid (100:25:20:1). The plates were then removed and allowed to air-dry at room temperature for 2 hr before development in the second dimension in acetonitrile–ethyl acetate–2 propanol–1-butanol–water–58% ammonium hydroxide (40:30:20:10:5:22) [6]. The position of each base was detected with a u.v. lamp (254 nm). The position on the chromatogram corresponding to each base was scraped off and the radioactivity was determined in a toluene:PPO:POPOP scintillation mixture using a Tracer Lab liquid scintillation spectrometer.

Determination of S-adenosyl homocysteine. Walker cell cultures were incubated with the concentrations of cyclic nucleotide analogues given in Table 2, centrifuged at 300 *g* for 3 min, washed with 0.9% NaCl and the cell pellet was disrupted in the presence of 200 μl 1N perchloric acid. The deprotinized supernatant was neutralized with 5N KOH and the insoluble $KClO_4$ was removed by centrifugation. This material was then analysed for SAH by high-performance liquid chromatography [7]. Analyses were performed using an Altex 100-A twin piston pump and a Pye Unicam detector.

RESULTS

Effect on growth rate. The effect of L-homocysteine on the growth inhibitory effect of mbcAMP and dbcAMP towards Walker carcinoma, estimated from the linear portions of the growth curves is shown in Figs. 1 and 2, respectively. L-Homocysteine alone at low concentrations had no effect on the growth rate of Walker cells, but showed a dose-related enhancement of the growth inhibitory effect produced by both mbcAMP and dbcAMP. Thus the LD_{50} for mbcAMP was reduced from 114 to 50 μg/ml in the presence of 0.66 mM L-homocysteine. There was no potentiation of growth inhibition produced by mbcAMP in the presence of 0.1 mM cysteine. Higher concentrations of cysteine were cytostatic. There was a potentiation of the growth inhibitory effect of a combination of mbcAMP and L-homocysteine in the presence of glycine (0.1–0.5 mM), the fractional growth inhibition being increased on average by a further 10%. There was no enhancement of the growth inhibition produced by exogenous cAMP or by PGE_2 which should elevate intracellular levels of cAMP. There was a slight enhancement of the growth inhibitory effect of the phosphodiesterase inhibitor methyl isobutyl xan-

Table 2. Effect of cyclic nucleotides and L-homocysteine on the intracellular level of SAH

Culture conditions	SAH (ng/10^6 cells) + S.E.M. 24 hr	48 hr
No addition	317 ± 24	346 ± 20
L-Homocysteine (Hcy) 0.1 mM	452 ± 40	510 ± 40
L-Homocysteine (Hcy) 0.4 mM	511 ± 30	486 ± 20
L-Homocysteine (Hcy) 0.66 mM	572 ± 20	454 ± 25
mbcAMP 0.24 mM	723 ± 30	570 ± 33
mbcAMP 0.24 mM + L-Hcy 0.1 mM	796 ± 45	796 ± 44
mbcAMP 0.24 mM + L-Hcy 0.4 mM	1025 ± 60	813 ± 35
mbcAMP 0.24 mM + L-Hcy 0.66 mM	824 ± 45	815 ± 53
dbcAMP 0.2 mM	622 ± 22	625 ± 45
dbcAMP 0.2 mM + L-Hcy 0.1 mM	687 ± 35	770 ± 33
dbcAMP 0.2 mM + L-Hey 0.4 mM	895 ± 43	1016 ± 40
dbcAMP 0.2 mM + L-Hcy 0.66 mM	987 ± 54	1023 ± 60

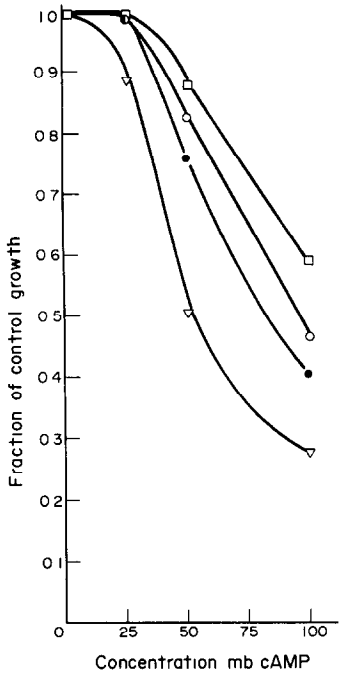

Fig. 1. Effect of L-homocysteine on growth inhibition by mbcAMP. Walker cells were grown in the presence of mbcAMP either alone (□——□) or in the presence of 0.1 mM (○——○), 0.4 mM (●——●) or 0.66 mM (▽——▽) L-homocysteine. Growth inhibition is calculated from the linear part of the growth curves.

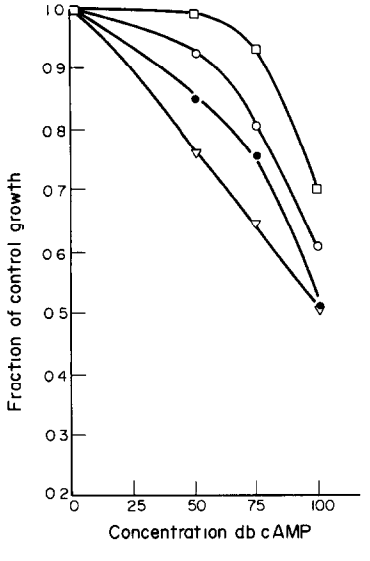

Fig. 2. Effect of L-homocysteine on growth inhibition by dbcAMP. Walker cells were grown in the presence of dbcAMP either alone (□——□) or in the presence of 0.1 mM (○——○), 0.4 mM (●——●) or 0.66 mM (▽——▽) L-homocysteine. Growth curves were constructed as described in Materials and Methods.

thine with the LD_{50} decreasing from 70 to 55 μg/ml in the presence of 0.3 mM L-homocysteine.

Effect on base methylation. The extent of methylation of some nucleic acid bases after treatment with L-homocysteine, mbcAMP, dbcAMP or a combination of cyclic nucleotide analogues and L-homocysteine is shown in Table 1. The major methylated bases found in the nucleic acid fraction were 5-methyl cytosine and N^6-methyl adenine. L-Homocysteine alone had no effect on the methylation of either 1-methyl adenine or 6-methyl cytostine, but there was extensive inhibition of methylation in the presence of the cyclic nucleotides alone and this was potentiated by concurrent administration of L-homocysteine. Incorporation of labelled methyl groups into N^6-methyl adenine and 7-methyl guanine was inhibited by both L-homocysteine and the butyrylated cyclic nucleotides and an enhanced inhibition was observed with the combination of agents.

Effect of SAH levels. Since the most likely mediator of the inhibition of methylation is SAH the intracellular concentration of this metabolite was measured in acid soluble extracts of Walker carcinoma cultured in the presence of various combinations of mbcAMP or dbcAMP and L-homocysteine. The results reported in Table 2 show that L-homocysteine, mbcAMP and dbcAMP alone increased the intracellular level of SAH, and that a further increase was observed in the presence of combinations of these agents. The results in Fig. 3 show the effect of mbcAMP and mbcAMP plus L-homocysteine on the accumulation of radioactivity in SAH after labelling Walker carcinoma with L-[2-^{3}H]methionine.

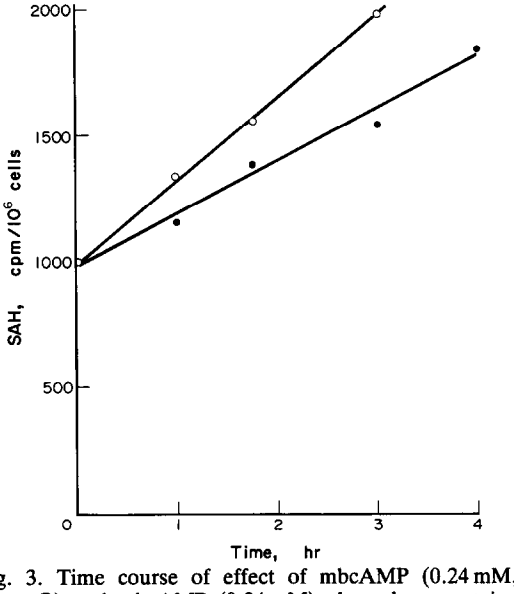

Fig. 3. Time course of effect of mbcAMP (0.24 mM, ●——●) and mbcAMP (0.24 mM) plus L-homocysteine (0.4 mM, ○——○) on metabolism of L-[2-^{3}H]methionine in Walker carcinoma. Walker cells were incubated with L-[2-^{3}H]methionine (1 μCi/ml) for 3 hr prior to the additions. At zero time either mbcAMP or mbcAMP + L-homocysteine were added and samples were removed at the time points indicated. The cells were sedimented by centrifugation and washed with 0.9% NaCl. The amount of radioactivity in SAH was determined from the HPLC effluent of a neutralised perchloric acid extract of the cells.

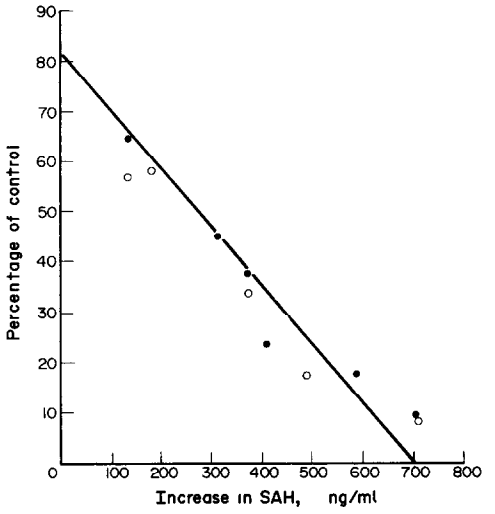

Fig. 4. Relationship between the intracellular concentration of SAH produced by the combinations of cyclic nucleotide analogues and L-homocysteine as indicated in Table 2 and inhibition of methylation of 5-methyl cytosine (●——●) and 7-methyl guanine (○——○).

Thus mbcAMP alone caused a rapid increase in the level of [^{3}H]SAH in Walker cells and this increase was enhanced in the presence of L-homocysteine. These results suggest that the increase in intracellular SAH observed under these conditions arises as a result of inhibition of SAH hydrolysis.

The results presented in Fig. 4 show a linear relationship between the intracellular concentration of SAH produced by combination of the butyrylated cyclic nucleotides and L-homocysteine and the extent of inhibition of [^{3}H]methyl group incorporation into 5-methyl cytosine and 7-methyl guanine. This suggests that inhibition of base methylation in the presence of mbcAMP or dbcAMP and L-homocysteine is due to the accumulation of SAH.

DISCUSSION

Adenosine 3′,5′ monophosphate (cAMP) has been postulated to play a role in the regulation of cell growth and differentiation and alterations in cAMP metabolism may be responsible for certain characteristics of the malignant state [8]. Most of the evidence relating to the growth inhibitory effect of cAMP has come from studies utilizing butyrylated derivatives [9, 10], which are thought to elevate intracellular levels of cAMP by inhibition of cAMP phosphodiesterase [11]. However, the concentrations of the analogues which are employed in growth inhibitory studies (10^{-3}M) are much greater than intracellular levels of cAMP (10^{-6}M). Such differences may reflect the somewhat high K_i values for inhibition of phosphodiesterase by such analogues. The possibility exists, however, that some of the effects could be due to the formation of butyrylated adenosine analogues due to cleavage of the cyclic phosphate ring by phosphodiesterase, which has been shown to occur both *in vitro* [12] and *in vivo* [13]. Such analogues would be resistant to adenosine deaminase and might be expected to be very effective mediators of adenosine toxicity.

The present experiments considered the possibility that the homocysteine enhancement of growth inhibition by cyclic nucleotide analogues may be due to elevation of the intracellular level of SAH with consequent inhibition of transmethylation reactions. The butyrylated cyclic nucleotide analogues are known to undergo rapid and extensive metabolism. Incubation of Chinese hamster ovary cells in culture with dbcAMP resulted in the accumulation of both dbcAMP and mbcAMP as well as N^6mbcAMP and mbc adenosine [12]. Also incubation of cultured hepatoma cells with [^{3}H]mbcAMP or dbcAMP resulted in greater than 65% of the label being associated with ADP and ATP with the rest distributed among the other nucleotides. Less than 15% remained as the cyclic nucleotides after 2 hr incubation [14]. This suggests that the butyrylated cyclic nucleotides act as precursors of adenosine and adenosine analogues.

Treatment of Walker carcinoma with the cyclic nucleotide analogues caused an inhibition of growth and a concomitant inhibition of nucleic acid methylation. Both of these effects were potentiated by L-homocysteine, probably due to an enhanced ability of the combination to elevate intracellular SAH levels. A role for SAH as the mediator of these effects is supported by the known ability of this compound to inhibit many SAM-dependent methylation reactions and the correlation observed between increases in intracellular SAH and inhibition of base methylation. While one particular methylation reaction may be especially sensitive to SAH it seems more likely that SAH toxicity results from the cumulative effects of partially inhibiting the methylation of many different kinds of molecules.

Acknowledgement—This work was supported by a grant from the Cancer Research Campaign.

REFERENCES

1. G. L. Cantoni, *A. Rev. Biochem.* **44**, 435 (1975).
2. R. T. Borchardt, in *The Biochemistry of S-adenosyl methionine* (Eds F. Salvatore, E. Borek, V. Zappia, H. G. Williams-Ashman and F. Schlenk), p. 151. Columbia University Press, New York (1977).
3. N. M. Kredich and D. W. Martin, Jr., *Cell* **12**, 931 (1977).
4. T. P. Zimmerman, R. D. Deeprox, G. Wolberg and G. S. Duncan, *Biochem. Pharmac.* **28**, 2375 (1979).
5. T. P. Zimmerman, C. J. Schmitges, G. Wolberg, R. D. Deeprose, G. S. Duncan, P. Cuatrecasas and G. B. Elion, *Proc. natn. Acad. Sci. U.S.A.* **77**, 5639 (1980).
6. T. W. Munns, K. C. Podratz and R. A. Katzman, *Biochemistry* **13**, 4409 (1974).
7. V. Zappia, P. Galletti, M. Porcelli, C. Manna and F. D. Ragione, *J. Chromatog.* **189**, 399 (1980).
8. M. J. Tisdale, *Cancer Treat. Rev.* **6**, 1 (1979).
9. Y. S. Cho-Chung, *Cancer Res.* **34**, 3492 (1974).
10. Y. S. Cho-Chung and P. M. Gullino, *Science* **183**, 87 (1974).
11. A. W. Hsie, K. Kawashima, J. P. O'Neill and C. H. Schroder, *J biol. Chem.* **250**, 984 (1975).
12. J. P. O'Neill, C. H. Schroder and A. W. Hsie, *J. biol. Chem.* **250**, 990 (1975).
13. M. Castagna, N. K. Palmer and D. A. Walsh, *Archs Biochem. Biophys.* **181**, 46 (1977).
14. D. K. Granner, L. Sellers, A. Lee, C. Butters and L. Kutina, *Archs Biochem. Biophys.* **169**, 601 (1975).