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Genetic and Biochemical Studies on Mutants of CHO Cells Resistant to 7-Deazapurine Nucleosides: Differences in the Mechanisms of Action of Toyocamycin and Tubercidin

Radhey S. Gupta and Kamal D. Mehta

Department of Biochemistry, McMaster University
Hamilton, Canada L8N 3Z5

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SUMMARY: From mutants of Chinese hamster ovary cells which are resistant to toyocamycin and tubercidin, second-step mutants which exhibit a further 8- to 9-fold increase in resistance to toyocamycin (Toy^{rII} mutants) but no change in resistance to tubercidin have been isolated. The Toy^{rII} mutants are similar to the first-step mutants in their levels of adenosine kinase activity (≃1%), as well as cellular uptake and phosphorylation of adenosine and its analogs. The increased resistance of the Toy^{rII} mutants to toyocamycin but not to tubercidin provides strong evidence that the mechanism of cellular toxicity of these two analogs is different from each other and suggests that these mutants may be affected in a cellular component which is specifically involved in the toxicity of toyocamycin. The Toy^{rII} mutants also exhibit increased resistance to sangivamycin and the tricyclic nucleoside pentaazacenaphthylene riboside (TCN, NSC 154020) indicating that the mechanisms of cellular toxicity of these two analogs may be similar to that of toyocamycin.

The pyrrolopyrimidine nucleosides toyocamycin, tubercidin and sangivamycin have stimulated considerable research because of their action against bacteria, schistosomes, mammalian cells in culture, RNA and DNA viruses, and in the treatment of cutaneous neoplasma in humans (see 1,2). Because of close structural similarity between these compounds and adenosine, the phosphorylated derivatives of these analogs can substitute for adenine nucleotides in a wide variety of cellular reactions (1-3). However, which particular cellular reaction(s) is primarily responsible for the cytotoxicity of the above analogs, and whether all of these analogs act in the same manner, is not clear at present. To understand the mechanisms of action of these nucleoside analogs we have been using a combined genetic and biochemical approach in which cellular mutants resistant to these analogs are initially isolated and subsequently the nature of the biochemical alteration in the mutant cells is examined. In earlier studies we have reported that when single-step mutants of Chinese hamster ovary (CHO) cells resistant to toyocamycin and tubercidin are selected, all such mutants are found to contain greatly reduced amounts of the

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enzyme adenosine kinase (AK) which phosphorylates these analogs before they become toxic to the cells (4,5). These mutants, however, provided no further information regarding the mechanism of action of these nucleoside analogs.

Since the mutants obtained after the single-step selection were killed at higher concentrations, we attempted to select second-step mutants which have become more resistant to these analogs. The present paper describes the isolation and some characteristics of second-step mutants resistant to toyocamycin (Toy^{rII} mutants). Very interestingly, these mutants do not exhibit any increased resistance towards the structurally related analog tubercidin which suggests that the genetic lesion in these mutants specifically affects the toxicity of toyocamycin. The cross resistance and other biochemical studies which are presented in this paper indicate that two additional 7-deazapurine ribosides, sangivamycin and the synthetic tricyclic nucleoside (3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentaazaacenaphthylene; TCN) may also act in the same manner as toyocamycin.

MATERIALS AND METHODS

Cell Lines and Culture Conditions: The parental CHO cell line from which the various toyocamycin resistant (Toy^r) mutants have been selected is auxotrophic for glycine, adenosine and thymidine and is referred to as Gat^- in these and earlier studies (4,6). Toy^{rI}-16 is a toyocamycin resistant mutant which has been obtained from the Gat^- line after a single-step selection in presence of toyocamycin (4). Toy^{rII}-1 and Toy^{rII}-2 are two second-step toyocamycin resistant mutants which have been obtained from Toy^{rI}-16 after selection in presence of higher concentrations of toyocamycin. The above cell lines were routinely grown in complete alpha medium supplemented with 5% fetal bovine serum by procedures described earlier (see 4). Studies of the effects of nucleoside analogs on cellular macromolecular synthesis were carried out with the proline requiring CHO cell line (referred to as wild type or WT in our work) (7). The cellular uptake and incorporation of [^{3}H]uridine and [^{3}H]thymidine was studied in alpha MEM medium supplemented with 5% dialyzed fetal bovine serum (DFBS) whereas studies on the uptake and incorporation of [^{3}H]leucine were carried out in alpha special medium lacking leucine and containing 5% DFBS (4,7). The plating efficiencies of various cell lines in medium containing different concentrations of the drugs were determined by procedures described earlier (4,7)

Drugs and Chemicals: Toyocamycin (NSC 63701), sangivamycin (NSC 65346) and the tricyclic nucleoside pentaazaacenaphthylene (TCN; NSC 154020) were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute, National Institute of Health, Silver Spring, MD; Tubercidin was purchased from Sigma Chemical Co., St. Louis, MO; [^{3}H]tubercidin was obtained by the custom labelling of tubercidin (New England Nuclear Corp., Boston, MA) and then purified by paper chromatography (specific activity 100 mCi/mmol). [^{3}H]adenosine (specific activity, 36 Ci/mmol), [^{3}H]thymidine (specific activity, 20.0 Ci/mmol), [^{3}H]uridine (specific activity, 38.8 Ci/mmol) and [^{3}H]leucine (specific activity, 47.0 Ci/mmol) were purchased from New England Nuclear, Canada.

Effect of Nucleoside Analogs on Cellular Macromolecular Synthesis: For these studies, exponentially growing CHO cells were suspended in the appropriate growth medium at a concentration of about 5×10^5 cells/ml. After removing the "0" time

samples which were pulse labelled in the manner described below, the various analogs were added to separate cultures to give final concentrations of 0.2 µg/ml (toyocamycin), 2.0 µg/ml (tubercidin), 2 µg/ml (sangivamycin) and 5 µg/ml (TCN). After various times, therefore, 2 ml aliquots of the cultures were removed and separately pulse labelled with either [^{3}H]leucine (5 µCi/ml), or [^{3}H]uridine (5 µCi/ml), or [^{3}H]thymidine (5 µCi/ml). After 30 min of labelling, an equal volume of cold 10% trichloroacetic acid was added to the cultures and the acid precipitable counts were determined in each case. The incorporation values obtained in the "0" time samples which were not treated with the drugs have been assumed as 100% in each experiment.

Other Procedures: The procedures for the measurement of adenosine kinase activity in cell extracts and for studying the cellular uptake of [^{3}H]adenosine were the same as have been described recently (4,5,7).

RESULTS

Figure 1A shows the dose response curves towards toyocamycin of the parental sensitive cells (Gat$^-$) and the Toy^{rI}-16 mutant. As can be seen, the Toy^{rI}-16 mutant is resistant up to about 50 ng/ml (other Toy^{rI} mutants behave similarly) and at

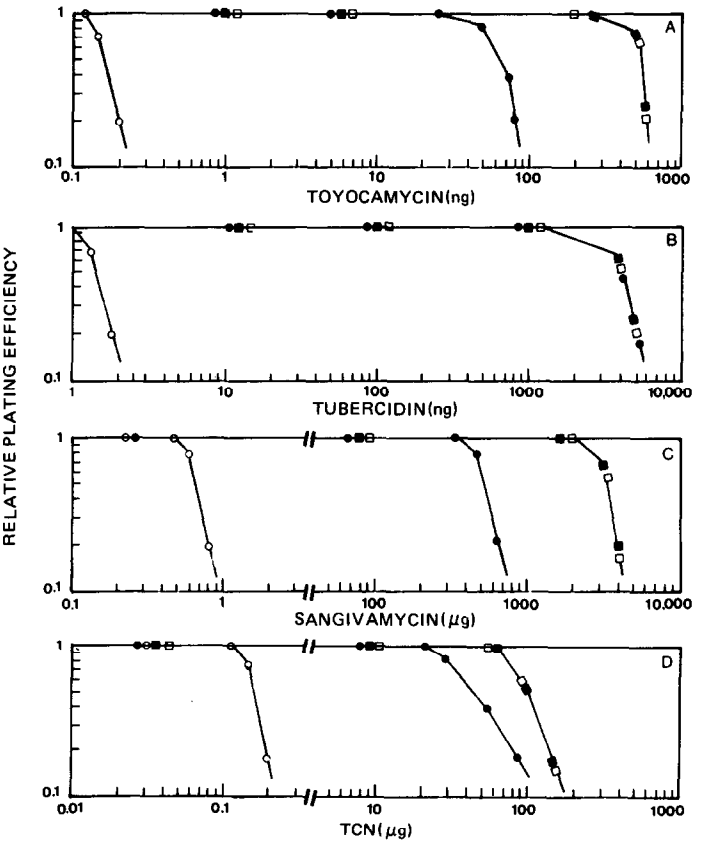

Figure 1: Dose response curves of the parental sensitive cells and the first- and second-step toyocamycin resistant mutants in presence of increasing concentrations of various 7-deazapurine nucleoside analogs. (A), toyocamycin; (B), tubercidin; (C), sangivamycin; (D), TCN. Symbols: ○—○, Gat$^-$; ●—●, Toy^{rI}-16; □—□, Toy^{rII}-1; ■—■ Toy^{rII}-2.

higher concentrations its plating efficiency decreased sharply. The selection of second-step mutants was carried out by plating a mutagen-treated (300 µg/ml of ethyl methanesulfonate for 20 hrs; plated on 7th day after the mutagen treatment) culture of the Toy^{rI}-16 cells in presence of 0.4 µg/ml of toyocamycin. The resistant clones were obtained at a frequency of about 1 in 10^6 in these experiments. Five of the resistant clones were picked in non-selective medium and subsequently their degree of resistance towards toyocamycin was determined. The dose response curves of two of the clones, Toy^{rII}-1 and Toy^{rII}-2 towards toyocamycin are also shown in Fig. 1A (the other clones examined behaved similarly). Based upon their D_{10} values, these mutants are about nine to ten fold more resistant to toyocamycin as compared with the Toy^{rI}-16 line. The drug resistance phenotype of these mutants has remained completely stable upon prolonged growth in non-selective medium.

To characterize these mutants, their cross resistance towards a number of other 7-deazapurine nucleosides which included tubercidin, sangivamycin and TCN (8) was determined. Results of these studies for the parental Gat^-, the Toy^{rI}-16 line and two second-step mutants are shown in Fig. 1B,C and D. As can be seen, the Toy^{rI}-16 mutant which lacks adenosine kinase showed greatly increased resistance to tubercidin, sangivamycin and TCN. This result indicates that the phosphorylation of all these analogs is an essential first-step in their toxic action and that the phosphorylation is carried out by the enzyme AK. Similar inferences regarding the toxicity and phosphorylation of toyocamycin, tubercidin, sangivamycin and TCN have been reached in earlier studies (see 1-3,9-11). Very interestingly, in contrast to the first-step mutant which showed corresponding increase to all of these analogs, the second-step mutants showed no further increase in their resistance for tubercidin but were about eight and two fold more resistant to sangivamycin and TCN, respectively. Similar results with these mutants have been obtained in a number of independent experiments.

The mutants of CHO cells resistant to various adenosine analogs, which are obtained after a single-step selection contain less than 1% of the AK activity in their cell extracts (4,5,12). However, such mutants can phosphorylate adenosine and tubercidin at a rate of between 5-15% of that seen with the parental sensitive

Table 1

Cellular Uptake of [^{3}H]Adenosine by the Parental and Mutant Cell Lines

Cell Line	Duration of uptake (min)	[^{3}H]Adenosine uptake/5x10^5 cells (cpm)	Uptake Relative to the Sensitive line (%)
Gat$^-$ (Toy^S)	10	3.2 x 10^5	100
Toy^{rI}-16	10	2.7 x 10^4	8.4
Toy^{rII}-1	10	2.5 x 10^4	8.2
Toy^{rII}-2	10	2.8 x 10^4	8.7

The cellular uptake and incorporation of adenosine by various cell lines was studied as described recently (7). The cellular uptake of [^{3}H]tubercidin in various Toy^{rI} and Toy^{rII} mutants was also found to be reduced to similar extent, i.e. between 5 and 6% of the sensitive cells (results not shown).

cells (7). It is not clear at present whether the observed phosphorylation in the mutant cells is carried out by the residual AK activity or by some other enzymic activity present in CHO cells. To find out whether the genetic lesion in our Toy^{rII} mutants has affected this residual phosphorylating activity, the level of AK activity as well as the cellular uptake and phosphorylation of [^{3}H]adenosine and [^{3}H]tubercidin in the mutant cells was studied. Studies on the measurement of AK activity in the mutant cells showed that the Toy^{rII} mutants contained similar levels of residual AK activity (≈1% of WT cells) as seen in the Toy^{rI} mutants (results not shown). The cellular uptake and phosphorylation of [^{3}H]adenosine and [^{3}H]tubercidin in the Toy^{rII} mutants was also found to occur to similar extent as seen in the first-step mutants (Table 1). These results provide evidence that the genetic lesion in the second-step mutants does not further affect the phosphorylation of adenosine or its analogs.

In earlier studies we have shown that in suspension culture of CHO cells, cellular DNA, RNA and protein synthesis were affected differently by toyocamycin and tubercidin (4). For example, toyocamycin affected RNA synthesis first and then DNA and protein synthesis, whereas in the case of tubercidin, protein and DNA synthesis were inhibited before any effect on RNA synthesis was observed. The increased cross resistance of Toy^{rII} mutants to sangivamycin and TCN indicated that the mechanism of cellular toxicity of these analogs may be similar to toyocamycin.

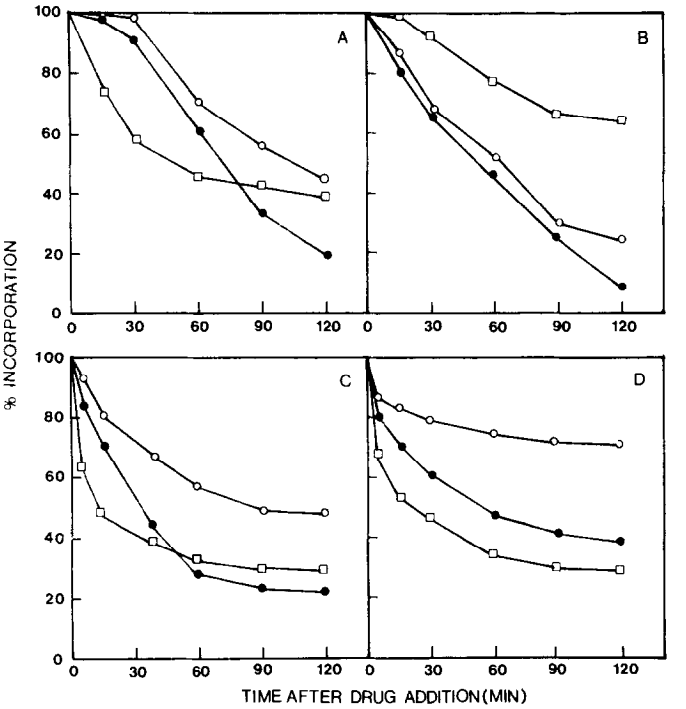

Figure 2: Effects of various 7-deazapurine nucleosides on the macromolecular incorporation of [^{3}H]uridine, [^{3}H]thymidine and [^{3}H]leucine in CHO cells. (A), toyocamycin; (B), tubercidin; (C), TCN; (D), sangivamycin. Symbols: O—O , [^{3}H]leucine; ●—● , [^{3}H]thymidine; □—□ , [^{3}H]uridine.

To obtain further evidence in this regard we have examined the effects of treatment with toyocamycin, tubercidin, sangivamycin and TCN on the macromolecular incorporation of [^{3}H]uridine, [^{3}H]thymidine and [^{3}H]leucine, in suspension culture of CHO cells. The results of these studies are shown in Figure 2. As can be seen, the effects of sangivamycin and TCN on macromolecular synthesis in CHO cells were analogous to those observed with toyocamycin, i.e. RNA synthesis was inhibited first and then DNA and protein synthesis were affected, but these differed from those of tubercidin.

DISCUSSION

This paper describes the selection and some biochemical characteristics of second-step mutants of CHO cells which display specific increase in their resistance towards a number of 7-deazapurine ribonucleosides. Although the biochemical function which is affected in the second-step mutants has not yet been identified, our results show that the lesion in these mutants does not further affect the level of phosphory-

lation of adenosine or its derivatives. Since mutants affected in the phosphorylation step are expected to exhibit cross resistance to all of the adenosine derivatives, the lack of increased resistance of the second-step mutants to the related 7-deazapurine nucleoside, tubercidin, also supports this inference. The above observations indicate that the molecular lesion in the second-step mutants is very specific and may occur at the cellular site which is inhibited by the phosphorylated derivatives of toyocamycin.

In earlier studies with emetine and podophyllotoxin resistant mutants we have shown that the cross resistance pattern of mutant cells can provide valuable information regarding other compounds which act in the same manner (13,14). In this context, the lack of cross resistance of the Toy^{rII} mutants to tubercidin provides strong evidence that the mechanisms of cellular toxicity of toyocamycin and tubercidin are not identical. At the same time, the proportionally increased cross resistance of the Toy^{rI} and Toy^{rII} mutants to sangivamycin and TCN provides suggestive evidence that the mechanism of cellular toxicity of these two adenosine analogs should be similar to that of toyocamycin. These inferences are supported by the inhibitory effects of the above nucleoside analogs on cellular macromolecular synthesis. Whereas toyocamycin, sangivamycin and TCN all showed greater inhibition of RNA synthesis initially, in the case of tubercidin, DNA and protein synthesis were inhibited first in comparison to the effect on RNA synthesis. These results show that the mechanism of cellular toxicity of toyocamycin, sangivamycin and TCN is different from that of tubercidin.

Earlier studies with toyocamycin and sangivamycin have shown that both these analogs are preferentially incorporated into RNA (1-3,15,16). On the other hand, in the case of TCN, its phosphorylation has been observed only to the 5'-monophosphate form and no incorporation into either RNA or DNA has been observed (11,17,18). These results indicate that the active form of TCN is its 5'-monophosphate which most likely inhibits an essential cellular reaction (11,17). Since the second-step toyocamycin resistant mutants are affected in a function which leads to increased resistance to toyocamycin, sangivamycin as well as TCN, it is expected that the resistance mechanism (i.e. affected function) should not involve either the higher

phosphorylated forms of these analogs or their incorporation into RNA. If this is true then the cellular toxicity of all three of these analogs (viz. toyocamycin, sangivamycin and TCN) should most likely be due to inhibition of an essential cellular reaction by their 5'-monophosphate derivatives. Further studies on the identification of the cellular component which is affected in the Toy^{rII} mutants should prove very useful in understanding the mechanisms of action of these nucleoside analogs.

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