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LACK OF MUTAGENICITY AND METABOLIC INACTIVATION OF APHIDICOLIN BY RAT LIVER MICROSOMES

Guido Pedrali-Noy, Giorgio Mazza*, Federico Focher and Silvio Spadari

Laboratorio di Genetica Biochimica ed Evoluzionistica, CNR, Pavia, Italy

and

* Istituto di Genetica, Università di Pavia, Pavia, Italy

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SUMMARY

In view of the possible utilization of aphidicolin, a specific inhibitor of DNA polymerase α, in the treatment of neoplastic diseases, it seemed important to assess the mutagenic effect of the drug and the possible modification induced by metabolic activation in the liver. This paper shows that aphidicolin lacks mutagenicity in the Ames' Salmonella-microsome test in agreement with our previous observation that it does not induce DNA repair synthesis in HeLa cells. During the studies of mutagenicity we have observed that aphidicolin is converted to inactive derivative(s) by rat liver microsomal oxidases. The reaction is dependent on time and temperature and requires $NADP^+$ and glucose-6-P. The metabolites are not mutagenic and they do not induce DNA repair synthesis in HeLa cells. Therefore the possible anti-cancer use of aphidicolin is not hampered by its partial metabolic inactivation in liver. Our results suggest however that aphidicolin will possibly be clinically useful at concentrations higher than those expected from our studies with human DNA polymerase α in vitro and human neoplastic cell lines in vivo. The metabolic derivative(s) of aphidicolin is inactive both against cellular DNA polymerase α and Herpes simplex viral DNA polymerase.

INTRODUCTION

Recent experiments in our laboratory have indicated that aphidicolin (1,2) has promising properties as a possible anti-cancer drug. These include : the inhibition of cellular and viral replicative DNA synthesis (2-5) due to the selective inhibition of human (3,4) and viral (3) replicative DNA polymerases (6-11); the failure to induce DNA repair synthesis in human cells which suggests the absence of a mutagenic activity (12); the lack of effect on DNA repair revealed by the capacity of human cells to repair damaged DNA in the presence of aphidicolin (12); the activity against several neoplastic animal and human cell lines - including leukemia and melanoma - (Pedrali-Noy, Belvedere, Focher and Spadari, manuscript in preparation). Since the failure of aphidicolin to induce DNA repair synthesis has been demonstrated in HeLa cells (12), which lack the enzymic machinery for activating mutagens and carcinogens, and since

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most anti-cancer drugs are mutagenic and therefore carcinogenic themselves, we have tested aphidicolin in the Ames' Salmonella -mammalian microsomes test where rat liver homogenates (S-9 mix) supply mammalian metabolism. Our results indicate that aphidicolin lacks mutagenicity, and, therefore, putative carcinogenicity, in this system. During these studies, however, we have found that rat liver microsomes can convert aphidicolin to inactive metabolite(s) which are non-mutagenic in the Ames' Salmonella test and do not induce DNA repair synthesis in HeLa cells. Thus aphidicolin may still offer advantages for clinical use over other anti-cancer drugs although amounts higher than those expected from our in vivo studies with human neoplastic cell lines will be required.

MATERIALS AND METHODS

Chemicals: Benzidine, 2-acetylaminofluorene (2-AF) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were obtained from Aldrich. Mitomycin C was from Serva, dimethylsulfoxide from Schwarz/Mann, ethidium bromide and methylmethansulfonate (MMS) from Eastman Kodak, glucose-6-phosphate and $NADP^+$ from Sigma. All other reagents were those previously described (3). Herpes simplex virus type I (HSV) DNA polymerase was purified from infected HeLa cells and was a DNA cellulose fraction with 17,000 units / mg.

Mutagenicity assay : The histidine requiring Salmonella typhimurium mutagenesis tester strains developed by B. Ames (13) were used. These strains include the standard set (TA 1535, TA 1537, TA 1538, TA 98, TA 100) lacking uvrB excision repair system and the set (his G46, his D3052, TA 92, TA 94) with a normal excision repair system. The plate test for reversion to histidine independence, the media employed and the tests for identifying the strains are those described (13). The rat liver oxidative microsomal enzymes used as metabolizing system were prepared from rat liver homogenates (9,000 x g supernatant or S-9) after induction by Aroclor 1254 (13). The S-9 mixture (S-9 mix) was prepared as described (13) with an amount of 0.1 ml of S-9 per ml. In the tests, controls with compounds of known mutagenic activity have been introduced to check the bacterial strains, their reversion specificity and the activity of rat liver homogenate. Aphidicolin was dissolved in dimethylsulfoxide and tested at 20, 200, 500, 1000 and 2000 µg / plate. No bactericidal effect has been observed even at the highest dose used.

Cell culture, preparation of templates, assay of DNA polymerases were those reported by Pedrali-Noy and Spadari (3).

RESULTS AND DISCUSSION

Lack of mutagenicity of aphidicolin in the Ames' Salmonella-microsome test.

We have assayed aphidicolin for its potential mutagenic activity with Salmonella typhimurium system using both the set of mutants lacking the excision repair uvrB system and the set of strains with normal excision repair. These mutants allow the detection of most mutagen compounds. Aphidicolin has been tested at different concentrations per plate, both in the presence and in the absence of the metabolizing system. A bactericidal effect observed at doses higher than 2000 µg / plate (250 µM aphidi-

TABLE I. Lack of mutagenicity of aphidicolin in Salmonella typhimurium standard strains.

compound	µg/plate	his$^+$ revertants per plate									
		TA 1535		TA 1537		TA 1538		TA 98		TA 100	
		– §	+	–	+	–	+	–	+	–	+
Aphidicolin	0	46	25	11	14	20	47	38	56	178	159
	20	42	21	18	24	22	46	28	58	173	145
	200	30	16	8	17	31	39	19	53	145	135
	500	37	20	8	14	21	52	35	49	184	168
	1000	48	13	3	20	24	51	33	48	179	145
	2000	45	21	5	12	23	40	38	50	201	127
MMS	500	36	32	18	17	25	60	28	58	1020	1704
Ethidium bromide	10	48	23	8	59	40	1188	30	1184	162	212
2-AF	50	45	29	14	34	45	1362	59	1430	114	1008
Benzidine	500	51	27	19	24	19	430	26	390	166	382
MNNG	1	>5000	>5000	16	24	21	49	53	62	>5000	>5000

§ Mutagenic activity tested in the presence (+) or in the absence (–) of S-9

TABLE ii. Lack of mutagenic activity of aphidicolin in Salmonella typhimurium strains with normal excision repair (uvrB$^+$).

compound	µg/plate	his$^+$ revertants per plate							
		his G46		his D3052		TA 92		TA 94	
		–§	+	–	+	–	+	–	+
Aphidicolin	0	4	8	34	37	48	48	26	43
	20	6	12	31	41	42	45	30	48
	200	7	10	21	46	32	43	33	53
	500	8	13	21	33	38	41	34	38
	1000	6	10	29	32	46	45	35	62
	2000	10	11	29	50	40	42	32	61
Mitomycin C	2.5	6	9	29	33	172	93	340	141

§ Mutagenic activity tested in the presence (+) or in the absence (–) of S-9

colin) indicates that the drug enters the bacterial cell. The results are reported in Table I and II and show the absence of a significant increase in the number of his^+ revertants over spontaneous reversion. We conclude that the compound is to be considered devoid of any mutagenic activity in the system tested.

Metabolic inactivation of aphidicolin by rat liver microsomes.

During the studies of mutagenicity we have found that rat liver microsomes can convert aphidicolin to inactive derivatives. Active aphidicolin can be detected even in the presence of inactive derivative with a very similar chemical structure, by following the inhibition of purified DNA polymerase α (3). The inhibition by aphidicolin *in vitro* is primarily non-competitive with respect to DNA, Mg^{++}, dGTP, dATP and dTTP but competitive with respect to dCTP (14,15); therefore the sensitivity of the assay depends on the concentration of dCTP in the reaction mixture. Figure 1 shows that when the reaction is carried out at 5 or 100 μM dCTP, the concentration of aphidicolin required to inhibit 50 % of the HeLa DNA polymerase α activity *in vitro* is 0.12 and 0.7 μg / ml respectively. Thus our reaction mixtures of 100 μl can easily detect amounts of active aphidicolin as low as 0.01 μg when 5 μM dCTP is used (Fig. 1). We prefer the use of (^{3}H)-dTTP in the assay to avoid the possible incorporation of radioactivity into acid-precipitable phospholipid precursor (16) which

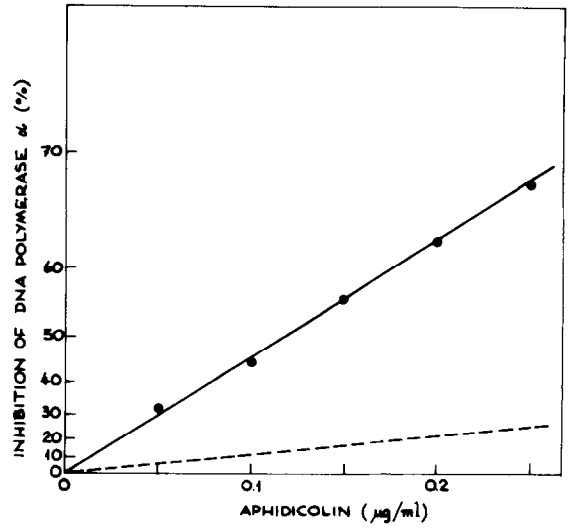

Figure 1. *In vitro* inhibition of HeLa cell DNA polymerase α by aphidicolin. DNA polymerase α was assayed in the presence of the indicated amounts of aphidicolin as described by Pedrali-Noy and Spadari (3) except that dCTP was present either at 5 μM (●——●) or at 100 μM (-----).

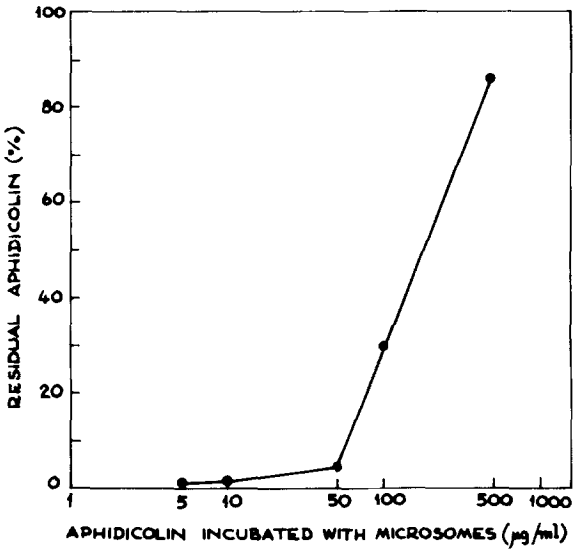

Figure 2. Inactivation of aphidicolin by rat liver microsomes. Increasing amounts of aphidicolin were incubated at 37°C for 30 min with 1 ml of S-9 mix containing 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-P, 4 mM $NADP^+$, 100 mM sodium phosphate, pH 7.4 and 0.1 ml of S-9. One ml of S-9 fraction (15 mg of proteins / ml) contained microsomes from 250 mg of wet liver. The concentration of residual active aphidicolin, following incubation with liver microsomes, was determined by measuring the inhibition of purified HeLa cell DNA polymerase α (see Fig.1).

may occur when (^3H)-dCTP is present and crude enzymic fractions - such as the microsomal preparation - are used.

Figure 2 shows that aphidicolin becomes inactive against DNA polymerase α following incubation with rat liver microsomes. The amount of microsomes obtained from 25 mg of induced rat liver is capable of inactivating 70 µg of aphidicolin within 30 min at 37°C.

Aphidicolin modified by liver microsomes does not induce DNA repair synthesis in HeLa cells.

We have recently shown (12) that aphidicolin does not induce DNA repair synthesis in HeLa cells. The metabolic inactivation of aphidicolin by liver microsomes raised the possibility that the metabolites may have acquired mutagenic properties on mammalian cells. Aphidicolin (50 µg / ml) was therefore incubated for 45 min with liver microsomes as described in the legend to Fig. 2. The inactivation was higher than 95 %. Growing HeLa cells were then exposed to the reaction mixture at final concentrations of aphidicolin plus derivative(s) of 20 and 40 µM - the K_i in vivo for active aphidicolin is 0.2 µM (3,12) - for 15, 30 and 60 min ; to each sample an excess of aphidicolin (20 µM) was added to assure complete inhibition of replicative

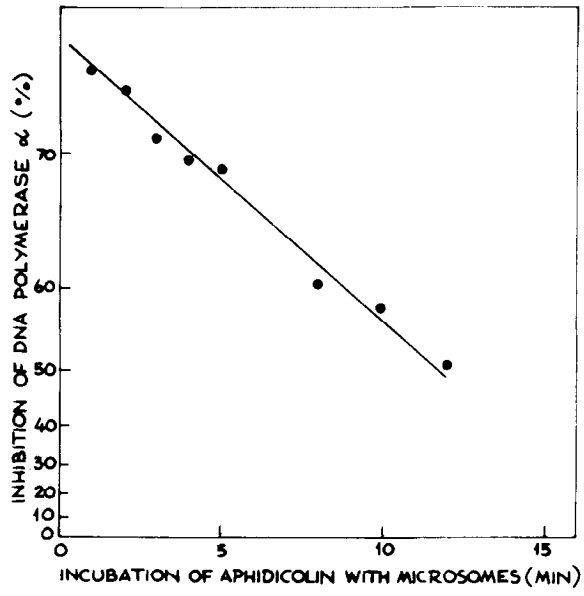

Figure 3. Inactivation kinetics of aphidicolin by rat liver microsomes. Aphidicolin (50 µg / ml) was incubated at 30°C with rat liver microsomes in the reaction mixture described in the legend to Fig. 2. At different times, aliquots of the reaction mixture were removed and assayed for the inhibition of DNA polymerase α (see Fig.1).

DNA synthesis. Both doses of metabolite(s) did not induce DNA repair synthesis. During these experiments we have also observed, as expected, that the metabolites do not inhibit the incorporation of (^{3}H)-thymidine, and therefore DNA synthesis, in HeLa cells. Thus, active aphidicolin can be detected in the presence of inactive derivative(s) also by simply following the inhibition of (^{3}H)-thymidine into DNA by cells in culture.

The inactivation of aphidicolin by microsomes is time- and temperature-dependent and requires $NADP^{+}$ and glucose-6-phosphate.

Figure 3 shows that the inactivation of aphidicolin by liver microsomal oxidases is dependent on time as expected for an enzymic reaction.

Table III shows that the reaction requires temperature, $NADP^{+}$ and glucose-6-phosphate. The presence of 0.02 % sodium azide, a known inhibitor of oxigenases , fully prevents the inactivation of aphidicolin by microsomal oxidases. Controls with DNA polymerase α used to calculate the per cent of inactivation of aphidicolin in the above conditions, contained equivalent amounts of microsomes as treated mixtures . Also a control with microsomes incubated at 37°C for 30 min without aphidicolin ruled out the production of inhibitors or activators of the DNA polymerase α during the incubation period.

TABLE III. Requirements for inactivation of aphidicolin by rat liver microsomes

Conditions	Inactivation of aphidicolin (%)
Complete system*	100
Incubation at 0°C	< 0.5
Microsomes pre-heated at 65°C for 20 min	< 0.5
minus $NADP^+$ and glucose-6-P	< 0.5
plus sodium azide (0.02 %)	< 0.5

* The complete system was as described in the legend to Fig.2.

Aphidicolin, following incubation with liver microsomes, is inactive also against the Herpes simplex DNA polymerase and cellular DNA polymerases β and γ.

We have recently shown that aphidicolin is not only a specific inhibitor of the α-polymerase of animal cells, but also a potent inhibitor of the DNA polymerase coded by Herpes simplex virus which is essential for the replication of the viral DNA (3). We have tested whether the treatment of aphidicolin with rat liver microsomal oxidases, which leads to a loss of activity against the cellular DNA polymerase α, would produce metabolite(s) still active against the Herpes simplex DNA polymerase because a derivative showing a differential inhibition of the viral and cellular α-polymerase would certainly have a clinical use. The results indicate that the modification of the basic structure of aphidicolin by liver microsomal oxidases leads to a loss of activity against both the cellular α- polymerase and the viral DNA polymerase and therefore had not been reported. The metabolic derivative(s) of aphidicolin was also found as inactive against HeLa cell DNA polymerases β and γ as intact aphidicolin (3-5).

Salmonella typhimurium and HeLa cells do not inactivate aphidicolin following incubation for several hours.

The significance of the mutagenesis results and the previous observation that aphidicolin inhibits in vivo the DNA synthesis of animal cells but not bacterial growth (14) is based on the assumption that both bacteria and HeLa cell cultures do not inactivate aphidicolin. This hypothesis was tested by preincubating Salmonella and HeLa cell cultures with predetermined amounts of aphidicolin (10 μg / ml) for 2, 4 and 10 hours. Each culture was then centrifuged and different quantities of supernatant, used in the reaction with DNA polymerase α, gave in both cases degrees of inhibition exac-

tly proportional to the expected amounts of aphidicolin. Thus, contrary to rat liver microsomes, bacteria and HeLa cells do not inactivate aphidicolin. Controls with culture media and supernatants of cultures which were not incubated with aphidicolin , gave no inhibition of the α-polymerase thus ruling out the presence in the media or the production, during cell growth, of inhibitors of the α- polymerase.

CONCLUDING REMARKS

Recent results obtained in our laboratory with aphidicolin, amongst which the selective inhibition of human and viral replicative DNA polymerases (3,4,12,14), the failure to induce DNA repair synthesis in HeLa cells (12), the lack of effect on DNA repair by human cells (12) and the activity against several neoplastic cell lines - including leukemia and melanoma - (Pedrali-Noy,Belvedere,Focher and Spadari, manuscript in preparation) suggested that aphidicolin is a potential anti-cancer drug. In this work we have shown that aphidicolin has another valuable property of a potential anti-cancer drug, because it is not mutagenic in the Ames' Salmonella - microsomes test, contrary to most anti-cancer drugs which are mutagenic and therefore carcinogenic themselves. We have also found that aphidicolin is converted into inactive derivative(s) by rat liver microsomes. These metabolites are however non-mutagenic in the Ames' test and do not induce DNA repair synthesis in human cells. Therefore the possible anti-cancer use of aphidicolin is not hampered by its partial inactivation in liver. This result, coupled with the observation that aphidicolin does not inhibit repairof damaged DNA in human cells (12) indicates that aphidicolin may still offer advantages for clinical use over mutagenic anti-cancer drugs. Also it is difficult from our in vitro results with induced rat liver microsomes to predict the extent of metabolization of aphidicolin in vivo by human liver. It has been reported however, that in the Ames' Salmonella- microsomes test the human S-9 fraction is less active than the S-9 fraction prepared from induced rats (17). Experiments are now taking place in our laboratory to determine the in vivo concentration and localization of active aphidicolin in different tissues and organs at various times following injection of the drug into animals. Our results suggest however that aphidicolin will possibly be clinically useful at concentrations higher than those expected from the studies with normal human and neoplastic cell lines and invite chemical modifications of the drug which might affect its sensitivity to liver microsomes (18), thus leading hopefully, to derivatives more active in vivo than aphidicolin.

A final comment concerns the use of aphidicolin for the evaluation of DNA repair synthesis in damaged human cells as recently proposed by Pedrali-Noy and Spadari (12). Our method relies on the complete inhibition of the replicative DNA synthesis by aphidicolin, thus allowing a simple detection of repair of DNA damaged by mutagenic agents. If the compound under study requires metabolic activation by liver microsomes to show mutagenicity, care should be taken to use amounts of aphidicolin large enough to avoid total inactivation by the microsomal oxidases, thus still assuring a complete inhibition of the replicative DNA synthesis. Alternatively the S-9 mix used for activation should be carefully washed away from cell cultures before addition of aphidicolin.

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