[CANCER RESEARCH 28, 166-169, February 1968]

The Induction of Tumors in the Hybrid *Nicotiana glauca* x *N. langsdorffii* Plants by 6-Azauracil and Its Reversal by Uracil and Actinomycin D[1]

M. Buiatti[2]

Biology Department, Brookhaven National Laboratory, Upton, New York 11973

SUMMARY

Azauracil was found to be the only one of several analogs of nucleic acid precursors tested in these studies which is capable of inducing tumors at a very early stage during seedling development in the genetically tumor-conditioned hybrid *Nicotiana glauca* × *N. langsdorffii*. Tumor induction by 6-azauracil was shown to be reversed by simultaneous treatments with uracil and actinomycin D as well as by posttreatments. Pretreatments with the same substances proved to be ineffective.

The possible implications of the findings in view of the possible involvement of an abnormal species of RNA in the process of tumor induction by 6-azauracil are discussed.

INTRODUCTION

Certain interspecific hybrids of Nicotiana spontaneously form tumors at late stages of development. However, tumors can be induced to appear at earlier stages by stress conditions (13), ionizing radiations (1), tumor tissue extracts[3], and chemical treatment (11).

The process of tumor induction and development has been shown to be under genetic control, and present available evidence suggests that it is due to abnormalities in gene function rather than somatic mutations of gene structure. More specifically, Schaeffer (11) has suggested that an altered nucleic acid metabolism might be responsible for the hormone-induced acceleration of tumor induction.

The present study deals with the action of substances involved in nucleic acid metabolism on tumor induction in the hybrid amphiploid *Nicotiana glauca* × *N. langsdorffii* and discusses implications relative to the possible involvement of a hypothetical abnormal RNA in the process of tumor formation.

MATERIALS AND METHODS

Seeds of the amphiploid hybrid *Nicotiana glauca* × *N. langsdorffii* were presoaked for 48 hr in distilled water and then treated for 24 hr with the following compounds: 6-azauracil (10^{-3} M), 2-aminopurine (10^{-3} and 10^{-2} M), 5-bromouracil (10^{-3} M and 10^{-2} M), 8-azaadenine (10^{-3} M), 5-fluorouracil (10^{-3} M and 10^{-2} M), uracil (10^{-3} and 10^{-2} M), and actinomycin D (20 mg/liter).

The commercial sources of the compounds used were as follows: Calbiochem (6-azauracil, 2-aminopurine, 5-bromouracil, and 5-fluorouracil); Nutritional Biochemical Corp. (8-azaguanine); Amend Drug and Chemical Co. (uracil); and Merck, Sharp & Dohme (actinomycin D in the form of Lyovac-Cosmegen). In addition to using these compounds individually, seeds were also treated with actinomycin D and uracil for 24-hr periods before, simultaneously with, and after treatments with 6-azauracil.

After treatment the seeds were thoroughly washed in running tap water for 30 min and then sown in Petri dishes (50 seeds per dish) on three layers of Whatman No. 1 filter paper moistened with distilled water. The Petri dishes were kept in a controlled environment chamber (continuous illumination, 600 foot-candles, 75°F). Each experiment was replicated three times, and at least two different experiments were carried out with each treatment. In the controls, seeds were treated with water alone for different periods according to the comparable chemical treatment.

Data on germination were taken 5, 10, 15, and 20 days after sowing. After 21 days the seedlings were harvested, scored for tumors, and fresh weights were measured.

RESULTS

In Table 1 the fresh weights and frequencies of seedlings with tumors following treatments with different analogs of nucleic acid precursors are presented. Chart 1 shows graphically the effects of the same treatments on germination. Only 6-azauracil was able to induce tumors under our experimental conditions (Table 1). With this compound tumors started to appear 10 days after treatment as green undifferentiated masses at the epicotyledonary region, and the frequency of seedlings with tumors continued to increase up to 16–18 days after sow-

---

[1] Research performed at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

[2] Present address: Instituto di Genetica dell'Universita, Pisa, Italy.

[3] M. Buiatti, C. R. Bhatia, and H. H. Smith. Effects of Tumor and Leaf Extracts on Tumor Induction in an Interspecific Hybrid of Nicotiana, manuscript in preparation.

Received April 6, 1967; accepted September 29, 1967.

Table 1

Treatment	Average fresh weight of 3 seedlings 21 days after treatment (mg)	Percentage of seedlings showing tumors 20 days after treatment
Control	7.28 ± 1.5	0.00
8-Azaadenine	5.64 ± 0.8	0.00
6-Azauracil	5.03 ± 0.7	55.86 ± 0.40
2-Aminopurine	7.11 ± 0.5	0.00
5-Bromouracil	9.08 ± 2.1	0.00

Fresh weight frequency of tumors in seedlings of the amphiploid *Nicotiana glauca* × *N. langsdorffii* treated with analogs of nucleic acid precursors.

ing. No tumors were observed in control seedlings, under our conditions, for periods up to 45 days after sowing. Also, when seeds of different genotypes (*Nicotiana glauca, Nicotiana tabacum,* and a mutant of the *Nicotiana glauca* × *N. langsdorffii* amphiploid hybrid which does not produce tumors) were subjected to 6-azauracil treatment, no tumors were observed. Hence, a tumor-conditioned genotype seems to be a prerequisite for 6-azauracil action. Particularly interesting in this respect is the lack of tumor formation following 6-azauracil treatment in the nontumorous mutant. According to recent evidence, this genotype is apparently intermediate, as far as tumor conditioning is concerned, between the parent species and the tumorous hybrid.[3]

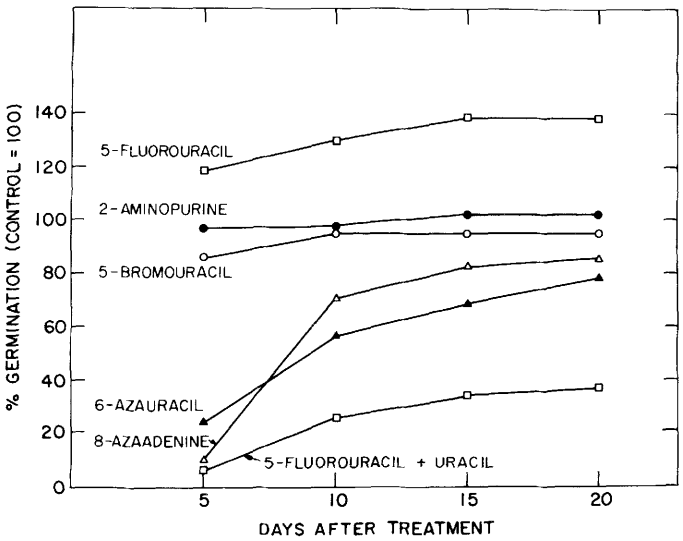

Chart 1. Effect of base analogs on germination of *Nicotiana glauca* × *N. langsdorffii.*

As shown in Chart 1, inhibition of germination occurred following treatment with 6-azauracil, with 8-azaadenine, and with 5-fluorouracil when given in combination with uracil. Fluorouracil alone showed a slight but statistically significant increase in germination, a phenomenon which is presently under investigation.

Neither 2-aminopurine nor 5-bromouracil showed any statistically significant effect on our material. This is in accordance with the lack of effect on similar material of another analog of DNA precursors, 5-iododeoxyuridine, shown by Schaeffer (11). It should be pointed out, however, that the possibility of permeability factors playing a decisive role in our case cannot, at present, be excluded.

As shown in Table 1, seedling growth (as measured by seedling fresh weight 21 days after treatment) was inhibited by 6-azauracil and, to a lesser extent, by 8-azaadenine. With 6-azauracil treatment, however, only those seedlings showing tumors had fresh weights lower than the control. This reduction in growth was mainly attributed, therefore, to the process of tumor formation *per se.* In general, inhibition of growth was more evident at early stages of development, as measured by seed germination, than at later stages, as measured by seedling weight. This demonstrated the ability of the seedlings to recover from the general growth inhibition induced during the brief seed treatments with analogs of nucleic acid precursors.

In an effort to clarify the mode of action of 6-azauracil in the process of tumor induction, a series of treatments in which this compound was combined with uracil and actinomycin D [known to inhibit DNA-primed RNA synthesis (3)] was designated as follows: (*a*) 24-hr treatment with 10^{-3} M azauracil in combination with either 10^{-2} M uracil or 20 mg/liter actinomycin D; (*b*) 24-hr treatment with 10^{-3} M azauracil followed by a 24-hr posttreatment with uracil or actinomycin D at the above concentrations; and (*c*) 24-hr treatment with either uracil or actinomycin D at the above concentrations followed by a 24-hr posttreatment with 6-azauracil 10^{-3} M. The results of such experiments are shown in Charts 2 and 3 and in Table 2.

As shown in Chart 2, uracil is able to partially reverse the germination inhibition (as shown by the data taken 5 days after treatment) when given at the same time as or after 6-azauracil treatment. Also, tumor induction by 6-azauracil

Table 2

	Azauracil (10^{-3} M)	Azauracil (10^{-3} M) + uracil (10^{-2} M)	Azauracil (10^{-3} M) followed by uracil (10^{-2} M)	Uracil (10^{-2} M) followed by azauracil (10^{-3} M)
% Seedling with tumors	55.98 ± 0.40	10.19 ± 2.48	11.06 ± 3.04	45.20 ± 5.89
	Azauracil (10^{-3} M)	Azauracil (10^{-3} M) + actinomycin (20 mg/liter)	Azauracil (10^{-3} M) followed by actinomycin (20 mg/liter)	Actinomycin (20 mg/liter) followed by azauracil (10^{-3} M)
% Seedling with tumors	55.98 ± 0.40	16.51 ± 1.54	2.68 ± 0.32	55.12 ± 7.20

Effect of 6-azauracil on tumor formation and the influence of combined treatments with uracil and actinomycin D.

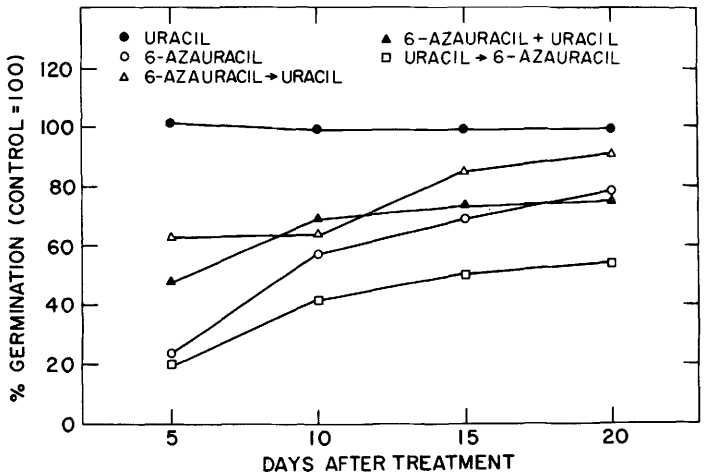

Chart 2. Effect of uracil on germination inhibition induced by 6-azauracil in *Nicotiana glauca* × *N. langsdorffii.*

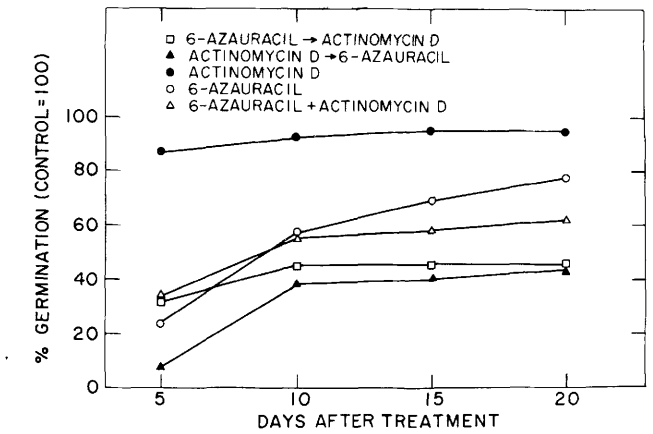

Chart 3. Effect of actinomycin D on germination inhibition induced by 6-azauracil in *Nicotiana glauca* × *N. langsdorffii.*

was reduced by approximately 80% by the same uracil treatments (Table 2). The reversal of azauracil-induced growth inhibition and tumor formation by uracil posttreatment rules out the possibility that uracil might be acting simply on 6-azauracil absorption into the seeds and suggests a subsequent competitive function.

As evident from Chart 3 and Table 2, actinomycin D, while having only a slight inhibitory effect on germination, when given alone not only failed to revert the germination inhibition induced by 6-azauracil, but, enhanced it, particularly when given as a pretreatment.

On the other hand, tumor induction, although not affected by pretreatment, was strikingly reduced when actinomycin was given together with 6-azauracil, and almost completely suppressed when the inhibitor was used as a posttreatment.

DISCUSSION

6-Azauracil has been shown to inhibit RNA synthesis by affecting orotidylic acid decarboxylase in normal and leukemic animal cells (2, 4) and in cocklebur leaf discs (9). Moreover, it has been shown to be incorporated in RNA in Arabidopsis (G. P. Rédei, personal communication) and cocklebur (10). Incorporation into RNA has been suggested as the possible cause for the phenotypic reversion of a variegated mutant in Arabidopsis (G. P. Rédei, personal communication). A further morphogenetic effect on fern gametophyte differentiation has also been reported (8). Other analogs of nucleic acid precursors are known to be carcinogenic in mammals and to stimulate tumor growth in genetic hybrids of Nicotiana (14). Thiouracil was shown to induce thyroid tumors in rats (7), and low dosages of 5-fluorouracil and 5-mercaptouracil were shown to stimulate tumor growth when included in the growth medium for plants of the hybrid *Nicotiana glauca* × *N. langsdorffii* (14). In studies on animal tumors, increasing evidence pointing to an involvement of abnormalities in RNA synthesis during the process of tumor induction is being accumulated. Kidson and Kirby (5) have shown altered patterns of messenger RNA synthesis during carcinogenesis by 4′-fluoro-4-dimethylaminoazobenzene. Binding of another carcinogen (2-acetylaminofluorene) to rat liver ribonucleic acid has also been repeatedly shown (6) and actinomycin D has been proven to inhibit induced carcinogenesis. Our experiments on genetic tumors show that an analog of uracil, known to be incorporated into RNA (G. P. Rédei, personal communication; and Ref. 10), is able to induce tumor formation in seedlings of the amphiploid *Nicotiana glauca* × *N. langsdorffii.*

This process is inhibited by simultaneous treatment and posttreatment with either uracil or actinomycin D. It seems reasonable to hypothesize that uracil exerts its action through competitive inhibition of the azauracil effect. A possible effect on absorption seems to be ruled out by the efficiency of uracil posttreatment. On the other hand, actinomycin D is supposed to inhibit DNA-primed RNA synthesis (3); we can then postulate as a working hypothesis that in our case actinomycin D is able to revert the induction of tumors by azauracil by inhibiting either the synthesis of an azauracil-containing abnormal RNA or the formation of a "tumorous" RNA in consequence of the action of azauracil on RNA synthesis. The latter hypothesis could be supported by the fact that actinomycin D is most active when given as a posttreatment. It is clear, however, that more detailed data are needed on the time-course of actinomycin action and on the effect of 6-azauracil on RNA synthesis in our experimental system. The overall impression of the data obtained to date, that RNA metabolism rather than DNA metabolism is involved, suggests that alterations in gene function rather than gene structure, such as somatic mutations, are necessary for tumor induction in genetically tumor-conditioned hybrids of Nicotiana. This notion is also supported by the great variation observed between electrophoretic patterns of protein from normal and tumorous tissues (C. R. Bhatia, M. Buiatti, and H. H. Smith. Electrophoretic Variation in Proteins and Enzymes of the Tumor Forming Hybrid *Nicotiana glauca* × *N. langsdorffii* and Its Parent Species, manuscript in preparation).

ACKNOWLEDGMENTS

Thanks are due to Dr. H. H. Smith for continuous encourage-

ment and helpful discussion, to Dr. Sanford Lacks for critical reading of the manuscript, and to Dr. Yoshihiko Hirono for discussion of the initial data.

REFERENCES

1. Ahuja, M. R., and Cameron, D. R. The Effects of X-irradiation on Seedling Tumor Production in *Nicotiana* Species and Hybrids. Radiation Botany, *3:* 55–57, 1963.
2. Bono, V. H. Jr., Weissmann, S. M., and Frei, E. III. The Effect of 6-Azauridine Administration on *de novo* Pyrimidine Production in Chronic Myelogenous Leukemia. J. Clin. Invest., *43:* 1486–1494, 1964.
3. Goldberg, I. H., Rabinowitz, M., and Reich, E. Actinomycin Action. I. Binding and Inhibition of RNA Polymerase Synthetic Reaction by Actinomycin. Proc. Natl. Acad. Sci. U. S., *49:* 2094–2101, 1962.
4. Handschumacher, R. E. Orotydilic Acid Decarboxylase Inhibition Studies with Azauridine-5′-Phosphate. J. Biol. Chem., *235:* 2917–2919, 1960.
5. Kidson, C., and Kirby, K. S. Selective Alteration of Rapidly Labelled Ribonucleic Acid Synthesis in Rat Liver during Azo Dye Carcinogenesis. Cancer Res., *25:* 472–476, 1965.
6. Marroquin, F., and Farber, E. The Binding of 2-Acetylaminofluorene to Rat Liver Ribonucleic Acid *in Vivo*. Cancer Res., *25:* 1262–1269, 1965.
7. Money, W. L., and Rawson, R. W. The Experimental Production of Thyroid Tumors in the Rat Exposed to Prolonged Treatment with Thiouracil. J. Nutrition, *40:* 321–335, 1950.
8. Raghavan, V. Differentiation in Fern Gametophytes Treated with Purine and Pyrimidine Analogue. Science, *126:* 1690–1691, 1964.
9. Ross, C. Influence of 6-Azauracil on Pyrimidine Metabolism of Cocklebur Leaf Discs. Biochim. Biophys. Acta, *87:* 564–573, 1964.
10. Ross, C. Metabolism of 6-Azauracil and Its Incorporation into RNA in the Cocklebur. Phytochemistry, *3:* 603–607, 1964.
11. Schaeffer, G. W. Tumor Induction by an IAA-Kinetin Interaction in *Nicotiana* Hybrids. Nature, *196:* 1326–1327, 1962.
12. Smith, H. H., and Kugelman, B. H. Autoradiographic Evidence for Incorporation of 2-Aminopurine into DNA and RNA of Plant Cells. Cytologia, *29:* 435–442, 1964.
13. Smith, H. H. Recent Cytogenetic Studies in the Genus *Nicotiana*. Advan. Genetics, 14.
14. Tso, T. C., and Burk, L. G. Effects of Certain Anti-Tumor Chemicals on a Tumorous *Nicotiana* Hybrid. Nature, *193:* 1204–1205, 1962.