ACTA SOCIETATIS
BOTANICORUM POLONIA
Vol. 52, nr 1: 61-69
1983

Effect of N-hydroxyurea, mitomycin C and actinomycin D on tumour formation on the leaves of *Kalanchoe daigremontiana*

JÓZEF KOWALCZYK

Institute of Physiology and Cytology, Łódź University,
Banacha 12/16, 90-237 Łódź, Poland *

(Received: January 28, 1982)

Abstract

The leaves of *Kalanchoe daigremontiana* wounded and infected with *Agrobacterium tumefaciens* were treated with single doses of inhibitors (hydroxyurea — 190, mitomycin — 0.5, actinomycin — 2 μg per leaf). After delaying the time of dosage of inhibitors during five days after inoculation, changes in susceptibility of the system to antitumorous activity of analysed compounds were observed. In several hours after inoculation (period of the bacteria metabolic activity in wounds) all the inhibitors prevent strongly the tumour formation. At the time between 14 and 72 hours after inoculation, including the phase of tumour induction, the system becomes sensitive to the DNA synthesis inhibitors, particularly hydroxyurea. The intensified action of actinomycin appears again only about 60 hours after inoculation and lasts till the end of experiment (the initiation of the transformed plant cell proliferation).

According to the literature the antitumorous effect of inhibitors could be connected with their action on the bacteria metabolism inside the host tissue. The activities of hydroxyurea and mitomycin in the second period correspond with the intensive DNA synthesis in plant cells, which is induced by wounding. The effect of actinomycin D in 60 hours after inoculation could depend upon the inhibition of the proliferation of the transformed host cells.

INTRODUCTION

Lots of experiments were held on the inhibition of crown-gall transformation caused by various antibiotics and metabolic inhibitors. The aim of all these studies was to find an effective antitumour agent and to describe the mechanisms of transformation of the normal plant cell into the tumour one under the action of *Agrobacterium tumefaciens*. However the results of these studies give only information whether

* present address: Łagniewniki 82, 98-310 Czarnożyły, woj. sieradzkie, Poland.

an inhibitor inhibits only the tumour initiation or the growth of already transformed plant cells (De Ropp 1948, Gribnau and Veldstra 1969, Abo-El-Dahab et al. 1977) and there is scarcely any more detailed data on the degree of the inhibitor action effectivity in the succesive steps of the crown-gall transformation.

Hydroxyurea (HU), the DNA synthesis inhibitor, inhibits the bacteria tumour formation on the sunflower stems and primary leaves of the "Pinto" bean (Rennert 1978, 1980). According to the results HU is active during the induction phase, when it inhibits the DNA synthesis in the host cells. The HU activity period during the tumorigenesis differs from the activity periods of other nucleic acid synthesis inhibitors. It allows to analyse the sequence of different molecular processes in the course of tumour formation. If it was to be true in the other host-pathogen systems HU could play the essential role in the crown-gall investigations.

MATERIAL AND METHODS

Bacteria number. *Agrobacterium tumefaciens* (Smith and Town.) Conn, the virulent strain CCM 1037 from the Czechoslovak Collection of Microorganisms in Brno, was cultured on an agar medium, in darkness, at 27°C (Bopp 1965). The 48-hour culture was used for the further experiments. The bacteria cell suspension was obtained by the appropriate dilution with sterile water. The cell number in 1 cm^3 of the sespension was determined by the method of series dilutions and plating on agar (Lippincott and Lippincott 1970).

Test plants. *Kalanchoe daigremontiana* Ham. et Perr. (Balley 1949) with 5-7 leaves pairs were cultivated and infected at the constant temperature (22°C) and relative air humidity (70-80%), in the day light and further fluorescence illumination (0.02 W cm^{-2}, 7^{00}-21^{00} h).

Inoculation. The plants were infected according to Beiderbeck's method (1970a). The pair of upper, well developed leaves (6-10 cm) was moistened with 1.5% Tween 80 water solution. Then each leaf half was pierced once with small brush with 30 needles, 1/3-mm long, set in 3 rows, 3.5 mm apart. After the wounding 0.1 cm^3 of the *A. tumefaciens* suspension was spread with a glass rod on the leaf surface. The bacteria density in the suspension was 5×10^7 cells/ml.

Leave treatment with inhibitors. Aqueous solutions of N-hydroxyurea (HU Schuchardt firm, München), actinomycin D (AD), and mitomycin C (MC) (both — Sigma Chemical Company), containing in 1 cm^3: HU — 3.8 mg, MC — 10 μg, AD — 40 μg were used. The drops of these solutions were spread with a glass rod on the leaf surface in amounts of: 190 μg HU, 0.5 μg MC, 2 μg AD per leaf. The leaf moistening

with HU, MC and AD solutions was performed once but at various times, before or after inoculation with *A. tumefaciens* in aim to determine the maximum activity periods of the tested compounds in the course of transformation. The tumour number was determined 14 days after inoculation with magnifying glass (at an eightfold magnification). The experimental variants (10 leaves each) were repeated 4-5 times. Transversal lines in diagrams indicate standard error of arithmetic mean calculated according to B r o d a (1976).

RESULTS

HU reduces considerably the number of tumours induced by *A. tumefaciens* on the *K. daigremontiana* leaves (Fig. 1). The susceptibility of the system to the HU doses used in the experiment occurs from 24 h before to 96 h after inoculation. During this time two periods of higher susceptibility are clearly distinguished. The first begins about 2 hours before inoculation and lasts during next 4-6 hours. During this time the HU treatment reduces the number of tumours to 30%. The second begins 12 hours later and lasts to 60 hours after inoculation. Then the HU treatment gives 60-70% decrease in the tumour number, which thereafter is constant (Fig. 1). After this period the effectivity of the HU action significantly decreases. The HU treatment 4 days after inoculation causes only the reduction of tumour number of a dozen or so per cent.

Figure 2 shows the results of the experiment with mitomycin (MC) used in the same way as HU. The general course of the curves for HU and MC is similar (Fig. 1). In the case of MC action there are also two periods of maximal susceptibility of the analysed crown-gall transformation system. As both HU and MC are the DNA synthesis inhibitors the similarity of the results seems to be evident. In the periods of their strongest influence the occurence of DNA biosynthesis indispensable to tumour formation can be expected.

Also actinomycin (AD) is the agent inhibiting crown-gall tumour formation (K u r k d j i a n et al. 1975, R e n n e r t 1980). As AD inhibits DNA transcription not replication (H a r b e r s and M ü l l e r 1962) its effect should be different from HU or MC. There is a data sugesting that this antibiotic acts in the last tumorigenesis phase connected with the beginning of proliferation of the already transformed plant cells (K u r k d j i a n et al. 1975, R e n n e r t 1980). Figure 3 illustrates the results confirming this view. Except the short period of AD action close to the inoculation, its stronger effect occurs but 60 hours after inoculation and lasts till the end of experiment (5 days after inoculation).

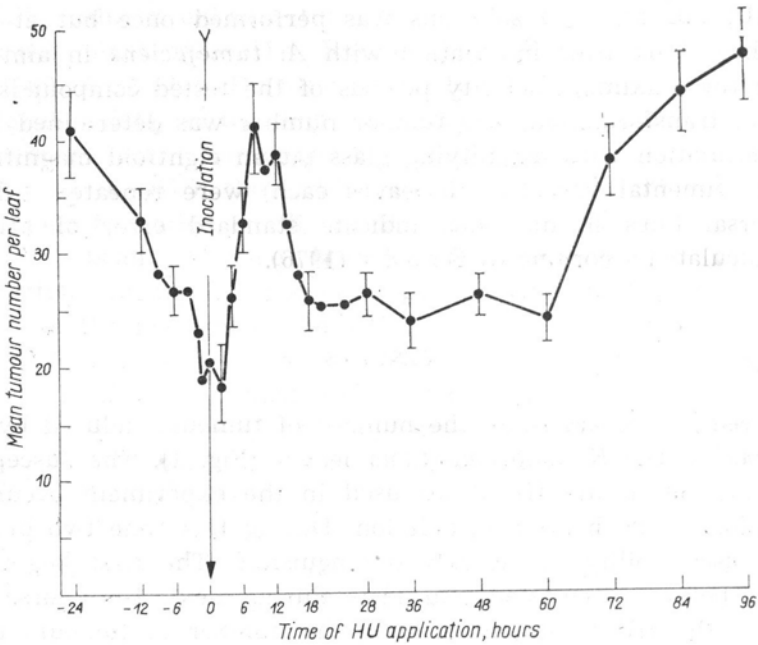

Fig. 1. Inhibition of tumour initiation on the leaves of *K. daigremontiana* treated with hydroxyurea (HU)

The *K. daigremontiana leaves* were moistened with HU solution (5×10^{-2} M), 0.05 ml per leaf. The treatment was performed once but at various times before and after leaf inoculation with virulent *A. tumefaciens* strain. The plants were prepared and the inoculation performed according to Beiderbeck's method (1970a). The tumour number was determined 14 days after inoculation. All the details are given above in the paper. The curve illustrates the mean results of 3 experimental series 10 replications (10 leaves) each. Transversal lines indicate standard errors of arithmetic means of the integrated experimental series, calculated according to Broda (1976). The mean number of control tumours is 57.7 ± 3.04

The HU and MC action on the crown-gall transformation on the *K. daigremontiana* leaves, when compared, show significant differences. The MC action is much stronger than the HU one in the period preceding leaf inoculation and a dozen or so hours after it. Between 16 and 60 hours after inoculation, when HU activity curve forms characteristic plateau (Fig. 1) the MC antitumoric effect is usually weaker than that of HU. The second period of stronger system susceptibility to MC activity occurs 20 hours later (36 hours after inoculation) and lasts considerably longer (to about 72 hours) than in the case of HU. The differences are probably caused by different action mechanisms of the analysed inhibitors.

It can be stated, when comparing the HU and MC effects with that of AD, that the degree of the tumour number reduction by AD is similar to that of HU in the period preceding inoculation. AD acts stronger just before and after inoculation. The different AD action occurs but in the further tumorigenesis stages. AD is only slightly effective in the period of HU plateau and where there is the peak of

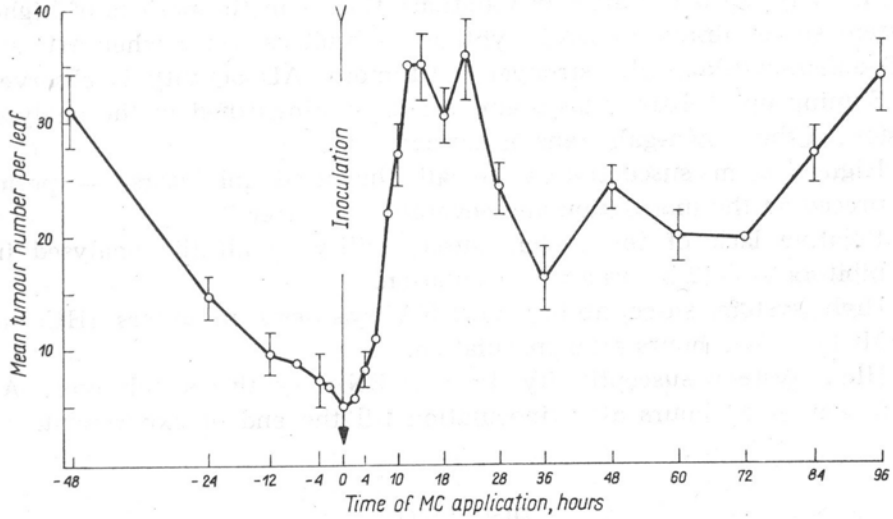

Fig. 2. Inhibition of tumour initiation on the leaves of *K. daigremontiana* treated with mitomycin C (MC)

The *K. daigremontiana* leaves were moistened with MC solution (10 μg/ml), 0.05 ml per leaf; other parameters as in Fig. 1. The mean number of control tumours is 55.0±3.07

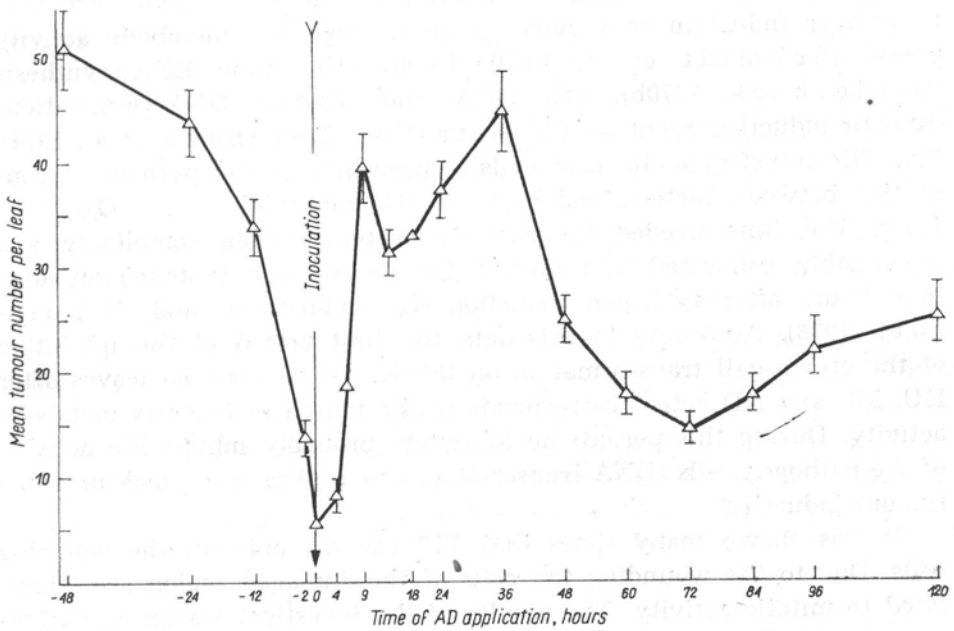

Fig. 3. Inhibition of tumour initiation on the leaves of *K. daigremontiana* treated with actinomycin D (AD)

The *K. daigremontiana* leaves were moistened with AD solution (40 μg/ml), 0.05 ml per leaf; other parameters as in Fig. 1. The mean number of control tumours is 55.9±3.06

MC activity (36 hours after inoculation), that is in the periods of higher system susceptibility to DNA synthesis inhibitors. Only when HU and MC action weakens the stronger antitumoric AD activity is observed.

Suming up, 4 different systems can be distinguished in the analysed system of the crown-gall transformation:

1. High system susceptibility to all the used inhibitors — period preceding the inoculation and several hours after it.
2. Relative lack of the system susceptibility to all the analysed inhibitors — 8-12 hours after inoculation.
3. High system susceptibility to DNA synthesis inhibitors (HU and MC) — 14-72 hours after inoculation.
4. High system susceptibility to the RNA synthesis inhibitor, AD action — 60 hours after inoculation till the end of experiment.

DISCUSSION

The full transformation cycle lasts for 5 days in the most of known systems of the crown-gall transformation testing (Klein 1957, Lipetz 1966). The susceptibility of *K. daigremontiana* leaves to antitumoric effect of the used doses of HU and MC occurs within the same period. Before the bacteria, living in the host tissues, are able to tumour induction they have to go through the metabolic activity period (Schmidt et al. 1969). During this time RNA synthesis (Beiderbeck 1970b), cell DNA and plasmid DNA replications (tumour induction agent — TIP) occur (Van Larebeke et al. 1974). The TIP transfer to the host cells is preceded by the permanent connection between bacteria and host cell (Głogowsky and Galsky 1978). The time needed by virulent bacteria to be completely and irreversibly connected (determined for the various systems) amounts to 6 hours after pathogen induction (Lippincott and Lippincott 1978). According to this data the first period of the inhibition of the crown-gall transformation on the *K. daigremontiana* leaves after HU, MC and AD action corresponds to the period of bacteria metabolic activity. During this period the inhibitors probably inhibit life activity of the pathogen cells (DNA transcription and replication) which prevents tumour induction.

It was shown many times that TIP can act only on the wounded cells. Due to the wounding the cells of the damaged region are stimulated to mitotic activity. The results of the investigations on *K. daigremontiana* indicate that the stage of maximum host susceptibility to the tumour transformation occurs before the first cell division induced by wounding (Lipetz 1966). The other data show that DNA synthesis phase is equally important (Bopp 1964, Kupila-Ahvenniemi

and Therman 1971). The activation of DNA synthesis was observed during the healing processes in many plant materials. It takes place between the first and the third day after wounding (Lipetz 1967, Kupila-Ahvenniemi and Therman 1971). It was also determined that DNA synthesis which is caused by wounding stimulus and preceds divisions occuring close to the wound is characterised by two waves (Lipetz 1967, Broekaert and Van Parijs 1973). That is why the second period of higher susceptibility of the analysed system of the crown-gall transformation to HU and MC corresponds with the period of DNA synthesis in the host cells. The characteristic plateau of the curve of tumour initiation inhibition by HU is in accordance with the first and second wave of DNA synthesis in the cells of *K. daigremontiana* stems (Lipetz 1967), stems of *Vicia faba* (Kupila-Ahvenniemi and Therman 1971), in the *Pisum sativum* seedlings (Broekaert and Van Parijs 1973) and in the carrot root discs (Hase et al. 1979). The synthesis begins 9-12 hours after wounding, falls gradually after 48 hours and declines on the third day of the experiment. However MC activity during this period begins a little bit later than HU activity. It is probably connected with the stronger MC effect on the divisions, close to the wound. Lipetz proved (1966) that in *K. daigremonitiana* the first divisions occur 36 hours after wounding. MC antitumorous effect is marked more clearly at this time. According to the classical division of the crown-gall transformation into three phases (Klein 1957), the second and the third day of this process corresponds with induction phase. The results of author's investigations indicate that this phase is characterized by the strong susceptibility to the DNA synthesis inhibitors and weak susceptibility to AD action.

The results presented in this paper are in accordance with the previous information on HU activity in the tumour transformation process. The only difference concerns the period of bacteria metabolic activity, when HU acted considerably more strongly in the *K. daigremontiana* leaves than in the other investigated biological systems (Rennert 1978, 1980). However in the previous investigations the inocula with higher densities of *A. tumefaciens* cells were used (Rennert 1978). It was also proved that effectiveness of HU action is inversly proportional to the inoculum density (Rennert 1980).

REFERENCES

Abo-El-Dahab M. K., El-Goorani M. A., El-Wakil M. A., 1977. Effect of certain chemicals on the *in vitro* growth of *Agrobacterium tumefaciens* and on the gall formation on artificially infected plants. Egyptian J. Pathol. 9: 43-57.

Balley L. H., 1949. Manual of cultivated plants. The Macmillan Company. New York, pp. 466-467.

Beiderbeck R., 1970a. I Quantitative Bestimmung des Infektionserfolge verschieden vorbehandelter Bakterien mit dem Igel-Test. Z. Naturforsch. 25b: 407-411.

Beiderbeck R., 1970b. Rifampicin und ein resistenter Klon von *A. tumefaciens* bei der Tumorinduction. Z. Naturforsch. 25b: 1457-1460.

Bopp M., 1964. Hemmung der Crown-Gall-Entstehung durch Fluorodeoxyuridine. Z. Naturforsch. 19b: 64-71.

Bopp M., 1965. Die Hemmung von *Agrobacterium tumefaciens* durch D-Aminosaüren. Z. Naturforsch. 20b: 899-905.

Broda B., 1976. Przewodnik do obliczeń statystycznych w biologii. 2 ed., vol. 88, AM w Łodzi, pp. 3-46.

Broekaert D., Van Parijs R., 1973. Crown-gall genesis in *Pisum sativum* L.: Histological observations and histophotometric DNA measurements. Medel Fak. Landbowetensch. Gent. 38: 343-360.

De Ropp R. S., 1948. Action of streptomycin on plant tumours. Nature 162: 459-460.

Głogowsky W., Galsky A. G., 1978. *Agrobacterium tumefaciens* site attachment as necessary prerequisite for crown-gall tumor formation on potato discs. Plant Physiol. 61: 1031-1033.

Gribnau A. G. M., Veldstra H., 1969. The influence of mitomycin C on the induction of crown-gall tumors. FEBS Letters 3: 115-117.

Harbers E., Müller W., 1962. On the inhibition of RNA synthesis by actinomycin. Biochem. Biophys. Res. Commun. 7: 107-110.

Hase Y., Yakaura K., Tanifuji, 1979. Differential replication of satelite and main band DNA during early stages of callus formation in carrot tissue. Plant Cell Physiol. 20: 1461-1469.

Klein R. M., 1957. The activation of metabolic systems during crown-gall tumour-cell formation. Proc. Natn. Acad. Sci. U.S. 43: 956-960.

Kupila-Ahvenniemi S., Therman E., 1971. First DNA synthesis around sterile and crown-gall inoculated wounds in *Vicia faba*. Physiol. Plant. 24: 23-26.

Kurkdjian A., Manigault P., Beardsley R. E., 1975. Transformation tumorale chez le pois: passage d'un etat precancereux a l'etat cancereux. Can. J. Bot. 53: 3002-3011.

Lipetz J., 1966. Crown-gall tumorigenesis. II. Relation between wound healing and tumorigenic response. Cancer Res. 26: 1597-1605.

Lipetz J., 1967. Wound healing in *Kalanchoe*. Ann. N. Y. Acad. Sci. 144: 320-325.

Lippincott J. A., Lippincott B. B., 1970. Enhanced tumor initiation by mixtures of tumorigenic and nontumorigenic strains of *Agrobacterium*. Infec. Immun. 2: 623-630.

Lippincott J. A., Lippincott B. B., 1978. Tumor initiation complementation on bean leaves by mixtures of tumorigenic and nontumorigenic *Agrobacterium* rhizogenes. Phytopathol. 68: 365-370.

Rennert A., 1978. Influence of N-hydroxyurea on the growth of seedlings and the process of crown-gall tumour formation on sunflower plants. Acta Soc. Bot. Pol. 47: 51-63.

Rennert A., 1980. N-hydroxyurea, mitomycin C and actinomycin D activity in the process of tumour formation on the primary leaves of the "Pinto" bean. Acta Soc. Bot. Pol. 49: 63-76.

Schmidt R. M., Lippincott B. B., Lippincott J. A., 1969. Growth requirement and infectivity of auxotrophic adenine-dependent mutants of *Agrobacterium tumefaciens*. Phytopathol. 59: 1451-1454.

Van Larebeke N., Engler C., Holsters M., Van den Elsacker S., Schilperoort R. A., Schell J., 1974. Large plazmid in *Agrobacterium tumefaciens* essential for crown-gall-inducing ability. Nature 252: 169-170.

Wpływ N-hydroksymocznika, mitomycyny C i aktynomycyny D na proces tworzenia tumorów na liściach Kalanchoe daigremontiana

Streszczenie

Przebieg transformacji tumorowej, wzbudzanej przez *Agrobacterium tumefaciens*, badano na liściach *Kalanchoe daigremontiana* stosując ilościową procedurę (Beiderbeck 1970). W celu określenia przeciwtumorowej aktywności niektórych inhibitorów, liście inokulowane zawiesiną wirulentnych bakterii traktowano pojedynczą dawką hydroksymocznika, mitomycyny C lub aktynomycyny D. Traktowanie to prowadziło do redukcji liczby powstających tumorów. Zmieniając czas stosowania inhibitorów w ciągu pięciu dni po inokulacji (czas transformacji crown-gall), stwierdzono zmiany wrażliwości systemu na antytumorowy wpływ badanych związków. W czasie pierwszych godzin po inokulacji wszystkie inhibitory silnie hamują tworzenie tumorów. W okresie między 14 i 72 godziną po inokulacji, obejmującym fazę indukcji tumorowej, system staje się bardziej wrażliwy na inhibitory syntezy DNA, szczególnie hydroksymocznik. Powtórne nasilenie działania aktynomycyny pojawia się dopiero około 60 godzin po inokulacji i utrzymuje się aż do końca eksperymentu.

Według danych literatury, antytumorowy efekt inhibitorów w pierwszym okresie można powiązać z bezpośrednim ich działaniem na metabolizm bakterii wewnątrz tkanki gospodarza. Aktywność hydroksymocznika i mitomycyny w drugim okresie zbiega się z intensywną syntezą DNA, wywołaną bodźcem zranienia w komórkach roślinnych, natomiast skuteczność działania aktynomycyny po 60 godzinach od inokulacji polega prawdopodobnie na hamowaniu etapu zapoczątkowania proliferacji przeobrażonych komórek gospodarza.