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Abstract

We describe the *in vivo* antitumor effect of the compound BG-8 on the induction and/or development of *Agrobacterium tumefaciens*- induced tumors on pea seedlings and *Chrysanthemum* plantlets. A single treatment applied before or after bacterial infection leads to a strong tumor-weight decrease without affecting the plant growth. The BG-8 inhibitory effect depends on the duration of treatment and compound concentration. *In vitro* this substance prevents cancer DNA synthesis and this explains the inhibition of tumor cell multiplication. There is little or no effect on healthy cell DNA *in vitro* synthesis.

Introduction

Vegetal cancer may have various origins but the most frequent of these is infection through widely spread bacteria, *A.tumefaciens* B_6 Many biological and biochimical studies have been performed in an attempt to grasp the mechanism whereby bacteria transform a normal cell into a cancerous one. In this respect, our contribution may be briefly summarized as follows . We first demonstrated that particular exogenous RNA may transform the oncogenic bacteria into non-oncogenic ones (Beljanski et al.,1972 a). We also demonstrated that a great variety of small size RNA initiate the appearance of tumors exclusively in the presence of the plant hormone (auxin)(Beljanski et al.,1974; Aaron-Da Cunha et al., 1975; Beljanski and Aaron-Da Cunha, 1976; Le Goff et al., 1976). Tumors originating with RNA have been shown to possess all the characteristics of crown-gall, i.e. overgrowth tissue is not self-limiting, can be indefinitely grafted onto healthy plants of the same species and may grow on a minimal culture medium that does not allow normal homologous cells to proliferate.

In our search for the mechanism of tumor induction and inhibition we first found special RNA-fragments which may very well inhibit plant cancer cells after infection with *A.tumefaciens*(Le Goff and Beljanski, 1979). If very important from a biochemical point of view, these results remain difficult to extend on a large scale and expensive to extrapolate to practical prevention or therapy. When looking for inhibitors of mammalian and plant tumors cells, we discovered that all DNA originating from cancer cells (both mammalian and plant DNA) do not behave as do DNA from normal cells when used as template in the *in vitro* synthesis of DNA. Cancer DNA respond very actively to many endogenous or exogenous substances while normal cell DNA responds only slightly. The mechanism of such differences was elucidated (Beljanski et al., 1981) and the perfect reproductibility of these differences was such that we devised a fast biochemical assay system (Beljanski,1979) called Oncotest for the screening of carcinogens and

anticancer substances. This test enabled us to select substances which recognize cancer DNA i.e. cancer cells either from mammalian or plant tissues. They bind to cancer DNA and thus prevent DNA replication and consequently cell proliferation without effect on normal cell DNA or normal cell development. So, in the extract of some *Apocynaceae*, we detected the presence of a compound which we termed BG-8 and which we have shown to correspond to alstonine (serpentine, an isomer of alstonine is also active as anticancer compound). The most remarkable property of BG-8 is that of distinguishing mammalian (Beljanski and Beljanski in press) and plant cancer cells DNA from DNA of healthy cells.

Material and methods

The BG-8 compound used in these studies was isolated from *Rauwolfia vomitoria* (Beljanski and Beljanski, in press). Its antitumor efficiency was tested on two different host plants : germinating pea seedlings obtained in laboratory conditions and young *Chrysanthemum* grown in green house conditions. The pathogenic agent used was *A.tumefaciens* B_6 (strain from our laboratory culture collection) aerobically grown overnight at 28°C on a synthetic medium (Manigault and Stoll, 1960) for pea test and maintained on nutrient broth agar slants for *Chrysanthemum* test. It is important to note that the activity of the water solution substance was pH-dependent with maximum response at pH 7.5.

Plant material

Pisum sativum cv Annonay seeds were surface-sterilized for 20 min in diluted bleach, washed several times with distilled water and allowed to germinate as described (Manigault and Kurkdjian,1967) when epicotyls reached 3-5 mm the seedlings were decapitated and bacterial suspension (about 10^8 B_6 cells/wound) was immediately applied. The plants were maintained in sterile water solidified with 0.7 % agar and keept at 24°C in darkness (conditions for optimal tumorisation). At the following stages of development : 24h, 48h, 4 days or 8 days, a range of concentrations (1 to 200 µg / wound) of BG-8 dissolved in distilled water (pH adjusted at 7.5) and filtered on millipore was placed in the preinfected wounds. A gentle scarification of surface cells makes substance penetration easier. Control seedlings received sterile distilled water under the same conditions. On the 12th day after bacterial infection , the tumors were excised and weighed and results from 30 plants per treatment were statistically analysed.

Chrysanthemum hortorum cv Padoux and Marquis used throughout these experiments were obtained from plant growers. They were planted out in enriched peat compost ("Zaainground")(it is clear that soil composition was of great importance for the experimental results). The pots were placed in a greenhouse under natural photoperiod at a temperature of 18°C and periodically watered using the "mist" system (relative humidity of the air 70-80%). Experimental tumors were obtained in two different ways : on a first set of plants, we cut three small chips on one side of the stem with a sterile blood lancet and applied a loopful of bacteria onto the wounds; On a second set of plants, tumors appeared on the roots after immersion of the plants in bacterial suspension (10^8 cells/ml)(1-2 min) and planting in bacteria 6 days-preinfected peat compost (10^9 cells/l of peat). This later method is closest to naturally occuring soilborn incculation. Several trials were performed

a) curative treatment : plants were inoculated with bacteria and three days later removed from the soil, then treated by dipping in a BG-8 water solution; we used two concentrations, 1 and 2 mg/ml and two dipping periods, 2 h and 6 h (liquid-level reaches the highest wound in the case of stem tumors); the plants were then replanted in the same pots.
b) preventive treatment : plants were treated by a solution of BG-8 as described above, 24 h before bacterial infection (along the stems or at root level).
c) concomitant treatment : BG-8 treatment and bacterial infection were occasionally made simultaneously.

In every case the tumors were excised 50 days later, weighed and the results compared to those obtained from control plants treated with distilled water

DNA *in vitro* synthesis

DNA from pea seedlings crown-gall tumors and healthy shoots were isolated by the phenol method (Thompson and Cleland, 1971). Purification was further performed by several chloroform treatments and alcohol precipitation. Integrity of DNA were controled before use (Beljanski et al.,1981) and hyperchromic effect (UV absorbance increase at 260 nm) on incubation with 0.1 M KOH solution was 40 and 35 % respectively. Only highly polymerized DNA were used. Conditions for DNA *in vitro* synthesis using ^{3}H-thymidine-5'-triphosphate as marker was described for mammalian cancer and healthy tissues DNA synthesis (Beljanski et al., 1981). Results are expressed by the amount of acid-precipitable ^{3}H-labelled DNA (TCA 5% solution).

Results

Inhibiting effect of BG-8 compound on tumor development in pea seedlings

The development of pea tumors induced by A.*tumefaciens* was observed in the absence and/or presence of various concentrations of the BG-8 compound.(see results in Fig.1 and Plate 1).It should be stressed that BG-8 (at indicated concentrations) does not influence the growth of pea shoots (lenght of control shoot : 25.5 cm $\pm$ 0.7 and this treated with 50 μg of BG-8/wound is 22.3 cm $\pm$ 0.9 , average of 30 plants) or of roots. Used at various concentrations from 5 to 200 μg per wound, BG-8 inhibits tumor development to completion or to a very high degree, independently if introduced at time 0 or later after bacterial infection of the wounds. However, it would appear that smaller concentrations of BG-8 are required if this compound is mixed with inoculum (time 0) than those introduced later in order to obtain very high levels of inhibition (90-100 %). It should be kept in mind that tumor weights were taken on the 12th day following infection, while BG-8 was introduced at time 24 or 48 h, 4 or 8 days after infection. In these two later instances the contact between BG-8 and tumorous cells was shorter than when introduced in the first hours of the cancerous process. In any case, the essential observation is that BG-8 "distinguishes " crown-gall tumorous cells from healthy cells. It kills the tumorous cells without affecting healthy plant cells. It should be noted that BG-8 has no effect on the growth of A.*tumefaciens* B_6

cultured in a nutrient broth medium containing 500 µg of BG-8/ml. Consequently, the inhibiting effect is manifest on cancerous cells and does not depend on the presence or absence of *A.tumefaciens* cells in the tumorous tissue. Moreover it is known that the presence of bacteria is no more required 24h following infection.

Inhibiting effect of BG-8 compound on *Chrysanthemum* tumor development

Results illustrating the tumor development on *Chrysanthemum* in the absence or presence of BG-8 compound may be found in Table 1 and Plate 1. Several conclusions may be drawn. Firstly, BG-8 (at indicated concentrations) has no influence on the growth of uninfected or infected plants. Secondly, the duration of treatment appears to be more important for curative and preventive treatments of plants carrying stem tumors than the relative concentration of BG-8. The percentage of inhibition (6h treatment) of tumor weight is 81 % and 86 % when plants are treated with a solution of 1 or 2 mg of BG-8/ml respectively. Thirdly, concomitant treatment of plants (first treated with BG-8 and immediately afterwards the bacteria were inoculated) results also in a significant inhibition of tumor weight (37-51 %). These values are lower than those obtained when curative and preventive treatments were applied. Forthly, curative or even concomitant treatment by BG-8 of plants carrying root tumors led to a drastic inhibition of tumor weight (83-93 %). This inhibition does not appear to be dependent upon the duration of treatment and the values are practically identical if the solution contains 1 or 2 mg of BG-8/ml. This excellent inhibition of tumors on plant roots may be due to the possibility that BG-8 occurying on the roots is partly retained in the soil from which it can be reutilized by tumorous cells. In the case of the stem tumors, the BG-8 compound which flows from root to stem may be more or less retained at the level of tumors and consequently the inhibiting action somehow decreased.

Effect of BG-8 compound on crown-gall and healthy DNA *in vitro* synthesis

The fact that DNA from cancer cells are generally destabilized compared to DNA from healthy tissues (Beljanski et al., 1981; Le Goff and Beljanski, 1981) encouraged us to investigate DNA *in vitro* synthesis in the absence or presence of the BG-8 compound. Results reported here show that this agent selectively inhibits crown-gall DNA *in vitro* synthesis and has little if any effect on the synthesis of DNA isolated from healthy plant cells (Fig. 2). It binds strongly to cancer DNA, not to healthy DNA (Beljanski and Beljanski, in press). It thus prevents the capacity of cancer DNA to act as template for DNA and RNA synthesis and consequently selectively prevents cancer cell proliferation.

Discussion and conclusion

Crown-gall disease can affect a wide range of susceptible dicotyledonous plants causing severe economic damage to growers. The possibility of protecting young plants has been investigated by many researchers. One of the most widely applied is Keer's technique (Kerr,1980)

which uses *Agrobacterium radiobacter* strain 84 (non-pathogenic bacteria) in combination with pathogenic *Agrobacterium tumefaciens* and results in reduced percentages of galls in various plants without however producing inhibition of all species galled plants (Panagopoulos, 1978; Süle and Kado, 1980). Strain 84 can only be used as a preventive agent and has no further effect on tumor regression; its effectiveness is depending of the pathogenic strain biotype, as shown with *Chrysanthemum* in particular (Faivre-Amiot et al.,1979). Different compounds have been used with the aim of preventing either tumor induction or proliferation. It was reported recently that d-glucosamine (Richardson and Morré, 1978) used at high concentrations induces regression of crown-gall tumors on *Phaseolus* leaves but at the same time it exhibits an inhibitory effect on growth of normal leaves; its preferential action on tumor cells was not elucidated. Along these lines, it was reported that cyclic-AMP (Babula and Galsky,1975) inhibits the number of crown-gall tumors developed on the primary leaves of pinto beans by 30 to 50 % ; similar results were observed on potato discs with prostaglandins E_1 and E_2 (Favus et al., 1977).Growth inhibition of *in vitro* grown crown-gall tissue by morphactin has been also reported (Raste and Johri,1979) but the level of inhibition is significant only with high concentrations of this compound. Studies in our laboratory enabled us either to inhibit crown-gall tumor induction by l-asparaginase (Beljanski et al., 1972 b) or to obtain crown-gall regression by U_2-RNA-fragments separately applied to the tumor surface (Le Goff and Beljanski, 1979). In the later case, significant necrosis was observed. Although U_2-RNA-fragment represent a very interesting series of small size RNA-fragments, they are costly and difficult to prepare for use on a large scale. In contrast, BG-8 is a compound easy to prepare in large quantities and we are to look for its application in field test. Moreover, it would be possible to combine BG-8 compound with K-84 strain to extend K-84 biological control to uncontrolled pathogenic *Agrobacterium* strains.

Data described here show that at given concentrations, BG-8 strongly inhibits crown-gall tumor development on pea seedlings treated as described in the text. Dipping *Chrysanthemum* plants in a solution containing the BG-8 compound, before or after infection with *A.tumefaciens* , leads to a considerable inhibition of the development of stem or root tumors ; in preventive treatment, BG-8 which is retained in the plant through the experimental period,is in place to be picked up by tumorous cells as they appear after bacterial infection. Tumorous cells are also its preferential target during curative treatment. In both cases, it is important to note that BG-8 compound has no perceptible effect on plant growth.

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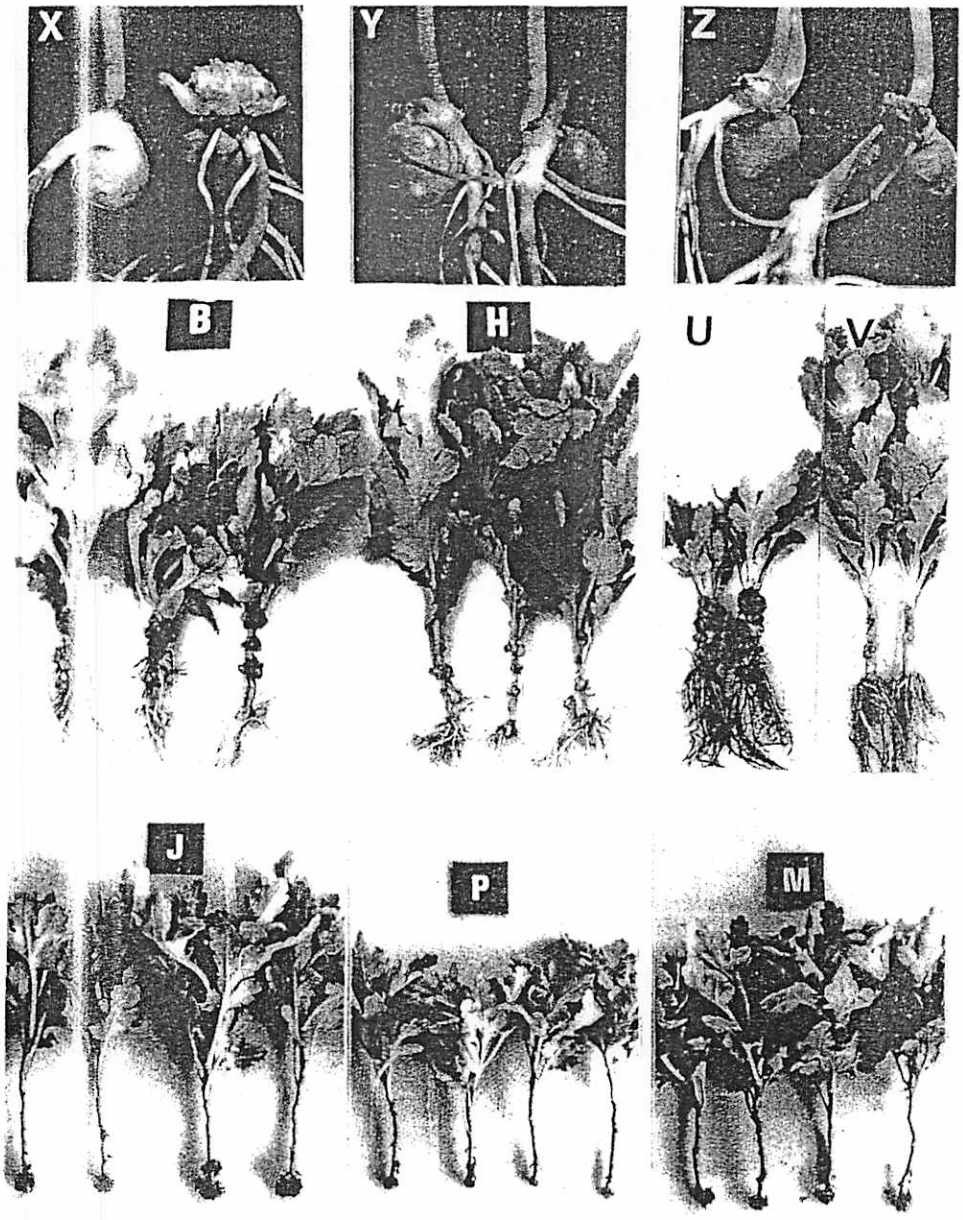
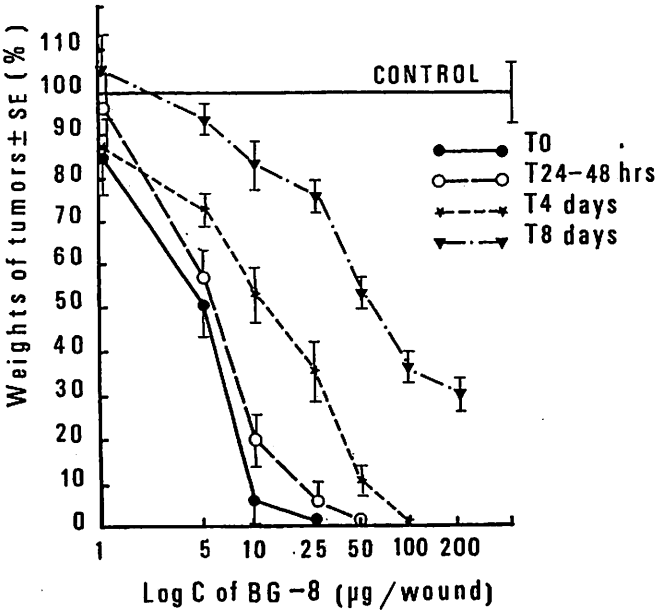
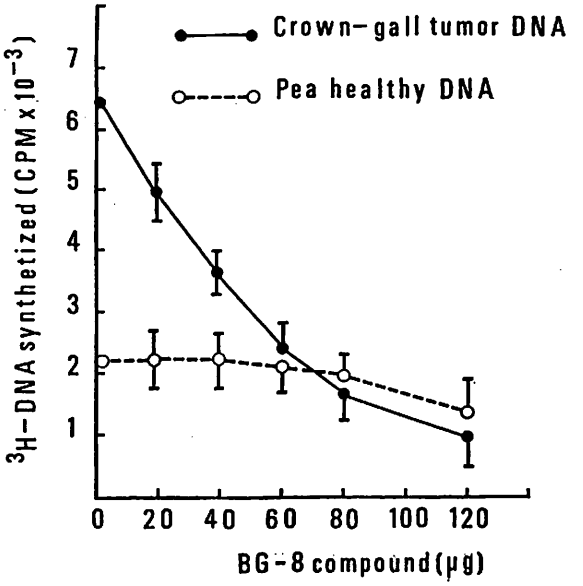


Plat 1- X: pea controls;Y and Z: infected pea wounds treated(BG-8,25µg /wound) at times 0, 24h, 4 and 8 days (from left to right). *Chrysanthemum* treated by BG-8(2 mg/ml, 6h). H and P: curative treatment; B and J : water treatment(control). V and M : preventive treatment; U and J : controls.
B, H, U and V :*Ch.* cv. Marquis . J, P and M : *Ch.*cv.Padoux.



At times indicated, BG-8 was applied to the wounds(to : BG-8 and B_6 were mixed just before application).Tumors fresh weight values were statistically compared by t-test and the averages from 2-3 experiments (30 plants/treatment) are expressed ± SE as percentage of the controls.

Fig.1- Inhibiting effect of BG-8 on pea B_6-induced tumors.



Dose-response curves represent cancer DNA synthesis and normal DNA synthesis. Results are expressed as CPM per assay

Fig.2 - Crown-gall and pea healthy DNA *in vitro* synthesis in the presence of BG-8.

Table 1- Preventive and/or curative inhibiting effect of BG-8 compound on *Chrysanthemum* crown-gall tumors. Inhibition % compared to control.

	Treatment applied	Control : water treated plants	Concentration of BG-8 solution and time of treatment: 1 mg / ml 2 h	1 mg / ml 6 h	2 mg / ml 2 h	2 mg / ml 6 h
STEM TUMORS	Curative*	1020	808 21 % NS	198 81 %***	647 36 %*	144 86 %***
	Preventive*	1087	714 34 % NS	551 49 %**	258 76 %***	311 71 %***
	Concomitant*	1087	678 37 %**	683 37 %*	582 46 %***	532 51 %***
ROOT TUMORS	Concomitant*	361	60 83 %**	29 92 %***	52 85 %**	23 93 %***
	Curative**	299	-	-	-	0 100 %
	Concomitant**	299	128 57 %**	-	65 78 %***	49 84 %***

*******Chrysanthemum* cv Padoux ***** *Chrysanthemum* cv. Marquis

For each type of treatment ten plants were used. In the case of stem tumors, each stem carried three independent tumors. The results are expressed as the mean value of the weight (mg) of three tumors per one stem. In the case of a single tumor on roots, the mean value of tumor weight (mg) per one plant is reported. We also present the percentage of tumor inhibition by the BG-8 compound compared to control tumors treated with water. The significance of comparison to control is given : NS not significant , * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$ (p values).