Cancer Chemother Pharmacol (1994) 34: 166–170

Cancer Chemotherapy and Pharmacology

Bullatacin – in vivo and in vitro experience in an ovarian cancer model

Christine H. Holschneider, Michael T. Johnson, Reba M. Knox, Ahmad Rezai, William J. Ryan, F. J. Montz

Gynecologic Oncology Service, Department of Obstetrics and Gynecology, UCLA Center for Health Sciences, CHS 24-127, 10833 Le Conte Avenue, Los Angeles, CA 90024, USA

Received: 9 July 1993/Accepted: 22 December 1993

Abstract. The cytotoxicity and antitumor effects of the acetogenin Bullatacin were evaluated in vitro in multiple ovarian cancer cell lines and in vivo in a murine ovarian teratocarcinoma (MOT) model in C3HeB/FeJ mice. The in vitro cytotoxicity of Bullatacin against four human ovarian epithelial tumor cell lines (OC-194, OC-222, OVCAR-3, and A-2780) was assessed in 48- and 72-h tetrazolium-dye (MTT) cytotoxicity assays. The percentage of cytotoxicity was determined on the basis of the mean optical density of the respective untreated cells and the dose effective against 50% of the cells (ED_{50}) was calculated for each cell line. In vivo experiments were performed on adult female C3HeB/FeJ mice, which were injected i.p. with 10^5 MOT cells and varying amounts of Bullatacin given either in a single dose or in 5 subsequent doses over 72 h. All mice were observed for survival relative to that of the control groups, which were injected either with 10^5 MOT cells with or without serial injections of vehicle or with vehicle only. All four epithelial ovarian cancer cell lines displayed sensitivity to Bullatacin. The relative cytotoxic effects were very heterogeneous, with the ED_{50} value ranging between 10^{-7} μg/ml for OC-194 and 4 μg/ml for the cisplatin-resistant cell line OVCAR-3 in a 72-h MTT cytotoxicity assay. All mice that had been injected i.p. with 10^5 MOT cells and 1.4 mg/kg or more of Bullatacin died within the first 24 h after injection, whereas all mice that had received 600 μg/kg of Bullatacin or less survived equally as long as the controls that had been injected with MOT only (21.1 ± 0.9 days). Mice that had received Bullatacin at a dose ranging from 600 μg/kg to 1.4 mg/kg either died during the 1st day postinjection or survived, but not longer than the MOT control group. Serial i.p. injections of Bullatacin again either led to death of the mice within 24–48 h of the last dose of Bullatacin or did not have any effect on the survival of the mice as compared with the respective control groups, which had been injected with the tumor and serial injections of vehicle (22.5 ± 2.2 days). In summary, Bullatacin showed no effect on MOT-caused animal death in C3HeB/FeJ mice at nonlethal dose ranges, whether it was given as a single i.p. dose or serially over 72 h. In vitro, however, it proved to be a very potent cytotoxic agent in a variety of ovarian cancer cell lines. As compared with other chemotherapeutic agents, which we accept as having clinically important antitumor efficacy against ovarian cancer, such as cisplatin or carboplatin, Bullatacin demonstrated a very favorable $ED_{50\ \text{in vitro}}/LD_{50\ \text{in vivo}}$ (the dose lethal to 50% of the mice) ratio.

Introduction

Ovarian cancer is the leading cause of cancer death from a gynecologic malignancy, with approximately 20,000 new cases and 12,000 deaths being reported annually in the United States [3]. This depressing reality exists despite the wide availability of extensive surgical procedures and multiple-drug chemotherapy. New substances must be identified that have high antitumor efficacy with acceptable toxicities.

In the late 1960s a large-scale screening program of plants was instituted through the National Cancer Institute (NCI) for the identification of naturally existing cytotoxic substances. This led to the identification of the recently (Food and Drug Administration, FDA) approved chemotherapeutic agent taxol as the active constituent of the bark extract of the Pacific yew *Taxus brevifolia* [10]. During such screening of plants at Purdue University, Hui and colleagues [6] isolated and characterized Bullatacin, another potentially promising cytotoxic substance. It is the most bioactive compound of a group of acetogenins ex-

This work was supported by the Bertha Warshaver Rubin Research Fund and was presented at the 21st annual meeting of the Western Association of Gynecologic Oncologists, May 19–23, 1993, Santa Monica, California

Correspondence to: F. J. Montz, M. D., Department of Obstetrics and Gynecology, UCLA, CHS 24-127, 10833 Le Conte Avenue, Los Angeles, CA 90024, USA

tracted from the bark of the *Annona bullata* tree [6]. Bullatacin has been found to be a very potent inhibitor of mitochondrial respiration with a specific site of action on complex I [reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase] of the respiratory chain [1]. In vitro testing at the NCI in cell growth-inhibition assays has found Bullatacin best active for certain CNS cancers, non-small-cell lung cancers, and leukemias [6]. In vitro cytotoxicity assays have demonstrated a remarkable potential against several human solid cell lines such as lung cancer cell line A549, breast cancer cell line MCF-7, and colon adenocarcinoma cell line HT-29 [8]. In vivo data obtained at Upjohn Company (Kalamazoo, Mich.) against L1210 leukemia in mice show increases in life spans of 38% for Bullatacin (at 50 μg/kg) and an apparent 300 times higher in vivo potency for Bullatacin than for Taxol [1].

In light of such promising data, this study was conducted to investigate the effects of Bullatacin on ovarian malignancies both in vitro using different epithelial tumor cell lines (OC-194, OC-222, A-2780, and the cisplatin-resistant cell line OVCAR-3) and in vivo using a murine ovarian teratocarcinoma model that closely resembles disseminated ovarian cancer.

Materials and methods

Bullatacin. Bullatacin, an acetogenin isolated from Annonaceae species, was kindly donated by Dr. J. L. McLaughlin at Purdue University (West Lafayette, Ind.). The purity of the substance was at least 95%. It was dissolved in dimethylsulfoxide (DMSO), maintained in a stock solution of 10 mg/ml at 4° C, and protected from light.

Cells. Human ovarian epithelial tumor cell lines OVCAR-3 and A-2780 were obtained from the American Type Culture Collection (Rockville, Md.). Ovarian epithelial tumor lines OC-194 and OC-222 were derived from patients at UCLA and maintained as continuous-culture cell lines. All cell lines were grown in RPMI 1640 (Cellgro, Washington, D. C.) supplemented with 10% heat-inactivated fetal bovine serum (Gemini, Calabasas, Calif.), 2 m*M* L-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml. Cells were grown in a humidified incubator containing 5% CO_2 at 37° C.

Cytotoxicity assay. For tetrazolium-dye (MTT) cytotoxicity assay [9], adherent epithelial tumor monolayers in culture were trypsinized and washed with culture medium. The cells were plated at 10,000 cells/well in 96-well flat-bottomed plates. After a 24-h preincubation period, Bullatacin was added to the appropriate wells in concentrations of 10-fold increments ranging from 10^{-8} μg/ml to 10 μg/ml. The plates were incubated for 48 or 72 h at 37° C. The supernatants were removed from all wells, 100 μl of MTT (Sigma. St. Louis, Mo.) solution (5 mg/ml) was added to each well, and the plates were incubated for 4 h at 37° C. The MTT solution was then removed and 100 μl of DMSO (Fisher, Fair Lawn, N. J.) was added to the wells to solubilize the MTT crystals. The plates were placed on a shaker for 30 min and absorbance was read at 540 nm on a multiwell spectrophotometer (Titertek). The percentage of cytotoxicity was calculated as (A-B)/A × 100 (%)), where *A* is the mean optical density of untreated wells and *B* is the optical density of Bullatacin-exposed wells.

Tumor. Mouse ovarian teratocarcinoma (MOT) is a syngeneic tumor that closely resembles disseminated ovarian cancer. The MOT cells were kindly donated by Dr. Robert Bast at the Duke Comprehensive Cancer Center (Durham, N. C.). This cell line does not grow in vitro. It was maintained by serial i.p. injections of 10^5 viable cells into C3HeB/FeJ mice every 2 weeks. Cell viability was assessed by trypan-blue exclusion prior to each i.p. transplantation. For the Bullatacin experiments, MOT cells at passages 2–8 were employed. An i.p. injection of as few as 10^3 MOT cells has previously been shown to kill all recipient mice [2].

Mice. All in vivo experiments were performed with adult, 8- to 10-week-old female C3HeB/FeJ mice obtained from Jackson Laboratories (Bar Harbor, Me.). The animals were fed standard laboratory diet.

Injections of MOT and Bullatacin. In the first experimental phase, 12 groups of 5 mice each were individually injected i.p. with a single dose of 10^5 MOT cells and with Bullatacin at different concentrations ranging from 72 μg/kg to 20 mg/kg in 0.25 ml of phosphate-buffered saline (PBS) as follows: group 1, 72 μg/kg; group 2, 150 μg/kg; group 3, 300 μg/kg; group 4, 480 μg/kg; group 5, 600 μg/kg; group 6, 720 μg/kg; group 7, 960 μg/kg; group 8, 1.25 mg/kg; group 9, 2.5 mg/kg; group 10, 5 mg/kg; group 11, 10 mg/kg; and group 12 20 mg/kg. In addition, one tumor-control group of mice (n = 7) was injected i.p. with 10^5 MOT cells in 0.25 ml of PBS and no Bullatacin. A second vehicle-control group was injected i.p. with 0.25 ml of PBS (n = 3) or DMSO/PBS (n = 3) only. In phase two of the in vivo experiment, groups of five mice each were injected serially every 18 h with five subsequent administrations of Bullatacin dosed at either 480 or 72 μg/kg per injection in 0.25 ml of PBS. A control group was injected i.p. serially every 18 h with 0.25 ml of PBS vehicle only. All mice were injected with 10^5 MOT cells concomitantly with the first injection of Bullatacin or vehicle. Again, mice were observed for abdominal distention and survival. The set endpoint for observation was 60 days in all phases of the experiment.

Results

Four epithelial ovarian cancer cell lines (OC-222, OC-194, OVCAR-3, and A-2780) were evaluated for in vitro sensitivity to Bullatacin. All cell lines displayed sensitivity to Bullatacin, although their responses were heterogeneous. In each case there was an apparent shoulder in the dose-response curve extending over several orders of magnitude. The relative sensitivity varied considerably, with the dose effective against 50% of the cells [ED_{50}] ranging from 10^{-7} μg/ml for OC-194 to 4 μg/ml for the cisplatin-resistant cell line OVCAR-3 in a 72-h MTT cytotoxicity assay (Fig. 1). In addition, the time of exposure appeared to have a crucial impact. In a comparison of 48-h and 72-h cytotoxicity assays, the ED_{50} value was approximately 10 (OVCAR-3) to 10^8 (OC-194) times lower when the 48-h incubation period was extended for another 24 h (Table 1).

The effect of Bullatacin on the growth of MOT cells in 8- to 10-week-old female C3HeB/FeJ mice was initially assessed in 12 groups of mice (n = 5/group) in which each animal received a single i.p. injection of Bullatacin at a

Table 1. Bullatacin-mediated cytotoxicity expressed as ED_{50} values obtained in 48- and 72-h MTT assays

Cell line	ED_{50} (μg/ml)	
	48-h MTT cytotoxicity assay	72-h MTT cytotoxicity assay
OC-194	10	10^{-7}
OC-222	5	5×10^{-4}
A-2780	10^{-2}	6×10^{-6}
OVCAR-3	50	4

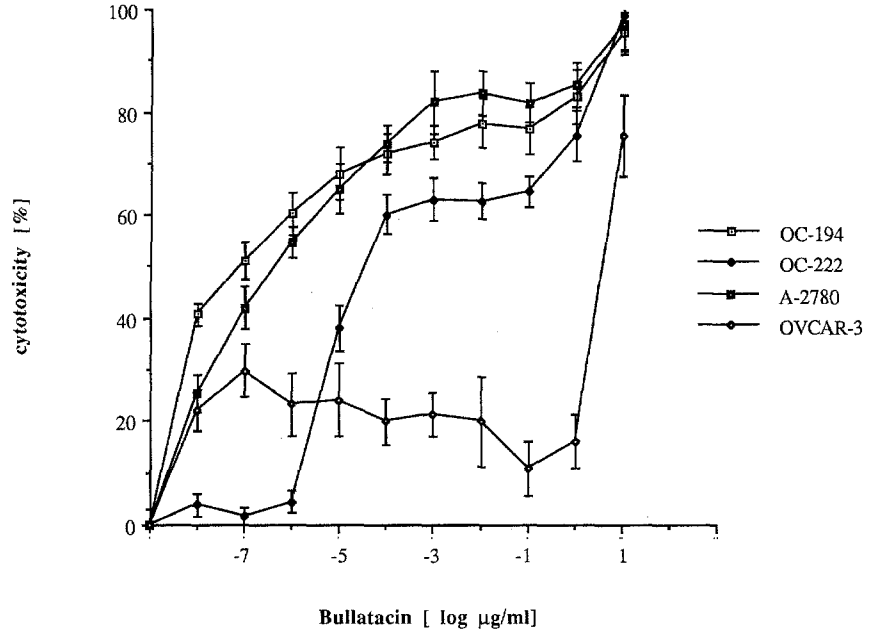

Fig. 1. Sensitivity of the human ovarian epithelial tumor cell lines OC-194, OC-222, A-2780, and OVCAR-3 to Bullatacin-mediated cytotoxicity in vitro in a 72-h MTT cytotoxicity assay

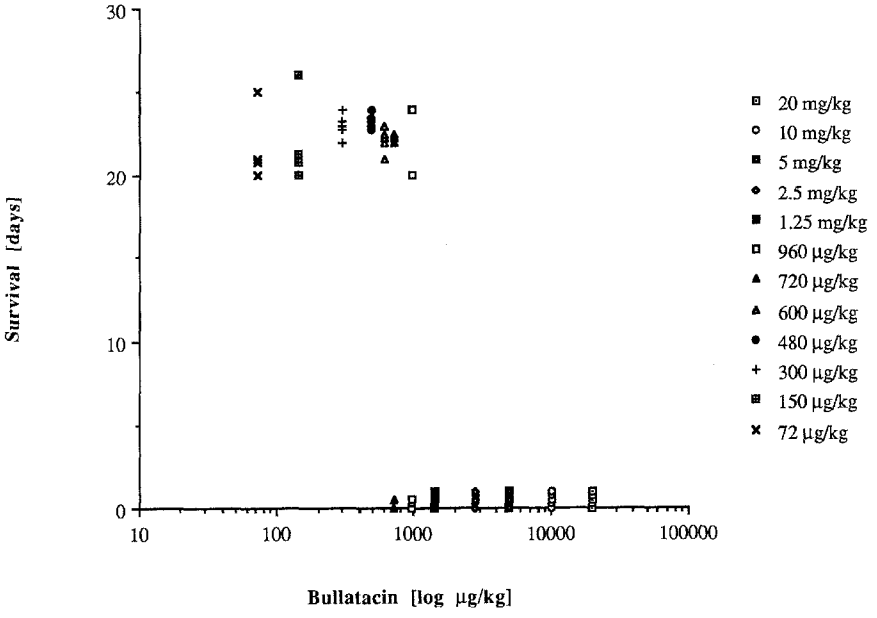

Fig. 2. Survival of mice injected i.p. with 10_5 MOT cells and differing single doses of Bullatacin ranging from 72 µg/kg to 20 mg/kg. For comparison, the mean survival of the control group of mice that receibved 10_5 MOT cells and vehicle alone was 21 ± 0.9 days

dose ranging from 20 mg/kg to 72 µg/kg in addition to the i.p. injection of 10^5 MOT cells. There were two control groups of mice. One group was injected with 10^5 MOT cells but no Bullatacin (n = 7), and the other was injected with vehicle only (n = 6; 3 mice received PBS, 3 mice received DMSO/PBS). In the five groups of mice that had received Bullatacin in the dose range of 20–1.4 mg/kg, all mice died within 24 h of the injection of Bullatacin and MOT cells. For the five groups of mice that had been given Bullatacin at doses ranging from 600 to 72 µg/kg, the mean survival of each group varied between 21.0±0.8 and 23.0±0.7 days, which corresponds exactly to the survival of the control group of mice that had been injected with MOT cells only (21.1±0.9 days). In the two groups of mice that had received 960 or 720 µg/kg of Bullatacin in a single i.p. dose at the time of MOT injection, the mean survival was 9.1±11.9 and 13.4±11.8 days, respectively. The high standard deviations reflect the following: in the 960-µg/kg group, these mice died within 24 h of the injection of Bullatacin and two mice survived to days 20 and 24, respectively. Very similar results were found in the 720-µg/kg group, in which two mice died within 24 h and all that survived subsequently died on day 22 after the injection of Bullatacin and MOT. All mice that had been injected with vehicle alone tolerated the injection well and were alive and active after 60 days. Thus, all mice that had received high-dose Bullatacin injections died within 24 h, presumably from drug toxicity. When Bullatacin was injected at a lower dose, it did not display sufficient toxicity toward the MOT cells and failed to increase the survival of the respective mice (Fig. 2).

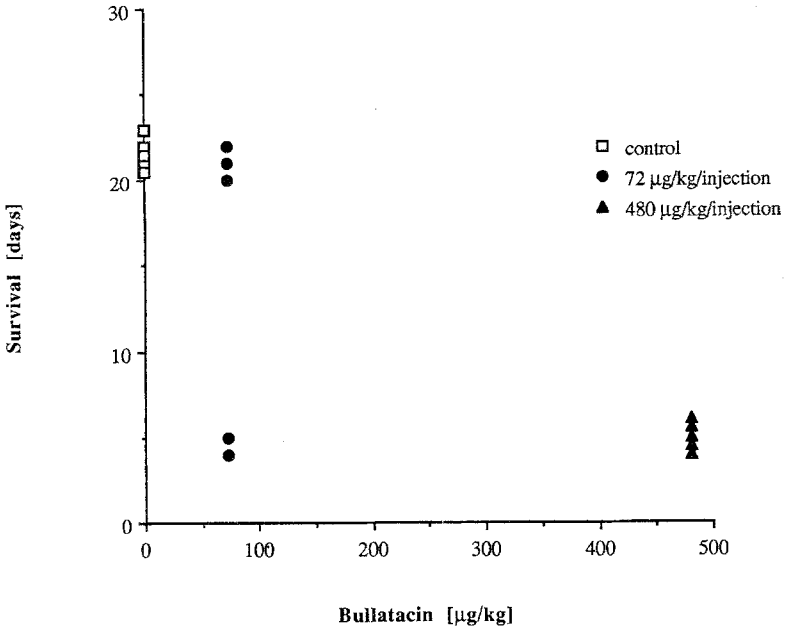

Fig. 3. Survival of mice inoculated i.p. with 10_5 MOT cells and 5 serial injections of either vehicle alone or Bullatacin at 72 or 480 µg/kg per injection over 72 h. None of the animals died during the injection period. Mice either died within 24–48 h of the last dose of Bullatacin, presumably from drug toxicity, or died of tumor at day 21, just as the control group, which had been injected with tumor cells and vehicle only

Considering the observation that a 72-h incubation period strongly enhanced the in vitro cytotoxicity of Bullatacin and markedly reduced the ED_{50} value, the in vivo experiment was continued into a second phase. Two groups of mice (n = 5 each) were injected i.p. serially every 18 h with five doses of Bullatacin. Each of the mice received 10^5 MOT cells along with the first drug injection. In the group that had been treated i.p. with 480 µg/kg of Bullatacin per injection, all mice died within 24–48 h of the last administration of Bullatacin. In the group that had been injected i.p. with 72 µg/kg of Bullatacin per dose, two mice died within 24 h of the last injection and three mice died of MOT at days 20, 21, and 22, respectively. This survival was equal to that noted in the control mice, which had been injected once with 10^5 MOT cells and serially with PBS vehicle only (mean survival, 22.5 ± 2.2 days). Thus, even after serial injections, we observed the same phenomenon: either the mice died of the cumulative toxicity of Bullatacin shortly after the treatment course had been completed or the Bullatacin did not display any antitumor effect measurable as an increase in survival (Fig. 3). Unfortunately, the possibility that MOT may be resistant to Bullatacin could not be tested in vitro since this cell line cannot be maintained in cell culture.

Mice that died shortly after the treatment with Bullatacin had been completed presumably died of drug toxicity. Since a formal necropsy/autopsy was not performed, we cannot comment on pathologic or histologic features such as end organ damage. However, clinical observations regarding the toxicity of Bullatacin are worth mentioning. The immediacy of lethal toxicity was most remarkable, with no other indicator of toxicity being present prior to death. In the 70 animals treated, including those that had undergone serial injections of Bullatacin, there was no case in which signs of toxic morbidity such as decreased activity, restlessness, hunched posture, decreased food or water intake, rapid or labored breathing, decreased alertness, or seizures were observed. Close clinical observation did not point to specific organ systems as sites of toxicity. Considering the mechanism of action, generalized peripheral suffocation at the cellular level due to inhibition of mitochondrial respiration appears most likely, which, as we know from other inhibitors of the respiratory chain, can manifest itself quite suddenly.

Discussion

Despite recent advances in surgical techniques and adjuvant therapies, ovarian cancer remains the most common cause of cancer death from a gynecologic malignancy in the United States. Therefore, a need remains for new agents with significant tumoricidal effects and acceptable toxicity. This demand has helped to direct the attention of researchers toward naturally occurring toxins. During a plant screening at Purdue University, Hui and colleagues [6] found the bark of the *Annona bullata* tree to be highly cytotoxic and identified Bullatacin as its most bioactive compound. In the present study we evaluated the cytotoxicity of Bullatacin in an ovarian cancer model in vitro and in vivo.

Our data show that in the MOT-C3HeB/FeJ in vivo model, Bullatacin failed to show any effect on tumor-caused animal death at nonlethal doses, independently of whether it was given as a single i.p. dose or serially in five doses over 72 h. In vitro, however, it displayed very potent cytotoxic effects on three of the four human epithelial ovarian cancer cell lines, with only the cisplatin-resistant cell line OVCAR-3 being less sensitive.

Interestingly, in all cell lines tested there was an apparent shoulder in the dose-response curve, which suggests three possibilities. First, a subpopulation of cells exists in each cell line that is relatively resistant to Bullatacin, requiring an up to 10^7-fold increase in Bullatacin concentration for cytotoxic effects. Second, there exists a certain metabolic state in which cells are more resistant to

Bullatacin, making two mechanisms of action likely. Taking into account the recently identified specific site of action on complex I (NADH-ubiquinone oxidoreductase) of the respiratory chain [1], the possibility that cells can be in an aerobic or anaerobic state of metabolism may account for such differences in sensitivity. Third, the shoulder in the dose-response curve may also represent an artifact of the assay, which appears unlikely since it is so readily reproducible. This "shoulder effect" could be a possible cause of the poor anti-MOT effect observed in vivo in the C3HeB/FeJ mice at doses that were not lethally toxic.

Unfortunately, we do not have data to explain the lack of antitumor effects of Bullatacin in the MOT-C3HeB/FeJ mouse model. In part this is due to the inability to maintain the MOT line in cell culture. Nevertheless, we can assess the significance – or insignificance – of our inability to demonstrate in vivo antitumor activity in the MOT model by comparing our findings with the results of similar experiments with other chemotherapeutic agents that we accept as having clinically important activity against ovarian cancer. The 72-h in vitro ED_{50} of Bullatacin for all cisplatin-sensitive cell lines (OC-194, OC-222, A-2780) was 1.44 (OC-222) to 7,200 (OC-194) times lower than the lethal single doses in C3HeB/FeJ mice. Similarly, the in vitro ED_{50} was 1.39 times higher (OC-222) to 3,600 times lower (OC-194) than the lethal cumulative doses of Bullatacin in mice (72 μg/kg × 5 doses). These $ED_{50\ in\ vitro}$/$LD_{50\ in\ vivo}$ (the dose lethal to 50% of the mice) ratios of Bullatacin compare most favorably with those of other frequently used chemotherapeutic agents. For example, cisplatin has an overall in vitro ED_{50} of 107 μg/ml for human ovarian cancer cell lines [4], with the LD_{50} in rats being 12 mg/kg [7]. Thus, the ED_{50} in vitro is 8.9 times higher than the LD_{50} in vivo. Similar results are found for carboplatin, where the overall ED_{50} for ovarian cancer cell lines is 490 μg/ml [4]. As compared with a carboplatin dose of 170 mg/kg, which is 100% lethal in mice [5], this gives an $ED_{50\ in\ vitro}/LD_{50\ in\ vivo}$ ratio of at least 2.88. In light of these ratios, it becomes very likely that the MOT tumor is resistant to Bullatacin. Thus, since Bullatacin promises to be an excellent substance with strong antitumor activity and a potential for a broad therapeutic window, it warrants further in vivo evaluation in other cancer animal models, e. g., against human epithelial ovarian cancer in nude mice, employing cancer xenografts known to be sensitive to Bullatacin in vitro.

References

1. Ahammadsahib KI, Hollingworth RM, Hui Y-H, McLaughlin JL (1993) Inhibition of NADH-ubiquinone reductase (mitochondrial complex-I) by Bullatacin, a potent antitumor and pesticidal annonaceous acetogenin. Life Sci 53: 1113
2. Berek JS, Lichtenstein AK, Knox RM, Jung TS, Rose TP, Cantrell JL, Zighelboim J (1985) Synergistic effects of combination sequential immunotherapies in a murine cancer model. Cancer Res 45: 4215
3. Boring CC, Squires TS, Tong T (1991) Cancer statistics 1991. CA 41: 19
4. Fanning J, Biddle WC, Goldrosen M, Crickard K, Crickard U, Piver MS, Foon KA (1990) Comparison of cisplatin and carboplatin cytotoxicity in human ovarian cancer cell lines using the MTT assay. Gynecol Oncol 39: 119
5. Gore ME, Hills CA, Siddik ZH, Sloane JP, Winkley AR, Smith IE, Millar JL (1987) Priming reduces the bone marrow toxicity of carboplatin. Eur J Cancer Clin Oncol 23: 75
6. Hui Y-H, Rupprecht JK, Liu YM, Anderson JE, Smith DL, Chang C-J, McLaughlin JL (1989) Bullatacin and Bullatacinone: two highly potent bioactive acetogenins from *Annona bullata*. J Natl Prod 52: 463
7. Kociba RJ, Sleight SD (1971) Acute toxicologic and pathologic effects of *cis*-diamminedichloroplatinum (NSC-119875) in the male rat. Cancer Chemother Rep 55: 1
8. Li X-H, Hui Y-H, Rupprecht JK, Liu Y-M, Wood KV, Smith DL, Chang C-J, McLaughlin JL (1990) Bullatacin, Bullatacinone, and Squamone, a new bioactive acetogenin, from the bark of *Annona squamosa*. J Nat Prod 53: 81
9. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55
10. Rowinsky EK, Cazenave LA, Donehower RC (1990) Taxol: a novel investigational antimicrotubule agent. J Natl Cancer Inst 82: 1247