

The effects of a Chinese herb formula, anti-cancer number one (ACNO), on NK cell activity and tumor metastasis in rats

Li Hong-Fen^a, Tal Waisman^b, Yair Maimon^a, Keren Shakhar^b, Ella Rosenne^b,
Shamgar Ben-Eliyahu^{b,*}

^a Complementary Medical Unit, Sourasky Tel Aviv Medical Center, and the International Chinese Medicine Cancer Research Center, Israel

^b Psychobiology Research Unit, Department of Psychology, Tel Aviv University, Tel Aviv 69978, Israel

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Abstract

The effects of anti-cancer number one (ACNO), a 19-herb Chinese formula used to treat cancer patients, were studied in F344 rats. In the first study, the number and activity of circulating NK cells were evaluated following 18 days of oral consumption of 0.1, 0.5, or 2 g/kg/day. The second study assessed the effect of ACNO on resistance to metastasis of the MADB106 tumor line, a syngeneic mammary adenocarcinoma that metastasizes only to the lungs and is highly sensitive to NK activity (NKA) *in vivo*. Resistance to metastasis was assessed under baseline conditions and following the administration of a β -adrenergic agonist, metaproterenol (MP). MP was used to simulate sympathetic response to stressful conditions, and was previously shown to suppress resistance to MADB106 metastasis. The results of the first study indicated a dose-dependent increase in NKA per ml of blood and per NK cell, with no significant changes in blood concentration of NK cells. In the second study, whereas MP caused a 4.5-fold increase in the number of metastases in untreated rats, only a 2.3-fold increase occurred in rats treated with ACNO. No significant improvement in baseline levels of resistance to metastasis was observed. These findings indicate the importance of studying ACNO under stressful conditions in patients with potentially metastasizing tumors. This may prove particularly important during the perioperative period, spanning from the detection of the primary tumor to postoperative treatment. During this critical period, psychological and physiological stress responses are known to cause massive immunosuppression, which was suggested to promote metastatic development. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Traditional Chinese medicine represents an accumulation of expert knowledge and has been practiced for at least 2000 years. Western medicine has tested

several herbal formulas of Chinese origin for their therapeutic value and active ingredients, and reported positive results in respect to some. For example, in Chinese medicine, *astragalus* has been used traditionally to strengthen the Qi, which is related to immunocompetence. Western research indicated that extracts from *Astragalus membranaceus* could stimulate immune functions and increase host versus graft response in cancer patients [1,2]. Several for-

* Corresponding author. Tel.: +972-3-640-7266; fax: +972-3-640-9547.

E-mail address: shamgar@post.tau.ac.il (S. Ben-Eliyahu).

mulas of Chinese herbs or specific extracts were also shown to increase immunity in humans [3–7], and protect against infectious diseases [4,8].

Several Chinese herbs were reported to increase survival rates of cancer patients, or used successfully as a prophylactic measure against cancer (for review see Refs. [9,10]). For example, the intake of *Ginseng* was dose-dependently associated with reduced cancer rates in humans [11] and rats [12,13]. Immunostimulation is one of the mechanisms suggested to underlie such effects. Indeed, a fractionated extract from *Astragalus* was shown to cause a 10-fold reduction in the doses of IL-2 required for in vitro induction of lymphokine activated killer cells (LAK) [1], and consumption or administration of red *Ginseng* were reported to increase natural killer cell activity in humans [3,4] and in rodents [14,15].

Natural killer (NK) cells are predominantly large granular lymphocytes (LGL) that, in humans, express CD16 and/or CD56 surface antigens and comprise 10–15% of peripheral blood lymphocytes [16]. NK cells are considered a key player in the defense against malignant cells and virally infected cells [17,18]. Studies in humans have found a positive correlation between levels of NK activity (NKA) and resistance to malignancy [19–23], and a causal relationship has been demonstrated using animal models [21,24–26]. Animal studies also indicated that NK cells are particularly important in controlling metastatic development [27].

Stressful conditions and stress hormones such as catecholamines were shown to suppress various immune functions, including NKA [28,29]. We have recently reported that various stress paradigms, including surgical stress and catecholamine infusion, increased susceptibility to metastasis by suppressing NKA [28–30]. The perioperative period in cancer patients is characterized by high levels of stress and a high risk for metastasis [31–34]. Thus, augmenting immune functions or preventing their suppression, using biological response modifiers (BRM), including Chinese herbs, may prove effective in such critical and vulnerable conditions.

Therefore, in the current study, we used a rat model to evaluate the effects of a particular formula, anti-cancer number one (ACNO), which is employed by Chinese medicine to treat cancer patients. The number and the activity of natural killer (NK) cells,

as well as resistance to tumor metastasis, were assessed in F344 rats following 18 days of oral consumption of ACNO. Additionally, we studied the effects of ACNO in preventing the metastasis-promoting effects of a β -adrenergic agonist, metaproterenol, which was shown in previous studies to increase metastatic dissemination [28]. This drug was used because it effectively simulates the impact of stress on NK activity and metastatic colonization [30]. To study host resistance to metastasis, we employed an animal model that we have previously found to be reliable in studying the influence of stress and surgery on metastatic development [29,35]: Fischer-344 rats were intravenously inoculated with syngeneic MADB106 adenocarcinoma cells, and lung metastases were counted 3 weeks later. These tumor cells are retained in the lungs and consequently develop metastases only in this organ [18,35]. Importantly, NK activity plays a crucial role in limiting lung colonization by MADB106 cells [18,36,37].

2. Materials and methods

2.1. Animals

Fischer-344 male and female rats (Harlan Laboratories, Jerusalem, Israel) were housed four in a cage with free access to food and water in a 12:12-h lighting regimen. Animals were acclimatized to the vivarium for at least 3 weeks before any experiment, and were handled three times during the week before each experiment. All experiments were conducted during the first half of the light phase. In any given experiment, all animals were of the same age (see Procedures and results).

2.2. Ingredients of ACNO (Latin) (patent #135325)

Panax ginseng (9.5%), *Poria cocos* (5.7%), *Atractylodes macrocephala* (5.7%), *Anglica sinensis* (5.7%), *A. membranaceus* (5.7%), *Curcuma zedoaria* (4.7%), *Scutellaria baicalensis* (5.7%), *Phellodendron chinense* (4.7%), *Coptis chinensis* (5.7%), *Glycyrrhiza uralensis* (5.7%), *Crataegus pinnatifida* (4.7%), *Hordeum vulgare* (1.9%), *Salvia miltiorrhiza* (4.7%), *Schisandra chinensis* (5.7%), *Hedyotis*

diffusa (6.6%), *Ophiopogon japonicus* (4.7%), *Lobelia chinensis* Lour (4.7%), *Scutellaria barbata* (5.7%), *Massa fermentata medicinalis* (1.9%).

P. ginseng was bought from Hui Nan Ginseng (Ji Lin province, China). *S. barbata* was purchased from KPC Products (Taiwan, China). All other compounds were bought from Mayway (Oakland, CA).

ACNO was dissolved in boiling water.

2.3. Feeding of the animals with ACNO

ACNO was mixed with a standard powdered rodent food; 100 g of dry food/kg body weight (of the total rats in each cage) were mixed with an equal weight of tap water (with or without ACNO) to form a “wet food mixture”. The wet mixture was placed in a 20-cm-long trough that was placed inside each cage at the beginning of the dark phase of each feeding day. To adjust animals to the feeding protocol, all rats received the wet mixture without medicine for 3 days before each experiment.

Rats from all groups consumed more than 95% of the wet mixture each day, approximately 70% of it during the dark phase. No additional food was available to animals consuming the wet mixture. Body weight was monitored twice a week along the experiment. Control rats received the same mixture without the medicine. In Experiment 2, an additional control group was maintained on the standard ad libitum feeding procedure with standard rodent dry food pellets.

2.4. The MADB106 tumor line

MADB106 is a selected variant cell line obtained from a pulmonary metastasis of a mammary adenocarcinoma (MADB100) chemically induced in the inbred F344 Fischer rat [18]. MADB106 cells were maintained as a monolayer cell culture in complete medium at 100% humidity, 5% CO₂ at 37 °C, and detached from the flask using 0.25% trypsin.

2.5. Induction and counting of tumor metastases

Rats were lightly anesthetized with halothane, and 10⁵ MADB106 tumor cells were injected into their tail vein in 0.5 ml of PBS supplemented with 0.1% bovine serum albumin. Rats were sacrificed with

halothane 3 weeks after tumor inoculation, and their lungs were removed and placed for 24 h in Bouin solution (72% saturated picric acid solution, 23% formaldehyde [37% solution] and 5% glacial acetic acid). After lungs were washed in ethanol, visible surface metastases were counted independently by two experimenters unaware of the group origin of each lung. In previous studies, we found a very high correlation between numbers of surface metastases assessed by different experimenters if this assessment was conducted at 3 weeks (or later) after tumor inoculation. At this stage, metastases are clearly differentiated from the lung tissue [35].

2.6. Metaproterenol

Metaproterenol (MP) (Sigma, Israel), a non-selective β -adrenergic agonist, with a higher affinity to β_2 receptors than to β_1 and a half-life of about 2 h in rats [38]. MP was dissolved in PBS for s.c. injection.

2.7. Flow cytometry

Fluorescence Activated Cell Sorter (FACS) analysis was used to assess the number of NK cells in the blood. NK cells were identified as NKR-P1^{bright} lymphocytes using FITC-conjugated anti-NKR-P1 mAb (BD PharMingen, San Diego, CA, USA). To assess the absolute number of NK cells per ml blood, a fixed number of polystyrene microbeads (20 μ m, Duke Scientific, Palo Alto, CA, USA) was added to the blood samples before they were prepared for cytometric analysis. For further details, see Shakhar and Ben-Eliyahu [28].

2.8. Whole blood NK cytotoxicity assay

The activity of NK cells was assessed using the whole blood cytotoxicity assay described in detail elsewhere [28]. Briefly, this procedure assesses anti-tumor cytotoxicity of NK cells per ml blood without prior purification of monocytes. One ml of blood was drawn by cardiac puncture, plasma was replaced with culture medium, and aliquots of the washed blood were then placed in the first row of a microtiter plate. To assess NK cytotoxicity at six different effector to target (*E/T*) ratios, successive

twofold dilutions of blood in complete media were performed. As target cells, a fixed amount of ^{51}Cr -radiolabeled YAC-1 lymphoma cells (6000/well) were added to the blood. Spontaneous and maximal releases of radioactivity from target cells were determined by substituting blood with the culture medium or Triton-X, respectively. Following a 4-h incubation period, samples of supernatant were recovered from each well for the assessment of radioactivity. Specific killing was calculated as $100 \times (\text{sample release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$. A correction for changing hematocrit and supernatant volume over different E/T ratios was included. Earlier studies have indicated that cytotoxicity measured using this procedure is attributable solely to NK cells, rather than other cell types or soluble factors [39–42].

2.9. Statistical analysis

One-way analysis of variance was used to analyze the number of NK cells per ml of blood. NK activity per ml blood was analyzed using repeated measures ANOVA for the different E/T ratios. To estimate NK activity on a per NK cell basis, two approaches were used: First, Lytic Units (LU) were calculated using the formula $100/ET_{45}$, where ET_{45} is the E/T ratio needed to lyse 45% of target cells. The regression exponential fit method was used to infer ET_{45} from the data [43]. If an animal did not reach the 45% level, the highest E/T ratio was assigned this value for the sake of calculating LU_{45} (a conservative approach that reduces differences between groups). The number of LU divided by number of NK cell was calculated as a measure of single NK cell cytotoxic activity. The second approach was to incorporate the number of NK cells per ml blood as an explaining variable. Repeated measure ANCOVA was used with the number of NK cells as a continuous independent variable and medicine as a factorial independent variable.

In Experiment 2 (see below), a two-way analysis of variance (ACNO/control by MP/saline) was used to analyze the number of lung tumor metastases. As ANOVA indicated significant group differences, post hoc Scheffé analysis was used for pairwise comparisons.

Data are expressed as mean \pm S.E.M., unless stated otherwise. $p < 0.05$ was considered significant in all comparisons.

3. Procedures and results

3.1. Experiment 1: the effect of ACNO on number and activity of NK cells

3.1.1. Procedure

Fischer-344 rats (59 male, 10–12 weeks old) were randomly assigned to a control group fed with the wet food mixture only ($n = 19$), or to one of three experimental groups consuming 0.1, 0.5, or 2 g/kg/day of ACNO for 18 days ($n = 13, 14, 13$, respectively). On the 19th day, rats were lightly anesthetized with halothane, and 1.5 ml of blood was drawn by cardiac puncture. The order of blood withdrawal was counterbalanced between all experimental groups. NK cytotoxicity was measured in a whole blood ^{51}Cr release assay, and number of NK cells assessed using flow cytometric analysis, as described above.

3.1.2. Results

Rats from all groups consumed more than 95% of the food each day throughout the feeding period and gained an average of 23.4 g/week with no significant group differences.

ACNO caused a significant dose-dependent increase in cytotoxicity of NK cells per ml blood ($F_{(3,55)} = 2.95$, $p < 0.05$) (Fig. 1). On the other hand, ACNO did not cause a significant change in the number of NK cells per ml blood, although a non-significant dose-dependent increase was evident, reaching an 18% increase in the highest dose. Nevertheless, introducing the number of NK cells per ml blood as a covariate in a repeated measure ANCOVA reduced the effect of medicine to a non-significant level ($p = 0.18$). This suggests that the increase in the number of NK cells in the highest dose of ACNO contributed, to some degree, to the evident increase in NK activity. Calculating LU_{45} per NK cell revealed that less than half as many NK cells from rats consuming the high dose of ACNO were needed to achieve the same levels of killing as in control rats. This index (LU/NK cell), which should

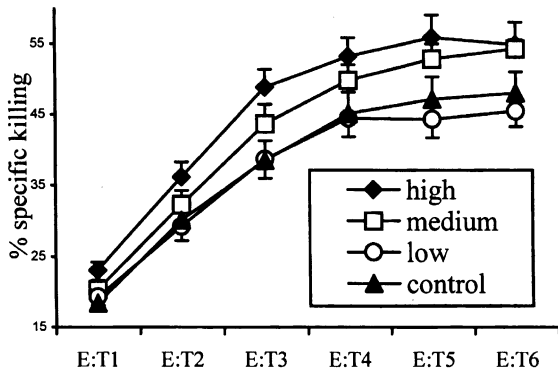


Fig. 1. The effects of increasing doses of ACNO (low, medium, high: 0.1, 0.5, or 2 mg/kg/day) on NK activity (% specific killing) in increasing effector to target (E/T) ratios. Data are presented as means \pm S.E.M. ACNO caused a significant dose-dependent increase in NK activity without significantly affecting number of NK cells (not shown).

allow the assessment of NK activity per NK cell, was not used statistically to assess differences between the groups; the cytotoxicity curves of the different groups were not parallel nor linear, and thus violated the prerequisites for using this index for statistical purposes.

3.2. Experiment 2: the effect of ACNO and of a β -adrenergic agonist on susceptibility to MADB106 metastasis

3.2.1. Procedure

Ninety-three Fischer-344 rats (8 weeks old) were used. Forty rats were fed with 2 g/kg/day of ACNO (a dose shown in the first study to cause a significant increase in NK activity), and 36 served as controls and were fed with the wet food mixture only. An additional control group ($n = 17$) was maintained on regular ad libitum dry food schedule throughout the experiment. Body weight was taken at the beginning of the feeding schedule and 3 weeks later. Following 3 weeks of feeding (3 days of adjustment to the wet food, and 18 days of ACNO treatment), half of the rats from each group were injected with the β -adrenergic agonist, MP (0.8 mg/kg s.c.), and the other half with saline; all rats were then injected i.v. with MADB106 tumor cells (forming a 3×2 design). The order of injection was counterbalanced between all experimental groups. Approximately half of the

rats in each group were male and half females (overall, 48 males and 45 females).

Feeding with ACNO was continued for another week, and thereafter all rats returned to ad libitum dry food schedule. Two weeks later, rats were sacrificed and their lungs were removed. Tumor metastases were counted independently by two experimenters unaware of the group origin of each lung.

3.2.2. Results

Rats from the two groups that were fed with wet mixture consumed more than 95% of the food throughout the 4 weeks feeding period and gained an average of 20.5 g/week (males) or 10 g/week (females), with no significant group differences and no significant difference compare to the third group maintained on ad libitum dry food schedule.

With respect to number of metastases, the correlation between the two counts of metastasis was 0.93, and an average count was used. No sex difference or interaction of sex with the effects of MP or ACNO was evident, thus males and females were combined for further statistical analysis. The baseline numbers (in saline-treated rats) of metastases were not affected by ACNO, and the number of metastases was very similar in the three groups (wet food only, dry food, and ACNO: means (\pm SD) = 69 (\pm 59), 57

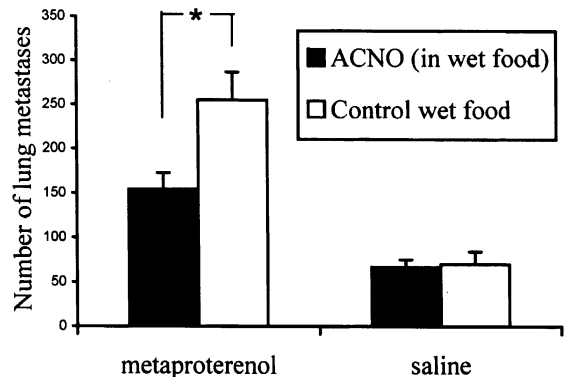


Fig. 2. The effects of ACNO (2 mg/kg/day) and of the β -adrenergic agonist, metaproterenol (0.8 mg/kg), on the number of MADB106 metastases. Data are presented as means \pm S.E.M. Metaproterenol injection significantly increased the number of metastases, and ACNO significantly reduced this effect of metaproterenol (*) without altering baseline levels of metastasis (in saline-injected animals).

(± 39), and 66 (± 38)). On the other hand, whereas MP caused a significant 4–5.5-fold increase in the number of metastases in the two control groups, it caused only a 2.3-fold increase in the ACNO group. A 2×2 analysis of variance conducted on the ACNO-treated groups and the wet food control groups indicated a significant interaction between the effects of MP and ACNO (Fig. 2) ($F_{(1,72)} = 7.01$, $p < 0.01$), and post hoc contrasts indicated that the control MP group had significantly higher number of metastases than the ACNO MP group. The two control groups (wet and dry food) that received MP were not significantly different from each other by planned or post hoc analysis. When combined to form one control group, the blocking effect of ACNO was even higher than the effect shown in Fig. 2 (statistics not reported), as the dry food control groups exhibited even greater effect of MP (although not significantly so) than the wet food control groups.

4. Discussion

The results indicate that the herbal formula ACNO caused a significant dose-dependent increase in NKA, reaching an approximately twofold effect in the highest dose when assessed at the end of the 18-day treatment period. The increase was evident in NKA per ml of blood and was accompanied by a non-significant 7–18% increase in the number of circulating NK cells. This suggests that the majority of the increase in NK activity is ascribed to increased cytotoxicity per NK cell, but some of it is related to the increase in the number of circulating NK cells. Other Chinese formulas were also reported to increase NKA [44–50]. ACNO also significantly reduced the metastasis-promoting effects of the β -adrenergic agonist metaproterenol (MP) but did not affect baseline levels of resistance to metastasis (in rats not treated with MP). Other Chinese formulas and specific extracts have been suggested to decrease cancer development either via a direct interaction with the malignant tissue [51–53], or indirectly by enhancing immunocompetence [54–56].

Importantly, previous research points to NK cells as a pivotal factor controlling lung tumor retention and lung metastases of the MADB106 tumor [35]: pulmonary NK cells were observed interacting with

MADB106 cells in situ [57]; selective depletion of NK cells typically increases MADB106 metastasis more than a 100-fold [28,37,58], and adoptive transfer of NK cells, but not other types of leukocytes, restores resistance to metastasis [18,59]; substances that enhance NK cell activity (e.g. LPS and Poly-IC) improve resistance to metastasis [35], and manipulations that compromise NK activity (e.g. prolonged hypothermia, alcohol consumption, swim stress, social confrontation, food deprivation, and surgery) interfere with it [29,37,60–62]. Notwithstanding, factors other than NK cells, immunological and non-immunological, are likely to also affect tumor metastasis [63]. Therefore, while systemic alterations in NK activity can be expected to affect the metastatic efficacy of MADB106 cells, not all changes in this index can be attributed to changes in NK activity.

The findings of the current study cannot identify a specific mechanism through which ACNO prevents MP from promoting metastasis. Nevertheless, based on the following considerations, we suggest that NK cells play a major role in this phenomenon. We have recently provided evidence that the increase in MADB106 metastasis induced by MP (using the same regimen used here) is mediated via the suppression of NKA: MADB106 metastasis were shown to be highly sensitive to NKA in vivo [35,59], MP caused a dose-dependent suppression of NKA, and, most importantly, the metastasis-promoting effects of MP were abrogated by a selective depletion of NK cells [28]. Thus, because the metastasis-promoting effects of MP are mediated by suppression of NKA, the protective effects of ACNO are most likely mediated by reduced susceptibility of NK cells to suppression by MP. Mechanisms other than NK cells that may affect tumor metastasis irrespective of the effects of MP are less likely to participate, as baseline levels of resistance to metastasis were not affected by ACNO.

The mechanisms underlying the suggested protective effects of ACNO may include an increase in the levels of NK-stimulating cytokines, such as IL-2, IL-12, or INF- γ . Several in vitro studies indicated that biological response modifiers and cytokines that increase NK activity (e.g. Poly-IC, LPS, IL-2, or INF- γ) also protect NK cells from suppression by ligands that elevate cAMP, such as β -adrenergic agents [64,65]. We have recently reported that inject-

ing rats with Poly-IC, which is known to increase levels of NK-activating cytokines (e.g. INF- γ , IL-1, 2 and 12) and NKA, reduced a 30-fold increase in lung tumor retention caused by MP to 2.5-fold effect [66]. Therefore, an increase in the in vivo levels of such cytokines, caused by ACNO, may underlie both the increase in circulating levels of NKA as well as the reduced susceptibility to the metastasis-promoting effects of β -adrenergic agonist MP. This hypothesis, not tested in the current study, should be tested in rats treated with ACNO by studying: (a) in vivo levels of cytokines, and (b) in vitro effects of MP on NK activity from rats treated or not with ACNO. If these studies are successful, active ingredients in the formula should be identified and their mechanism of action should be investigated. These goals were beyond the scope of the current study.

Because metastases of the MADB106 line are sensitive to NKA [18,28,35,36,59], the findings that ACNO increased circulating levels of NKA, but did not improve baseline levels of resistance to MADB106 metastasis, appear inconsistent. However, as MADB106 form metastases only in the lungs, NK activity in the lungs' capillary beds and interstitial tissue may play a greater role in determining the number of lung metastasis than do levels of NKA in the circulation. The current study did not assess levels of NKA in immune compartments other than the circulating blood. It is therefore unclear whether the increase in NKA induced by ACNO is restricted to some immune compartments (e.g. the circulation), or whether it is ubiquitous, but, in the current experimental settings, insufficient to improve baseline levels of resistance to MADB106 metastasis.

An additional non-exclusive hypothesis explaining the apparent inconsistency between the in vitro and in vivo findings relates to technical aspects of the in vitro assessment of NK activity. To assess NKA in vitro, blood is drawn following a minute or two of handling the rat and lightly anesthetizing it. Thus, blood catecholamine levels (e.g. adrenaline) at the time of blood withdrawal are most likely raised. In vivo, these hormones would be quickly degraded in various organs, but after blood is collected for assessment of NK activity, these hormones maintain their impact on NK cells in vitro for longer periods, or until serum is replaced by artificial medium (30–

90 min later). This delay is common in most studies assessing NK activity in animals or in humans. However, as adrenaline and β -adrenergic agonists are known to suppress NK activity in vitro [67–69], we suggest that the above-mentioned 30–90 min exposure to catecholamines, which occurred in all groups of the in vitro study, may have suppressed NKA in the control group more than in the ACNO-treated groups. Blood from rats treated with ACNO may have higher resistance to such suppression, as suggested by the in vivo study (see two paragraphs above). This rather speculative hypothesis should be directly tested. If this hypothesis holds, it unifies the effects of ACNO on NK activity, with its protective effects on resistance to the NK-sensitive MADB106 metastasis evident under the condition of prolong in vivo exposure to the β -adrenergic agonist (second study).

Although ACNO is used by cancer patients, its impact on NKA, on resistance of NK cells to β -adrenoceptor stimulation, or on development of metastasis, have not been studied systematically in cancer patients nor in healthy humans. If the observed effects of ACNO also occur in cancer patients that are in risk of metastasis, then ACNO should be especially beneficial in stressful periods. Surgery for the removal of the primary tumor is obviously stressful, has been shown to cause marked immunosuppression, and was suggested to promote metastasis via suppression of NKA [29,70,71] and via additional mechanisms [31–34]. Thus, scheduling ACNO to the perioperative period may benefit cancer patients by preventing metastatic development that is promoted by surgery- and stress-induced immunosuppression. The potential advantage of using ACNO compared to standard BRM is its long-term tolerance by patients. This formula can be used not only during the perioperative period, but also continuously following surgery and throughout chemotherapy.

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