Antioxidant oils and *Salmonella enterica* Typhimurium reduce tumor in an experimental model of hepatic metastasis

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Abstract: Fruit seeds high in antioxidants have been shown to have anticancer properties and enhance host protection against microbial infection. Recently we showed that a single oral dose of *Salmonella enterica* serovar Typhimurium expressing a truncated human interleukin-2 gene (*SalpIL2*) is avirulent, immunogenic, and reduces hepatic metastases through increased natural killer cell populations in mice. To determine whether antioxidant compounds enhance the antitumor effect seen in *SalpIL2*-treated animals, we assayed black cumin (BC), black raspberry (BR), and milk thistle (MT) seed oils for the ability to reduce experimental hepatic metastases in mice. In animals without tumor, BC and BR oil diets altered the kinetics of the splenic lymphocyte response to *SalpIL2*. Consistent with previous reports, BR and BC seed oils demonstrated independent antitumor properties and moderate adjuvant potential with *SalpIL2*. MT oil, however, inhibited the efficacy of *SalpIL2* in our model. Based on these data, we conclude that a diet high in antioxidant oils promoted a more robust immune response to *SalpIL2*, thus enhancing its antitumor efficacy.

Keywords: antioxidants, colorectal cancer, tumor models, metastasis

Introduction

Attenuated strains of *Salmonella enterica* (*S. enterica*) serovar Typhimurium were first explored as delivery vectors for anticancer therapeutics in the mid 1990s. Since then, *S. enterica* tumor vaccines have shown efficacy in experimental animal models against multiple tumor types including colorectal, melanoma, breast, bone, spine, liver, pancreatic, prostate, and lung. In our experimental models, we have focused on the efficacy of an avirulent and highly immunogenic strain, *S. enterica* Typhimurium χ4550, that was engineered to express a human interleukin-2 (IL-2) gene in primary and experimental metastasis models. In these studies, *S. enterica* χ4550 expressing a human IL-2 gene was effective at reducing the number and volume of hepatic metastases both when administered before and after tumor introduction. Decreased tumor burden in these models was inversely correlated with increased hepatic natural killer (NK) cell populations and when depleted, *S. enterica* χ4550’s antitumor properties were abolished. Most recently, we have demonstrated that *S. enterica* Typhimurium expressing a C-terminal truncated human IL-2 gene (*SalpIL2*) reduced osteosarcoma lung metastases and significantly increased the number of detectable NK cells in the lung as compared to *S. enterica*-vector control or saline gavaged animals. In addition, in the serum of animals with pulmonary osteosarcoma metastases, a single oral dose of *SalpIL2* altered the detection of several proinflammatory cytokines associated with tumor progression. In *Rag-1* knockout mice, which do...
not have functional T-cells, SalpIL2 continued to decrease tumor burden. Thus, SalpIL2’s antitumor properties appear to be independent of T cell contribution.

Adjuvants, such as cyclooxygenase-2 (Cox2) inhibitors appeared to enhance the antitumor efficacy of our attenuated S. enterica strain. When administered with Cox2 inhibitors, S. enterica significantly reduced the number and volume of hepatic metastases as compared to all other treatment types. However, due to insolubility of Cox2 inhibitors in water, controlled dose delivery of these drugs in the drinking water of mice required multiple suspension doses daily. Similar to pharmaceutical Cox2 inhibitors, fruit seed extracts from black cumin (BC; Nigella sativa), black raspberry (BR; Rubus occidentalis), and milk thistle (MT; Silybus marianum) have been shown to contain high levels of antioxidants and have inhibitory effects on Cox2.

In addition, BC and BR seed extracts suppress activity of NFκB, a proinflammatory transcription factor shown to be hyperactive in many cancers. Anticancer properties of extracts from BC, BR, and MT oils have been demonstrated by observation of decreased tumor burden in oil treated animals. The application of antioxidant compounds also appears to be clinically beneficial. Freeze-dried BR gels, when applied directly to premalignant oral lesions, induced regression of cancerous tissues. However, to the best of our knowledge, BR oil has not been reported to affect the growth of syngeneic tumors in organs, nor have BC, BR, and MT oils been shown to be efficacious as adjuvants with immunomodulatory anticancer therapies.

In these studies, we sought to test the hypothesis that BC, BR, and MT antioxidant oils would enhance the efficacy of SalpIL2 in an experimental murine model of hepatic metastases. In this report, we show that several antioxidant compounds augment the cell-mediated immune response to SalpIL2 in nontumor bearing mice. In addition, antioxidant oils provide adjuvant antitumor properties to SalpIL2 and appear to directly decrease the growth of experimental hepatic metastases. In contrast to BC and BR oils, MT oil abrogated the ability of SalpIL2 to reduce the number of hepatic tumors in our model.

Materials and methods

Quantification of lipid-soluble antioxidants in selected oils

Fruit seed oils provided by Botanic Oil Innovation (Spooner, WI) were examined for lipid-soluble antioxidant capacities using an ACL kit to measure the capacity of lipophilic substances following the directions as described by the manufacturer (Analytik Jena, Spring, TX). Briefly, fruit seed oils were dissolved in hexane (20%) and methanol (79%) and run in triplicate to determine Trolox equivalents, a derivative of vitamin E, by chemiluminescence using the Photochem instrument (Analytik Jena).

Bacteria

The avirulent, but highly immunogenic S. enterica serovar Typhimurium strain χ4550, a well-characterized adenylatecyclase, cyclic adenosine monophosphate receptor protein, and aspartate semialdehyde dehydrogenase (asd) deletion mutant, was a kind gift from Dr Roy Curtiss III (Washington University, St Louis, MO). Plasmid pIL2, constructed by insertion of a C-terminal truncated human IL-2 cDNA immediately downstream from the trc promoter in pYA292 which contains an S. enterica asd cDNA for stable maintenance, was electroporated into S. enterica Typhimurium χ4550 and the resultant strain was renamed SalpIL2. SalpIL2 was cultured overnight at 37°C in Luria-Bertani broth (Difco Laboratories, Detroit, MI), washed and reconstituted to approximately 10⁶ colony forming units (CFU) in Hank’s Balanced Salt Solution (HBSS). Bacteria aliquots were stored as a 15% glycerol stock in liquid nitrogen until used in experiments. On the day of oral inoculation, bacterial stocks were allowed to thaw to room temperature and CFU concentration was confirmed by serial dilution on MacConkey agar plates (Becton, Dickinson, and Company, Bedford, MA). The University of Minnesota Institutional Biosafety Committee approved the use of SalpIL2 in all experiments as described.

Mice

Female 6 to 8 week old C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and allowed to acclimate for a minimum of 7 days in specific pathogen free conditions. After inoculation with SalpIL2, all experimental animals were transferred to biosafety level two for the remainder of the study. Antioxidant oils were mixed with ground standard mouse chow (Harlan) to make a homogenous 10% w/w meal. Experimental mice were given ground meal or meal mixed with antioxidant oil daily and drinking water was supplied ad libitum. All animals were housed in microisolator cages and cared for by trained personnel from Research Animal Resources. The University of Minnesota Institutional Animal Care and Use Committee approved all protocols.
Murine adenocarcinoma tumor model
MCA-38 syngeneic tumors were propagated in naïve C57BL/6 mice as previously described. Briefly, 4 × 10^6 tumor cells were injected subcutaneously into the hind flank and allowed to grow until a palpable mass was present. Anesthetized animals were euthanized and the tumor mass was aseptically removed, minced and digested in 0.1% DNase, 0.1% hyaluronidase, and 0.1% collagenase (Sigma Co, St Louis, MO) in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (Sigma) for 2 hours with gentle agitation. The tumor solution was filtered through 150 µm Nitex mesh (Sefar America, Kansas City, MO), centrifuged at 500 × g for 10 minutes and resuspended in HBSS twice before enumeration by trypan blue exclusion. 5 × 10^4 cells were injected into the spleen of anesthetized animals via transabdominal incision and allowed to circulate for 3 minutes. The spleens were removed and the animals were allowed to recover on warming pads until ambulatory. Animals were sacrificed and hepatic metastases were visually enumerated 14 days posttumor injection.

Splenic and hepatic lymphocyte enrichment
Spleens from nontumor bearing animals were collected for splenic lymphocyte population analysis on day 3, 7, 14, and 21-post oral gavage and start of antioxidant oil. After 14 days of tumor growth, tumor-burdened livers were ground in DMEM with 10% fetal goat serum using sterile glass stoppers. The tissue slurry was filtered through 150 µm Nitex mesh, layered on top of lymphocyte separation medium (Mediatech, Inc, Herndon, VA) and centrifuged at 300 × g for 1 hour. Splenic, hepatic, and tumor lymphocytes were collected from the Ficoll-hypaque layer and serially washed with phosphate buffered saline with 1% bovine serum albumin and 0.1% sodium azide (Sigma).

Flow cytometry
Splenic and hepatic lymphocytes were examined for cells reactive with anti-NK1.1, anti-CD4, and anti-CD8 fluorochrome-conjugated anti-mouse monoclonal antibodies (BD Biosciences Pharmingen, San Diego, CA). Lymphocytes were incubated with the antibodies at 4°C for 30 minutes. After washing, analysis was performed with FACS cancytofluorometer (Becton-Dickinson, Grenoble, France) using Cell Quest Pro software (Becton-Dickinson). Lymphocytes were gated by side and forward scatter profiles. Data presented is based on 10,000 gated events.

Statistical analyses
Tumor number, volume, and splenic and hepatic lymphocyte populations were recorded for each mouse and used to calculate the mean values for each experimental cohort. All differences between groups were determined by Fisher's exact test and ANOVA using StatView software (v 5.0.1; SAS Institute, Cary, NC). In the following sections of the manuscript we describe the results as significant (P < 0.05) or "tended/marginally" significant as (P < 0.10).

Results
The lipid-soluble antioxidant capacities of BC, BR, BC+BR, and MT oils were examined. The combination BC+BR seed oil displayed higher antioxidant capacities (25.9 ± 0.2) in equal combination as compared to individual oils (BC, 21.0 ± 0.1 and BR, 19.8 ± 0.6). In contrast, MT seed oil displayed the lowest levels of lipid-soluble antioxidants (1.3 ± 0.2) of the oils tested.

BC and BR enhance cellular immune response to SalpIL2
On day 14, NK cell populations were increased (P < 0.10) in animals administered SalpIL2 with or without BC+BR oil diet (10% w/w) compared to saline and oil-only groups (Figure 1A). On day 21, SalpIL2 with BC+BR significantly increased NK cell populations over control and combination oil-only groups. SalpIL2 with or without BC+BR increased CD8+ T cell populations significantly by day 7 over saline control and oil-only groups (Figure 1B). By day 21, however, all experimental groups had significantly lower splenic CD8+ and CD4+ T cell populations than control animals (Figure 1B and 1C).

BC and BR enhance the NK cell response to SalpIL2 in tumor burdened animals
We next used a hepatic metastasis model to screen whether NK cell populations, increased from BC+BR and SalpIL2 administration (Figure 1A) would reduce tumor burden in vivo. Consistent with our previous studies, SalpIL2 tended to reduce the volume of hepatic metastases by 78% in our model (P < 0.10). All experimental treatments, for example, BC+BR oil with and without SalpIL2, did not have significantly reduced tumor number and volume as compared to control animals (Figure 2A and 2B). Hepatic NK cell populations were significantly increased by SalpIL2 alone (272%) or SalpIL2 with BC+BR combination oil (412%) as compared to the controls (all P < 0.05). Mice given the combination of
SalpIL2 with BC+BR oil tended to have increased NK cell populations compared to animals only given SalpIL2 (Figure 2C; \( P < 0.10 \)). CD8\(^+\) and CD4\(^+\) T cell populations were not significantly different across the experimental groups (Figure 2D and 2E).

**BC oil diet reduces hepatic metastases**

Although the combination BC+BR seed oils did not significantly decrease tumor number and volume in our hepatic metastasis model, we examined whether either BC or BR oil alone may enhance the antitumor efficacy of SalpIL2. Similar to the BC+BR combination oil diet, a BC seed oil diet did not significantly decrease the number of hepatic metastases. A diet with BC oil alone, however, reduced tumor volume by 74\% and when administered with oral SalpIL2, significantly decreased tumor volume 75\% as compared to control animals (Figure 3B; all \( P < 0.05 \)). The reduction of tumor volume was similar to SalpIL2-only treated animals (91\%). The antitumor effect observed by BC oil appeared to be independent of increased NK and CD8\(^+\) T cell populations. Only SalpIL2-treated animals, with or without BC oil, displayed significantly higher NK cell populations as compared to controls (Figures 3C and 3D; \( P < 0.05 \)). Lastly, SalpIL2 with BC oil treated animals displayed a significant 29\% reduction in CD4\(^+\) T cell population at the experimental endpoint (Figure 3D; \( P < 0.05 \)).

**BR oil diet has treatment and preventative potential on hepatic metastases**

In our model, a diet high in BR seed oil significantly decreased tumor volume, not tumor number, by 47\% as compared to controls (Figure 4A and 4B; \( P < 0.05 \)). No significant additional benefit in reduction of tumor volume was observed in animals administered SalpIL2 with (61\%) or without BR seed oil (64\%). In hepatic lymphocyte populations, however, only animals treated with SalpIL2 and BR oil had significantly increased NK cells (219\%) in the tumor-burdened animals when compared to control (Figure 4C; \( P < 0.05 \)). There were no significant changes in T cell populations between treatment groups. Previously, we showed that inoculation of mice with *S. enterica* 7 days prior to tumor injection provided protection against the establishment of hepatic metastases. Similar to administering a diet of BR oil 3 days after tumor injection, BR oil alone significantly reduced the volume not number of hepatic metastases by 45\% as compared to controls (Figures 5A and 5B; \( P < 0.05 \)). Animals given SalpIL2 with BR oil had similar reduction in
tumor number and volume to SalpIL2 alone, 81%, 98% and 70%, 96% respectively as compared to controls \((P < 0.10)\). Only animals administered SalpIL2 with BR oil showed a significant 298% increase in NK cell populations as compared to control animals \((P < 0.05)\). SalpIL2-only animals had a marginally significant increase of 260% in NK cell populations compared with controls \((P < 0.10)\). Only CD8\(^+\) T cell populations were significantly altered between BR oil SalpIL2 treatment groups \((P < 0.05)\).

**BR oil diet augmented cellular immunity to SalpIL2**

To further elucidate the antitumor efficacy of BR seed oil, we examined the effect of BR oil diet on the splenic lymphocyte populations in SalpIL2-treated mice. BR oil with SalpIL2 increased NK cells by day 3 over control animals \((5.7 \text{ to } 4.7)\) respectively; Figure 6A; \(P < 0.05\), which has not previously been observed in SalpIL2-treated animals.\(^{13}\) By days 7 and 14 however, increased NK-cell populations by SalpIL2 were independent of BR oil treatment. With respect to CD8\(^+\) T cell populations, SalpIL2 with BR oil significantly increased CD8\(^+\) T cell populations on days 3 and 14 compared to SalpIL2-treated and control animals \((P < 0.05)\). SalpIL2 and BR oil treatment also affected CD4\(^+\) T cell populations. By day 14, SalpIL2 significantly decreased CD4\(^+\) T cell populations \((P < 0.05)\), while SalpIL2 with BR oil appeared to negate the SalpIL2-dependent drop in CD4\(^+\) T cells (Figure 6C).

**MT seed oil abrogates the antitumor efficacy of SalpIL2**

As demonstrated in Figure 5, the antitumor effect of SalpIL2 is most evident in animals when delivered prior to...
tumor administration. MT oil, however, appeared to inhibit SalpIL2's ability to reduce tumor number (98% to 37%; P < 0.10; Figure 7A) and volume (99% to 7%; P < 0.01; Figure 7B). MT oil appeared to reduce the NK cell response to SalpIL2 as NK cell populations were significantly increased in animals given SalpIL2 with MT oil (255%) and SalpIL2 alone (335%; Figure 7C). Interestingly both MT oil (315%) and SalpIL2 alone (387%) increased CD8$^+$ T cell populations as compared to controls (Figure 7D), whereas, SalpIL2 with MT oil (176%) showed a significant reduction in CD8$^+$ T cell populations as compared to SalpIL2 alone. SalpIL2 increased the number of hepatic CD4$^+$ T cells (376%) as compared to controls, which was significantly increased with respect to all other experimental groups.

**Discussion**

In this report, we investigated whether several known antioxidant seed oils could reduce hepatic metastasis or enhance the antitumor efficacy of attenuated *S. enterica* Typhimurium. Consistent with previous reports on synergistic antioxidant capacities upon mixing oils, we show, compared to individual oils, the combination of BC+BR had increased antioxidant capacity. When given to mice without tumors, the combination of BC+BR oil with SalpIL2 resulted in an increase in splenic NK cells at day 21. The BC+BR combination oil was synergistic with SalpIL2 and increased hepatic NK cells populations, however, the number and volume of hepatic metastases were not significantly decreased. Perhaps due to the increased cell-mediated immune response to SalpIL2, the addition of BC+BR oil to the diet may have provided an increased clearance of SalpIL2. Thus, additional doses may be required to maintain the same level of SalpIL2 infection and demonstrate a significant difference between SalpIL2 with and without BC+BR oil diet.

Since, BC and BR oils individually have displayed significant antitumor properties in other models, we next asked whether individual BC or BR oils have the ability to reduce tumor burden in our hepatic metastasis model. BC
Effect of antioxidant oils and *S. enterica* on hepatic metastasis

On Day 0, $5 \times 10^4$ MCA-38 cells were injected intrasplenically to naïve animals. A single oral administration of SalpIL2 and initiation of BR (shaded area) seed oil diet 10% w/w was given to animals on Day 3. Animals were maintained for a total of 14 days prior to collection of tumor and hepatic lymphocyte data (see diagram above). Tumor number (A) and tumor volume (B) in animals fed a diet consisting of BR with and without SalpIL2. Hepatic natural killer (NK) (C), CD8$^+$ T (D), and CD4$^+$ T cell response (E) to SalpIL2 with and without BR oil on Day 14.

Notes: Bars represent statistical significance between groups. Data was normalized to percent of control and presented as ± standard error of mean from three independent experiments with $n = 5$ mice per group.

Similar to BC oil, BR oil alone significantly reduced tumor volume and appeared to enhance the NK cell response to SalpIL2 in mice with established metastases. Since tumor number was not significantly reduced, we interpreted these data to suggest that BR oil reduced the growth rather than induced regression of tumor. Although SalpIL2 alone marginally increased hepatic NK cells, the addition of BR oil significantly increased NK cells in tumor bearing mice, suggesting BR oil enhanced the NK cell response to SalpIL2. This observation was consistent in our experiments in nontumor burdened animals, as BR oil appeared to also increase the NK cell response to SalpIL2 (Figure 6). Oral administration of SalpIL2, with and
Figure 5 Effect of black raspberry (BR; *Rubus occidentalis*) seed oil and SalpIL2 on the prevention of experimental hepatic metastases in mice. A single oral administration of SalpIL2 and initiation of BR (shaded area) seed oil diet 10% w/w was given to animals on Day -7. On Day 0, 5x10⁴ MCA-38 cells were injected intrasplenically and animals were maintained for 14 additional days. Tumor and hepatic lymphocyte data collected on Day 14 (see diagram above). Tumor number (A) and tumor volume (B) in animals fed a diet consisting of BR with and without SalpIL2. Hepatic natural killer (NK) (C), CD⁸⁺ T (D), and CD⁴⁺ T cell response (E) to SalpIL2 and BR oil in tumor burden mice on Day 21. Notes: Bars represent statistical significance between groups. Data was normalized to percent of control and presented as ± standard error of mean from three independent experiments with n = 5 mice per group.

Figure 6 Effect of black raspberry (BR; *Rubus occidentalis*) seed oil on the splenic lymphocyte response to SalpIL2 in mice. Splenic natural killer (NK) (A), CD⁸⁺ T (B), and CD⁴⁺ T cell populations (C) as determined by flow cytometry in response in animals fed a diet consisting of BR seed oil 10% w/w (O), administered as single oral dose of SalpIL2 (☐) or SalpIL2 + BR oil (▲) starting on day 0 as compared to control animals (☒). Notes: The table insert indicates statistical significance between groups. Data are mean ± standard deviation from one experiment, n = 5 mice group.
without BR oil, induced a significant increase in splenic NK cells after as short a time period as 3 days and peaked 7 days after oral inoculation (Figures 1A and 6A). Tumor delivery at the peak of the NK response to SalpIL2 and BR (Figure 5) resulted in greater than 90% reduction of tumor, though there was no statistical difference between the two Salmonella groups. BR oil alone, however, significantly reduced tumor volume suggesting BR oil can induce a preventive effect to the liver within 7 days to reduce tumor growth.

Not all oils tested in our models were shown to have measureable antitumor effects. MT oil, which had the least detectable amount of lipid soluble antioxidants, did not provide any protection against hepatic metastasis. Extracts from milk thistle seeds have shown antiproliferative effects with colon cancer cell lines and showed synergism with the chemotherapeutics doxorubicin and paclitaxel in vitro.\(^40\) Most importantly milk thistle extracts have demonstrated promise as adjuvant treatment with minimal adverse effects in human clinical trials as reviewed by Tamayo and Diamond.\(^41\) Silibinin, a flavinoid contained in MT seeds has been shown to inhibit tumorigenesis in rats\(^42\) and promotes cell cycle arrest of human colon cancer cells.\(^43\) Our results with MT oil seem to contradict previous reports, however, mice were only fed the diet of MT oil 1 week prior to tumor injection, whereas others dosed silibinin for 3 weeks prior to prostate tumor xenograft.\(^44\) Even though our tumor cells must survive metastasis from the spleen to the liver, the exposure to MT oil appeared to have little effect on tumor cell death or survival. In contrast, MT oil treatment alone slightly increased tumor burden and reversed the antitumor efficacy of SalpIL2 in our model. Silibinin has been described to be hepatoprotectant against infection and may have blocked the ability of SalpIL2 to mount an antitumor response, perhaps by enhancing host resistance to infection in the liver.\(^45,46\)

In these studies, we show antioxidant oils with SalpIL2 induce a robust NK cell-mediated response and reduction of tumor burden. By administering SalpIL2 and the antioxidant oil.
diet 3 days after establishment of hepatic metastases, we model the clinical situation in which patients are diagnosed with distant metastases. In the clinical setting, soon after surgical resection, systemic chemotherapy is used to reduce undetectable micrometastases or unresectable metastatic lesions. Despite these efforts, patients diagnosed with metastatic colorectal cancer have an average 3-year survival rate of 11%.47 Because there is a propensity for malignant colon cancers to form hepatic metastases, the use of *S. enterica*, whose primary site of infection is within the liver, is an ideal vector for colorectal hepatic metastases. Furthermore, based on the data in this report, the administration of antioxidant oils with SalpIL2 induces a more robust NK cell response as compared to SalpIL2 alone; even when tumors are not present. Hence, when administered prior to tumor development or under low metastatic tumor burden, SalpIL2 with antioxidant oils may create an environment in the liver that is abundant with NK cells and thus inhospitable for tumor metastasis. By reducing the occurrence of hepatic metastasis, SalpIL2 and antioxidant oils may provide a significant survival benefit, perhaps similar to the 90% 5-year survival of colon cancer patients without metastatic disease.47 Therefore, additional studies on the mechanism by which antioxidant oils and SalpIL2 suppress metastatic tumors is warranted.

**Disclosure**

BS, AL, and DS are stockholders in Botanic Oil Innovation. All other authors certify that they have no commercial associations that may pose a conflict of interest in connection with this manuscript.

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