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Effect of Homeopathic Treatment on Gene Expression in Copenhagen Rat Tumor Tissues

Rajesh L. Thangapazham, MS, N. V. Rajeshkumar, PhD, Anuj Sharma, MS, Jim Warren, MS, Anoop K. Singh, PhD, John A. Ives, PhD, Jaya P. Gaddipati, PhD, Radha K. Maheshwari, PhD, and Wayne B. Jonas, MD

Background: Increasing evidence suggests that the inability to undergo apoptosis is an important factor in the development and progression of prostate cancer. Agents that induce apoptosis may inhibit tumor growth and provide therapeutic benefit. In a recent study, the authors found that certain homeopathic treatments produced anticancer effects in an animal model. In this study, the authors examined the immunomodulating and apoptotic effects of these remedies. **Materials and Methods:** The authors investigated the effect of a homeopathic treatment regimen containing *Conium maculatum*, *Sabal serrulata*, *Thuja occidentalis*, and a MAT-LyLu Carcinosis nosode on the expression of cytokines and genes that regulate apoptosis. This was assessed in prostate cancer tissues, extracted from animals responsive to these drugs, using ribonuclease protection assay or reverse transcription polymerase chain reaction. **Results:** There were no significant changes in mRNA levels of the apoptotic genes *bax*, *bcl-2*, *bcl-x*, *caspase-1*, *caspase-2*, *caspase-3*, *Fas*, *FasL*, or the cytokines interleukin (IL)-1 α , IL-1 β , tumor necrosis factor (TNF)- β , IL-3, IL-4, IL-5, IL-6, IL-10, TNF- α , IL-2, and interferon- γ in prostate tumor and lung metastasis after treatment with homeopathic medicines. **Conclusions:** This study indicates that treatment with the highly diluted homeopathic remedies does not alter the gene expression in primary prostate tumors or in lung metastasis. The therapeutic effect of homeopathic treatments observed in the in vivo experiments cannot be explained by mechanisms based on distinct alterations in gene expression related to apoptosis or cytokines. Future research should explore subtle modulations in the expression of multiple genes in different biological pathways.

Keywords: homeopathy; Carcinosis; MAT-LyLu; Copenhagen rats; apoptosis; cytokines; metastasis

Apoptosis and cell cycle progression are 2 intimately linked phenomena. The ability of tumor cells to respond to damage and eventually activate the apoptotic cascade determines the ultimate success of cancer therapy.¹ Tang and Porter² summarized the data on the relationship between apoptosis and prostate

cancer and concluded that identification of new therapeutic agents can be achieved by analyzing apoptotic changes. The key regulators involved in apoptosis are well characterized and include caspases, Bcl-2 family, tumor necrosis factor (TNF) receptor family, and other adapter proteins.³ Androgen-dependent prostate tumors undergo apoptosis in response to androgen ablation and expression of Bcl-2, and caspases correlate with the prostate cancer cell's sensitivity to the therapy. Prostate cancers respond to hormone ablation through apoptosis, which is regulated by several genes including the tumor suppressor gene p53 and proto-oncogene Bcl-2.⁴ Bcl-2 confers negative control in the pathway of cellular suicide machinery. A Bcl-2 homologous protein, Bax, promotes cell death by competing with Bcl-2. While Bax-Bax homodimers act as apoptosis inducers, Bcl-2-Bax heterodimer formation evokes a survival signal for the cells. Both Bcl-2 and Bax are transcriptional targets for the tumor suppressor protein, p53, which induces cell cycle arrest or apoptosis in response to DNA damage. Thus, evaluation of novel therapies such as those in complementary and alternative medicine should examine these factors as possible mechanisms.

In a previous study conducted in our laboratory, we investigated the antitumor effect of a homeopathic treatment comprising *Conium maculatum*, *Thuja occidentalis*, *Sabal serrulata*, and homeopathic *Carcinosis* on the prostate cancer line MAT-LyLu cells growth in Copenhagen rats. We found significant reduction in the tumor incidence (23%), tumor volume (45%), and tumor weight (33%) in the homeopathy-treated group as compared with the control group. The study also revealed increased apoptosis and decreased

RLT, NVR, AS, JW, AKS, JPG, and RKM are in the Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda, Maryland. RLT and AS are also at the Birla Institute of Technology and Science, Pilani, India. JAI and WBJ are at the Samuelli Institute, Alexandria, Virginia.

Correspondence: Wayne B. Jonas, MD, Samuelli Institute, 1700 Diagonal Road, Suite 400, Alexandria, VA 22314. E-mail: wjonas@siib.org.

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proliferation in MAT-LyLu cells inoculated into Copenhagen rats in comparison to untreated controls.⁵ In the present study, we have analyzed the effect of homeopathic treatment on the expression of genes involved in apoptosis and cytokines in tumor and lung tissues from the homeopathy-treated animals to explore possible mechanisms underlying homeopathic treatment. A few available studies have suggested immunoregulation by homeopathy in high dilutions.⁶

Materials and Methods

Homeopathic Medicines

Homeopathic medicines *C maculatum*, *S serrulata*, *T occidentalis*, *Asterias*, and *Phytolacca* with 30 c, 200 c, and 1000 c concentrations were obtained from Boiron (Simi Valley, Calif). *Carcinosins* (1000 c) were prepared from MAT-LyLu and MDA-MB-231 cells by Washington Homeopathic Products Inc (Bethesda, Md), as previously described.⁷

Animal Treatment and Tissue Collection

Tissues collected from the animals, in our in vivo study with Copenhagen rats described earlier,⁷ were used in the present study. Briefly, Copenhagen rats inoculated with MAT-LyLu cells were fed with 100 μ L of a homeopathic remedy regimen or succussed water as controls once daily. The homeopathic treatment regimen contained *T occidentalis* (100 c) on the first and fourth day of the week, *C maculatum* (1000 c) on the second and fifth day of the week, *S serrulata* (200 c) on the third and sixth day of the week, and MAT-LyLu *Carcinosin* (1000 c) on the seventh day of the week. Five weeks after cell inoculation, the experiment was concluded and animals were killed. At the time of killing, tumors and lungs were removed, weighed, and immediately frozen for further analysis.

mRNA Analysis by Ribonuclease Protection Assay

To determine the effect of homeopathic treatment on mRNA expression, tumor and lung tissue specimens were homogenized, and the total RNA was isolated using TRIzol (Invitrogen, Carlsbad, Calif). The isolated RNA was quantitated by spectrophotometry and equalized, and the purity was checked on 1% formaldehyde agarose gel. mRNA expression of treated and untreated cells was determined by ribonuclease protection assay (RPA). mRNA levels for apoptotic genes and cytokines were estimated using RiboQuant multiprobe sets rAPO-1 (bax, bcl-2, bcl-x, caspase-1, caspase-2, caspase-3, Fas, FasL, L32, and glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) and rCK-1 (interleukin [IL]-1 α , IL-1 β ,

TNF- β , IL-3, IL-4, IL-5, IL-6, IL-10, TNF- α , IL-2, interferon [IFN]- γ , L-32, and GAPDH) using a kit from BD Biosciences (San Diego, Calif), respectively. The protocols used for the RPA were according to the manufacturer's instructions. Briefly, 20 μ g of each RNA sample was hybridized at 56°C for 12 to 14 hours with a ³²P-UTP-labeled probe. The probe was prepared by transcribing the rat apoptosis template set using T7 RNA polymerase. After hybridization, samples were subjected to RNase digestion for 45 minutes at 30°C. The ribonuclease-protected bands were then resolved on denaturing urea-polyacrylamide gels, followed by autoradiography. L32 and GAPDH mRNAs served as housekeeping gene controls in the assay to ensure equal loading of RNAs.

mRNA Estimations by Reverse Transcriptase Polymerase Chain Reaction From Laser-Controlled Microdissected Tumor Tissue From Lung Specimens

We also performed mRNA analysis based on laser-controlled microdissection (LCM) of defined regions of the tissues. The LCM technique allowed for efficient isolation of tumor regions with no or very low contamination of surrounding tissue components, simultaneously leaving the intracellular structure and molecules intact. Frozen lung tissue specimens from animal experiments⁷ were used for LCM using the Arcturus PixCell Iie Laser Capture Microdissection System and the Histogene LCM Frozen Section Staining Kit (Arcturus, Mountain View, Calif). Total RNA was isolated from microdissected regions using the PicoPure RNA Isolation Kit (Arcturus, Mountain View, Calif). Complementary DNA was synthesized using total RNA and the SuperScript First Strand Synthesis System for reverse transcriptase polymerase chain reaction (RT-PCR; Invitrogen, Carlsbad, Calif). Primers for caspase-1 and GAPDH were obtained from Maxim Biotech Inc (South San Francisco, Calif). Caspase-3 primers were synthesized 5' ACG GTA CGC GAA GAA AAG TGA C 3' for sense and 5' TCC TGA CTT CGT ATT TCA GGG C 3' for antisense. The PCR reaction mixture contained 10 pmol/L of each primer pair and 2.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, Calif). The linear range of amplification of each gene was determined by carrying out 18 to 35 cycles in increments of 3 cycles each. Based on the results, amplification reactions were carried out in 25 to 33 customized cycles for each gene (95°C for 30 seconds, 55°C-60°C for 45 seconds, and 72°C for 60 seconds) using 10% of cDNA. Annealing temperatures used were 3°C to 5°C less than the primer's melting temperature. GAPDH was used as an internal control. PCR products (10 μ L each) were analyzed by electrophoresis on 2%

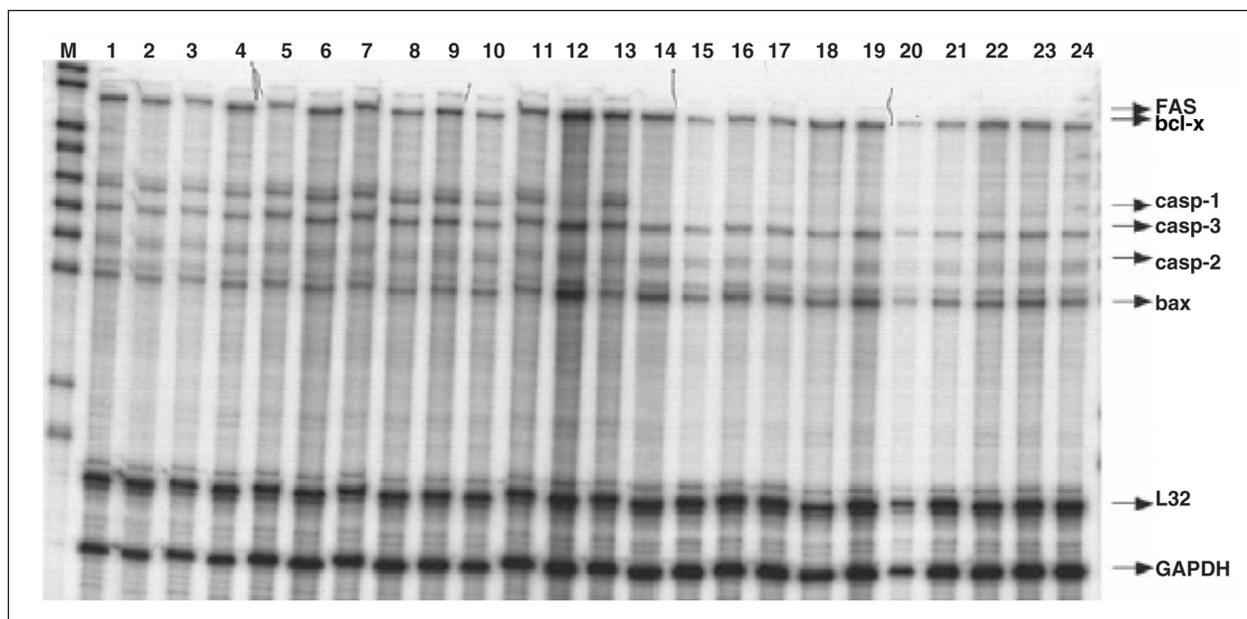


Figure 1 mRNA expression of apoptotic genes as analyzed by ribonuclease protection assay. Lanes: M = marker; 1-4 = normal; 5-9 = lung specimens from MAT-LyLu-injected animals; 10-14 = lung specimens from MAT-LyLu-injected and homeopathy-treated animals; 15-19 = tumor specimens; 20-24 = tumor specimens from homeopathy-treated animals.

agarose gels containing ethidium bromide. Densitometric analysis of PCR products was performed with Scanalyze software, and the quantitations were normalized to GAPDH.

Results

Effect of Homeopathic Treatment on Apoptotic Gene Expression in Tumor Tissues

MAT-LyLu cells inoculated in rats treated with the different homeopathic medicines, *C maculatum*, MAT-LyLu *Carcinosin*, *S serrulata*, and *T occidentalis* were analyzed for modulations in gene expression and were compared with the untreated controls. The study was done by RPA specifically for genes involved in the apoptotic process. The analysis with the multiprobe set for apoptosis (rAPO-1) containing Fas antigen, bclXL, bclXS, FasL, caspase-1, caspase-3, caspase-2, bax, and bcl-2 showed no significant differences in mRNA levels for any of these apoptotic genes between treated and water-treated controls in tumor and lung tissues (Figure 1). However, marked differences were observed in the caspase-1 mRNA levels between lung and tumor tissue specimens. Primary tumors lacked the expression of caspase-1, whereas lung tumors and normal animal tissue showed caspase-1 expression. Homeopathy treatment did not alter the expression of this gene.

As gene expression analysis using total RNA of bulk tissue did not show specific differences in the gene expression of homeopathy-treated and water-treated tissues, we extended the analysis to LCM-captured

lung tumor tissue that facilitates elimination of skewed data from contamination of tumor tissue with surrounding normal tissue. Expression analysis for the apoptotic genes caspase-1 and caspase-3 from such microdissected tissue also did not show any significant differences between homeopathy-treated and water-treated lung tumor specimens (Figure 2).

Effect of Homeopathic Medicines on Inflammatory Cytokines

We investigated the cytokine profiles in the lungs and tumors of Copenhagen rats inoculated with MAT-LyLu cells and treated with the homeopathic treatment regimen. The analysis was done by RPA using a multiprobe panel (rCK-1) having IL-1 α , IL-1 β , TNF- β , IL-3, IL-4, IL-5, IL-6, IL-10, TNF- α , IL-2, and IFN- γ . No significant differences were observed for any of the cytokines analyzed between homeopathy-treated and water-treated controls (Figure 3). L-32 and GAPDH mRNAs served as housekeeping gene controls in the assay to normalize the data for equalizing the RNAs.

Discussion

Homeopathy is one of the most widely used complementary and alternative medicines in the world,⁸ and many consider it as a placebo treatment because of the extremely low doses used.⁹ The physical nature of homeopathic remedies and also the precise role of succussion in the remedy preparation process are unknown. However, evidence presented in our previous study indicates effectiveness of homeopathic

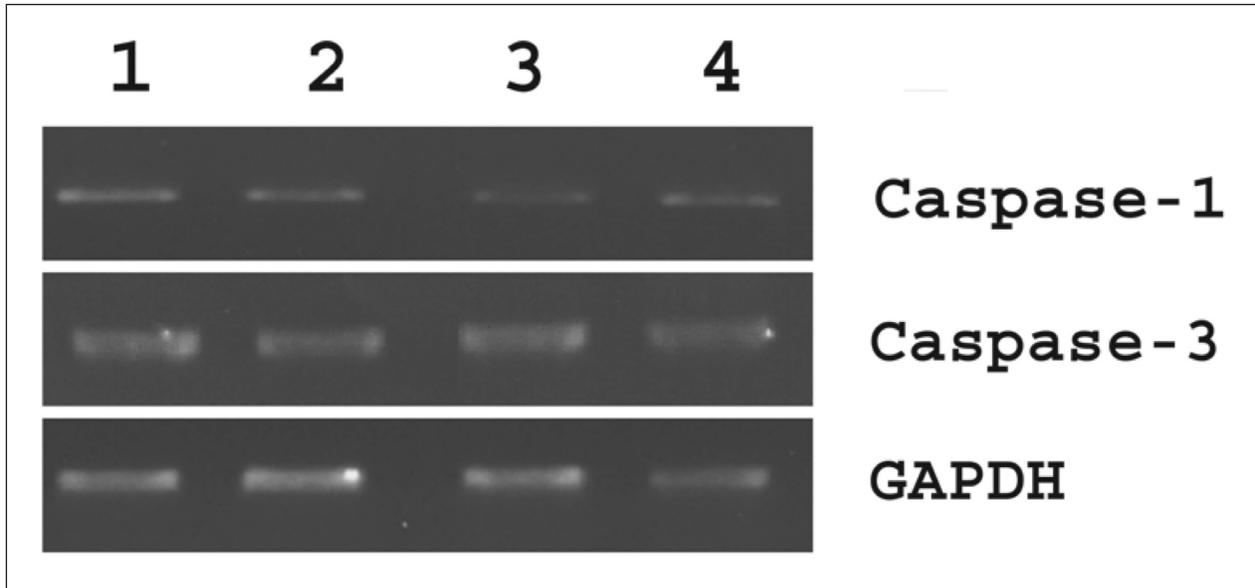


Figure 2 Reverse transcriptase polymerase chain reaction analysis of caspase-1 and caspase-3 mRNAs expressed in laser microdissected lung tumor tissues. Lanes: 1 and 2 = MAT-LyLu-injected animals; 3 and 4 = MAT-LyLu-injected and homeopathy-treated animals. GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

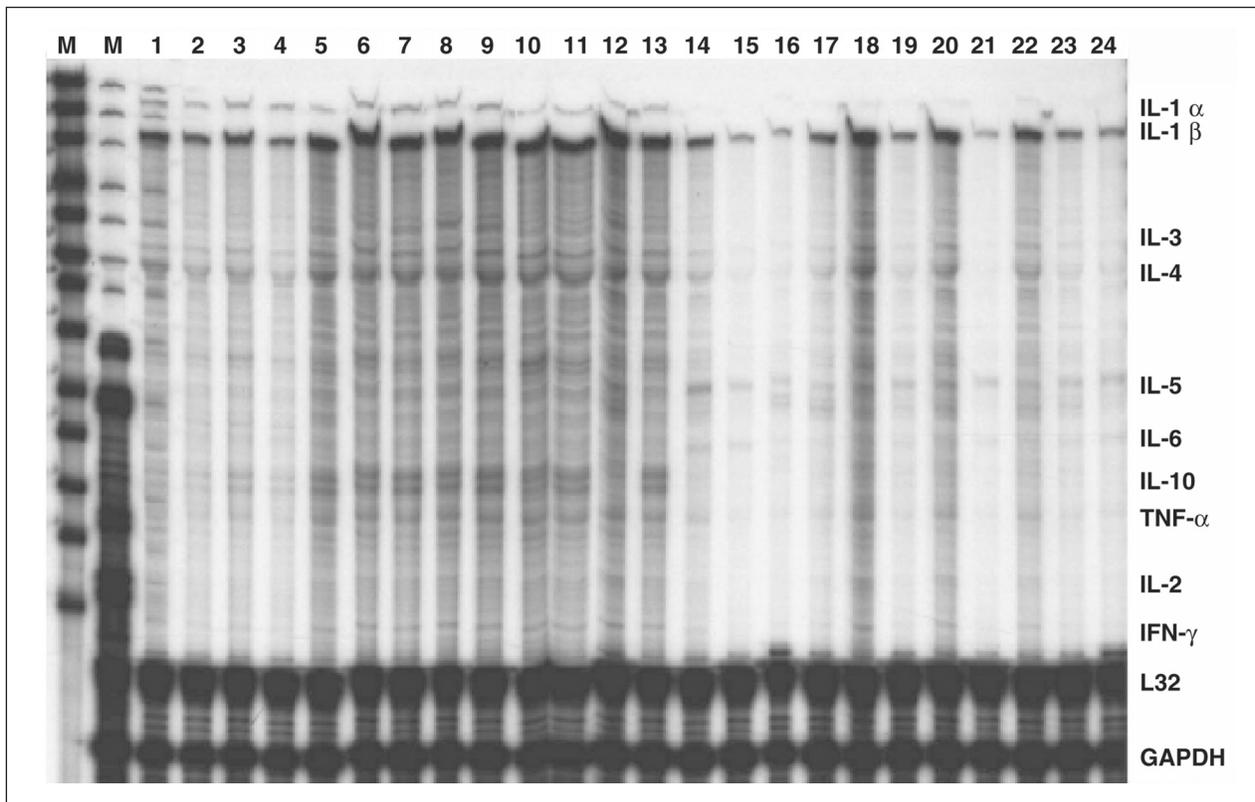


Figure 3 mRNA expression of cytokine genes as analyzed by ribonuclease protection assay. Lanes: M = marker; 1-4 = normal; 5-9 = lung specimens from MAT-LyLu-injected animals; 10-14 = lung specimens from MAT-LyLu-injected and homeopathy-treated animals; 15-19 = tumor specimens; 20-24 = tumor specimens from homeopathy-treated animals. IL = interleukin; TNF = tumor necrosis factor; IFN = interferon; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

medicines in reducing tumor incidence, tumor growth, and metastasis in rats.⁷ Given these observations, we explored possible mechanisms for these effects

by investigating the regulation of apoptotic genes and cytokines in the prostate tumors of Copenhagen rats that responded to homeopathy.

Studies from several independent laboratories have also indicated enhanced cellular adaptive process and anticancer effects with low doses of chemicals including some homeopathy medicines.¹⁰⁻¹² Similarly, ultralow doses of cadmium have been shown to induce the expression of protective proteins¹³ and render the protection of prostate cells from neoplastic transformation to higher dose exposures.¹⁴ Arsenic trioxide has been widely used in homeopathic medicine and has been shown to induce incomplete differentiation, apoptosis, and degradation of oncogenic protein in acute promyelocytic leukemia.¹⁵⁻¹⁷ Ultralow doses of TNF and adriamycin/cisplatin have been reported to induce apoptosis in resistant human ovarian cancer cells.¹⁸ Immunotherapy of hepatocellular carcinoma patients using an ultralow dose of IL-2 (1 MIU/d) induced tumor regression and prolonged the patients' survival.¹⁹ Recently, the toxicity of ultrahigh dilutions of 3,5-dichlorophenol showed a significant inhibitory effect from these preparations using luminescent bacteria testing.²⁰ Analyses of reports from clinical trials of homeopathy have been mixed.^{9,21} While these studies address the clinical effects of homeopathic medicines, they do not address mechanisms of action.

Treatment with high dilutions in our study did not show any significant effect on the expression of genes analyzed. In a separate study, treatment of human as well as rat prostate cells with highly diluted homeopathic medicines also did not show any alterations in their mRNA expression.²²

During prostate tumorigenesis, losses in the protein expression of apoptotic genes such as caspase-1 and caspase-3 have been reported.²³ In the same study, RT-PCR analysis of mRNA expression did not show reduced expression in all the specimens analyzed, suggesting posttranscriptional deregulation. However, in our study, we found a reduction in the mRNA expression of caspase-1 only in all primary tumor tissues and not in lung tissues. The discrepancy in the expression between primary tumors and lung tumors may indicate tissue-specific differences and probably different mechanisms involved in the caspase-1 regulation. Also, homeopathic medicines did not alter caspase-1 expression in either of these tissues.

Conclusions

In conclusion, our experiments show that the investigation of specific gene expression analysis for apoptosis and cytokine alteration may not provide sufficient insights into the mechanisms of homeopathic treatment of prostate cancer. Investigation of the homeopathic treatment of cancer may require novel and sensitive methodologies such as global gene array analysis to detect subtle differences to

account for the beneficial effects of homeopathic treatment *in vivo*. We are planning to study the changes in protein levels using proteomics in the future.

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References

1. McCloskey D, Armstrong D, Jackisch C, Davidson N. Programmed cell death in human breast cancer cells. *Recent Prog Horm Res*. 1996;51:493-508.
2. Tang DG, Porter AT. Target to apoptosis: a hopeful weapon for prostate cancer. *Prostate*. 1997;32:284-293.
3. Boedefeld WM II, Bland KI, Heslin MJ. Recent insights into angiogenesis, apoptosis, invasion, and metastasis in colorectal carcinoma. *Ann Surg Oncol*. 2003;10:839-851.
4. Johnson MI, Hamdy FC. Apoptosis regulating genes in prostate cancer. *Oncol Rep*. 1998;5:553-557.
5. Jonas WB, Gaddipati JP, Rajeshkumar NV, et al. In vitro and in vivo assessment of homeopathic treatment for prostate cancer. Paper presented at: Society of Integrative Oncology First International Conference; November 18, 2004; New York, NY.
6. Heine H, Schmolz M. Immunoregulation via "bystander suppression" needs minute amounts of substances—a basis for homeopathic therapy? *Med Hypotheses*. 2000;54:392-393.
7. Jonas WB, Gaddipati JP, Rajeshkumar NV, et al. Can homeopathic treatment slow prostate cancer growth? *Integ Cancer Ther*. 2006;5:343-349.
8. Jonas WB. The homeopathy debate. *J Altern Complement Med*. 2000;6:213-215.
9. Shang A, Huwiler-Muntener K, Nartey L, et al. Are the clinical effects of homeopathy placebo effects? Comparative study of placebo-controlled trials of homeopathy and allopathy. *Lancet*. 2005;366:726-732.
10. Cambar J, Delbancut A, Barrouillet M. Effects of metal dilutions on cells and integrated systems. In: Taddei-Ferretti C, ed. *High Dilution Effects on Cells and Integrated Systems*. Hong Kong: World Scientific Editions; 1998:45-62.
11. Wiegant F, van Rijn J, van Wijk R. Enhancement of the stress response by minute amounts of cadmium in sensitized Reuber H35 hepatoma cells. *Toxicology*. 1997;116:27-37.
12. Linde K, Jonas W B, Melchart D, Worku F, Wagner H, Eitel F. Critical review and meta-analysis of serial agitated dilutions in experimental toxicology. *Hum Exp Toxicol*. 1994;13:481-492.
13. Goering P, Waalkes M, Klaassen C. Cadmium toxicity. In: Goyer R, Cherian M, eds. *Handbook of Experimental Pharmacology; Toxicology of Metals, Biochemical Effects*. New York, NY: Springer-Verlag; 1994.
14. Cambar J, Delbancut A, Barrouillet M. Effects of cadmium very high dilutions in renal tubular cell cultures. In: Taddei-Ferretti C,

- ed. *High Dilution Effects on Cells and Integrated Systems*. Hong Kong: World Scientific Editions; 1998:107-115.
15. Soignet SL, Maslak P, Wang ZG, et al. Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. *N Engl J Med*. 1998;339:1341-1348.
 16. Che G, Zhu J, Shia X. In vitro studies on cellular and molecular mechanisms of arsenic trioxide in the treatment of acute promyelocytic leukemia: As₂O₃ induces NB4 cells apoptosis with down regulation of Bcl2 expression and modulation of PML-RAR alpha/PML proteins. *Blood*. 1996;88:1051-1061.
 17. Shao W, Fanelli M, Ferrara FF, et al. Arsenic trioxide as an inducer of apoptosis and loss of PML/RAR alpha protein in acute promyelocytic leukemia cells. *J Natl Cancer Inst*. 1998;90:124-133.
 18. Tsuchitani T, Zigelboim J, Beker J, Bonavida B. Potentiation of cytotoxicity against human ovarian cell lines with combination of sub toxic concentrations of tumor necrosis factor and adriamycin or cisplatinium. *J Cell Pharmacol*. 1991;2:1-11.
 19. Palmieri G, Montella L, Milo M, et al. Ultra-low-dose interleukin-2 in unresectable hepatocellular carcinoma. *Am J Clin Oncol*. 2002;25:224-226.
 20. Brack A, Strube J, Stolz P, Decker H. Effects of ultrahigh dilutions of 3,5-dichlorophenol on the luminescence of the bacterium *Vibrio fischeri*. *Biochim Biophys Acta*. 2003;1621:253-260.
 21. Linde K, Jonas W. Are the clinical effects of homeopathy placebo effects? *Lancet*. 2005;366:2081-2082, 2083-2086.
 22. Thangapazham RL, Gaddipati JP, Rajeshkumar NV, et al. Homeopathic medicines do not alter growth and gene expression in prostate and breast cancer cells in vitro. *Integ Cancer Ther*. 2006;5:356-361.
 23. Winter RN, Kramer A, Borkowski A, Kyprianou N. Loss of caspase-1 and caspase-3 protein expression in human prostate cancer. *Cancer Res*. 2001;61:1227-1232.