AZURIN DERIVED CANCER DIAGNOSTIC AND THERAPEUTIC AGENTS

Shortened version of title: Azurin Cancer Agents

Abstract. Azurin is a naturally occurring cupredoxin of the oxidative reduction cycle in plants and some bacteria. *In vitro* cultures of human melanoma cells exposed to azurin demonstrate stabilized concentrations of suppressor protein p53. Azurin demonstrates significant toxicity to cancerous cells, but significantly less toxicity to healthy cells. The effect of azurin and suppressor protein p53 within cancerous cells depends upon azurin modifications. Azurin's selective movement across a cell surface is the cytotoxic feature which distinguishes its effect in normal cells from cancerous cells. Therapeutic and diagnostic molecules linked to azurin are potentially useful for diagnostic and chemotherapeutic purposes.

Key words: azurin, melanoma; p53; receptors; chemotherapy; cytotoxicity

INTRODUCTION

CDG Therapeutics, Inc. [hereinafter CDG] is a Delaware corporation established in 2001, and which develops remarkable new cancer pharmaceuticals and diagnostic aids. Founder, shareholder and inventor, Dr. Tapas Das Gupta, M.D., Ph.D. is an internationally known investigator and clinician for cancer chemotherapy. In addition to these responsibilities, Dr. Das Gupta is Chairman of the Department of Surgical Oncology at the University of Illinois School of Medicine in Chicago, Illinois. Co-

founder, shareholder and inventor Dr. Ananda Chakrabarty, Ph.D. is a leading investigator and authority on genetic engineering of microorganisms.[1] Dr. Chakrabarty is a Distinguished Professor at the University of Illinois School of Medicine in Chicago.

Dr. Craig Beattie, Ph.D. is the chief scientific officer of CDG Therapeutics, Inc. He has extensive research experience as a lead investigator in cancer chemotherapy, as well as structural and functional genomics, genome mapping, and regulation of gene expression. Dr. Beattie is currently a Professor in the Department of Animal Biotechnology at the University of Nevada in Reno, Nevada.

CDG is closely associated with research and clinical resources of the University of Illinois School of Medicine. CDG's mission is development of biochemical agents which selectively enter human cancerous cells and transport cancer therapeutic or diagnostic agents therein. This article examines earlier work which led to the inventions described in the investigator's recent published U.S. utility patent application. The article will also review CDG's new chemotherapy and diagnostic techniques, and why they are so crucial to advances in cancer treatment.

Early Investigations

Scientists and physicians have recognized the tumor-reducing properties of bacterial substances since the nineteenth century.[2] One group of these tumor-reducing substances, known as cupredoxins, participates in energy production within bacteria and plants in nature.[3] The CDG investigators have focused their studies upon naturally occurring and

modified azurin, one category of cupredoxins, became of its therapeutic potential in cancer treatment and diagnosis.[4],[5]

Azurin and Suppressor Protein p53

In an early investigation Dr. Das Gupta and Dr. Charabarty, together with other scientists, studied the cytotoxic effect of bacterial azurin. In these experiments they initially exposed melanoma cells to naturally occurring bacterial azurin and azurin fragments. The investigators were previously aware of a naturally occurring cellular substance known as suppresser protein p53 [hereinafter p53].[6] They also knew that the presence of p53 within cells is associated with shrinkage of cancerous tumors. However, the effectiveness of p53 in causing cell death depends upon its intracellular concentration.[7]

The investigator's experiments indicated that p53 physically associates with naturally occurring bacterial azurin within melanoma cells. The investigators concluded that this azurin/p53 complex results in higher concentration and stability of p53 within these cancerous cells.[8] In contrast, a non-naturally occurring azurin could not complex with p53, and consequently did not stabilize p53 levels within melanoma cells. Consequently, with this non-naturally occurring azurin the investigators observed significantly less cytotoxicity within human melanoma cells.[9]

Also of tremendous interest to the investigators in this study were results of *in vivo* histological and immunochemical experiments in live nude mice. These experiments demonstrated that azurin, together with intracellular p53, correlated with tumor regression of melanoma transplants in nude mice. Furthermore, this regression was

remarkably similar to regression of primary human cutaneous melanoma.[10],[11] The investigators were excited because there was no significant death of normal mouse cells. These surprising results suggested that azurin's cytotoxicity is directed selectively towards human cancer cells and not healthy cells.

How Tumors Shrink

The investigators also studied how azurin influences p53's action by either (a) initiating cell death; or (b) terminating cell growth.[12] Their results established that naturally occurring bacterial azurin in the presence of p53 induces cell death, but not termination of cell growth. In contrast, non–naturally occurring azurin did not induce cell death in the presence of p53, although this azurin did significantly halt cell growth in cancerous cells.[13]

The investigators concluded that the effect of azurin combined with p53 within cancerous cells -- death or growth termination -- depends upon specific amino acid modifications to azurin.[14] They also observed that naturally occurring bacterial azurin, in the intracellular presence of p53, induces significantly less cytotoxicity within normal human mammary epithelial cells than human breast cancer cells.[15] These observations were analogous to the results of the melanoma tumor regression in nude mice. Similar, consistent and supporting results were obtained with human melanoma and normal fibroblast cell cultures.[16]

How Azurin Enters Cells

The investigators also demonstrated that azurin movement into a cell, and across a cell surface, is necessary for an intracellular cytotoxic effect. In this study the investigators

microinjected azurin into normal cells and cancerous cells under identical experimental conditions. This approach eliminated azurin movement across and through a cell surface, so any cytotoxic effect did not depend upon a selective entry mechanism into either cell type. Results demonstrated significant death of both normal and cancerous cells after microinjection with identical types and amounts of azurin.[17]

The investigators concluded that azurin's selective movement across a cell surface was the crucial feature for cytotoxicity within human cancerous cells, and significantly less cytotoxicity within normal cells. The investigators predicted that azurin initially selectively attaches to cancerous cell surfaces, based upon azurin's structural similarity to specific mammalian cell surface proteins. The structurally similar azurin then binds to enzymes which are hyperexpressed upon cancerous cell surfaces.[18]

Azurin Movement is an Energy-Dependent Process

The investigators also addressed whether this selective azurin movement into cancerous human cells is an energy dependent process.[19] They confirmed that a pre-selected protein which was unlinked to azurin, did not enter cancerous cells at 37 degrees C. or 4 degrees C. However, this pre-selected protein attached to naturally occurring bacterial azurin, did significantly enter cancerous cells at 37 degrees C, although there was significantly less entry at 4 degrees C.[20]

Based upon these results investigators concluded that azurin moves into a cell by an energy dependent and temperature sensitive process. They also predicted that azurin

movement, across and through the cell surface, required a process in which internal cell components participate.[21]

Temperature Sensitive Entry is Specific to Azurin

Further experiments shed light on the rate of azurin entry into cancerous cells and the manner in which azurin enters with an attached, or linked protein (i.e., cargo protein).[22] The investigators increased concentrations of non-identifiable (unlabled) azurin, within a cancerous cell solution containing identifiable (labeled) azurin with an attached protein. As the concentration of unlabeled azurin in solution increased, the amount of labeled azurin and protein decreased within these cancerous cells.[23]

The investigators concluded from these results that both azurin, and azurin with an attached protein, are competing for the same transporting biochemical groups along cell surfaces. These transporting groups, presumably enzymes or receptors, are responsible for azurin transport across and through the cell surface.[24]

In related experiments the investigators increased the concentration of rustocyanin, another cupredoxin, within the cancerous cell solution. The results established that neither the presence of, nor increasing the concentration of rusticyanin, changed the entry rate of azurin into cells.[25] The investigators concluded that azurin entry receptors are specific for azurin, and these receptors do not recognize nor transport rusticyanin. More importantly for clinical purposes, further experiments demonstrated that rusticyanin did not exhibit azurin's selective entry in human cancerous cells and not normal cells.[26]

U.S. UTILITY PATENT APPLICATION NO. 11/244,105

The above investigations culminated in CDG's U.S. utility patent application entitled "Cupredoxin derived transport agents and methods of use thereof."[27] The application proposes inventions (although not exclusively) as follows:

1. Proteins which are structurally similar to naturally occurring cupredoxins or H.8 protein.[28] In a narrower characterization of the invention, the application designates azurin which naturally occurs within a prokaryotic bacterium; [29]
2. Proteins which are structurally similar to the large category of cupredoxins, and which attach to additional molecules for transport into cancerous cells.[30]
3. A method of contacting cells with proteins similar to cupredoxins, and which proteins link to additional molecules for transport into cells.[31] The linked molecules function as a detection tool for diagnostic aids such as: x-rays, contrast agents, MRI imaging, and ultrasound, although not exclusively.[32] Nanoparticles are representative of cargo compounds which are transported into mammalian cells by azurin for diagnostic purposes.[33]
4. Diagnostic or therapeutic kits which contain cupredoxin attached to the appropriate diagnostic or pharmaceutical compound. The kit also includes an appropriate delivery means, such as capsules or ampules and syringes, for clinical administration.[34]
5. Methods for enabling nucleic acids to produce wild type azurin with an attached protein for use as therapeutic agents or diagnostic aides.[35]

U.S. Application No. 11/244,105 also characterizes as part of the invention, that portion of the azurin molecule required for entry into a cancerous mammalian cell. This amino acid sequence, in either genetically altered or naturally occurring azurin, is known as a cupredoxin entry domain (or a protein transduction domain, i.e., PTD).[36] The inventors initially recognized that modifying the PTD amino acid length can alter azurin entry into certain human cancerous cells. Now they realized that changing the identity, and chemical characteristics, of amino acids within the entry domain sequence also affect azurin's entry into cancerous cells.[37]

Azurin Linked Substances

Molecules linking to azurin entry domain sequences are also a component of CDG's invention. For example, nanoparticles labeled with identifying substances detect disease.[38] For other clinical applications such as ultrasound diagnosis, microspheres**,** nanospheres and liposome vesicles are useful.[39]

For cancer chemotherapy, the attached non–azurin molecules must (i) kill cancer cells, or (ii) retard malignant tumor cell division, or (iii) both processes simultaneously.[40] For example the attached compound can be a cell cycle control protein such as p53, *supra*, a suicide protein, immune modulating protein, or a bacterial toxin.[41] Other cargo compounds include nucleic acids which code for chemotherapy proteins.[42]

In other embodiments the attached molecule is a pharmaceutical which kills cancerous cells after transport therein by its linked azurin.[43]

Gene Therapy

As an additional invention component, Application No. 11/244,105 describes nucleic acid molecules which code: (i) azurin and/or (ii) a protein attaching to an azurin sequence. To replicate these nucleic acids the CDG investigators create genetically altered cell components and viruses known as vectors.[44]

To create vectors, the investigators insert bacterial nucleic acid, which codes for azurin and a linked protein, into the original cellular or viral genetic material. The investigators then clone (grow) the viruses or cell components which contain the inserted nucleic acid. In this manner the inserted nucleic acid replicates as if it was an original component of the vector genome. The investigators then introduce the (now more numerous) vectors into cancerous human cells.[45],[46] The inserted nucleic acid can now produce within the cells, thereby producing azurin and the preselected protein intracellularly.

In other embodiments, the appropriate genetic nucleic acid uptake by cancerous cells is increased by coating DNA onto small particles such as biodegradable beads. The linked azurin then transports these beads into cancerous cells.[47]

CONCLUSIONS

Selective transport into cancer cells is the first most crucial feature off azurin's immense potential, because its cytoxicity for specific cancer cells does not affect healthy human cells. The result for patients is fewer toxic side effects than with administration of

conventional cancer chemotherapy. As a result, a debilitated patient's normal cells are not victims of azurin's inducement of cell death, thereby conserving that patient's bone marrow and immunologic cell resources.

The second most potentially crucial feature of azurin is the tremendous number of diagnostic and therapeutic substances which can link to azurin molecules. These compounds can "piggyback" with azurin into cancer cells under circumstances in which otherwise they could not enter cancerous cells to be clinically effective.

Based upon these two features of azurin, the investigators are currently developing azurin variants which further maximize specificity but further minimize clinical allergic reactions and side effects.[48] In addition to these carriers, we also look forward to CDG's development of gene therapies for azurin and protein nucleic acids to administer as chemotherapy with fewer toxic side effects.

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The author wishes to express her appreciation to Dr. Craig Beattie for his technical assistance during the preparation of this article.

[1] Dr. Chakrabarty has created genetically novel micro-organisms which digest material of oil spills. The patent for these micro-organisms became the subject matter of the U.S. Supreme Court decision Diamond v. Chakrabarty, 447 U.S. 303 (1980). In that decision, the U.S. Supreme Court held that genetically engineered micro–organisms are proper subject matter for U.S. utility patents.

[2] *See* Yamaha, T.; Goto, M. ; Punj, V.; Zaborina, O.; Chen, M.L.; Kimbara, K.; Majumdar, D.; Cunningham, E.; Das Gupta, T.K.; Chakrabarty, A.M. Bacterial redox protein azurin, tumor supressor protein p53 and regression of cancer. Proceedings of the National Academy of Sciences(2002) 99:22, 14098-14103, 14098.

[3] De Rienzo, F.; Gabdoulline, R.R.; Menziani, M.C.; Wade R.C. Blue copper proteins: a comparative analysis of their molecular interaction properties. Protein Science (2000) 9:1439-1454, 1439. For example, cupredoxins participate in electron transport during photosynthesis.
[4] Azurin producing bacteria include *Pseudomonas aeruginosa*, *pseudomonas floresences* and *Pseudomonas putida*. *Id.* at 1446 (Table I). "Wild type" refers to naturally occurring azurin from *Pseudomonas aeruginsa*, a prokaryotic bacterium.
[5] Naturally occurring cupredoxins contain a single copper atom per molecule; hence the designation "blue copper protein." *Id.* at 1439.
[6] Yamada, T. et al. Bacterial redox protein azurin, tumor suppresser protein p53 and regression of cancer at 14100. Apoptosis is defined as cell death during which DNA and other nuclear material coalesces. Protein p53 is a nucleoprotein which moves from a cell nucleus into the cytosol.
[7] *Id.* at 14099, 14102. The investigators' results suggested that naturally occurring bacterial azurin (from *Pseudomonas aerginosa*) stabilizes p53, thereby raising the intracellular level of this cell-death inducing protein. *Id.* at 14102.
[8] *Id.*
[9] *Id.* A stabilized intracellular p53 level precedes significant production of reactive oxidizing compounds which result in apoptosis (cell death). Chakrabarty, A.M. Micro-organisms and Cancer: Quest for a Therapy. Journal of Bacteriology(May 2003) 2683-2686, 2684. Under certain circumstances, protein p53 prevents cell cycle progression (i. e, cells no longer divide) thereby resulting in termination of tissue or tumor growth. Hiraoka, Y.; Yamada, T.; Masatoshi, G.; Das Gupta, T.K.; Chakrabarty, A.M. Modulation of mammalian cell growth and death by prokaryotic and eukaryotic cytochrome c. Proceedings of the National Academy of Science(April 27, 2004) 101:17, 6427-6432, 6429.
[10] *Id.* at 14102.
[11] Yamada, T.; Hiraoka, Y; Ikehata, M.; Kimbara, K.; Avener, B.S.; Das Gupta, T.K.; Chakrabarty, A.M. Regulation of mammalian cell growth and death by bacterial redox proteins: relevance to ecology and cancer therapy, Cell Cycle(June 2004) 3:6, 752-755, 753.
[12] Yamada, T.; Das Gupta, T.K.; Chakrabarty, A.M. Apoptosis or growth arrest: Modulation of tumor suppresser p53 specificity by bacterial redox protein azurin. Proceedings of the National Academy of Sciences (April 6, 2004) 101:14, 4770-4775, 4774. There are two genes--p21 and baz-- which regulate cell growth and death which are activated by protein p53, because they have p53 responsive promoter elements. *Id.* at 4772.
[13] *Id.* at 4771-72.
[14] *Id.* at 4771.
[15] Yamada, T.; Fialho, A.M.; Punj, V.; Bratescu, L.; Das Gupta, T.K.; and Chakrabarty, A.M. Internalization of bacterial redox protein azurin in mammalian cells: entry domain and specificity. Cellular Microbiology (2005) 7:10, 1418-1431, 1419.
[16] *Id.* at 1420.
[17] Punj, V.; Bhattachargyga, S.; Saint-Die, D. ; Vasu, C.; . Cunningham, E.A.; . Graves, J.Bacterial cupredoxin azurin as an inducer of cell death and regression in human breast cancer. Oncogene(2004) 23:2367-78 . The investigators predicted that naturally occurring azurin possessed a structural similarity to naturally occurring compounds known as ligands on cancerous cell surfaces.
[18] Yamada, T. et al. Internalization of bacterial redox protein azurin in mammalian cells; entry domain and specificity at 1424.
[19] *Id.* at 1421. Amino acid residues 50-77 of naturally occurring bacterial azurin are crucial to the movement across and through a cell surface membrane under any experimental conditions. *Id.* However, shorter naturally occurring azurin sequences demonstrated relatively greater impairment of cell entry at 4 degrees C. than did longer azurin amino acid sequences. This observation suggests that the amino acid sequence length of a naturally occurring azurin molecule may contribute to a different, although less effective, azurin surface binding and subsequent movement at 4 degrees C. *Id.*
[20] *Id.*
[21] *Id.* at 1423.
[22] *Id.* at 1424. A fusion protein, also known as a cargo protein, is transported into a cell while attached to an azurin molecule.
[23] *Id.* at 1424. Labeled compounds which are used for intracellular identification of azurin include, although not exclusively: radioactive molecules, fluorescent compounds, or colored components such as dyes.

[24] *Id.* The azurin amino acid sequence 50 through 77 contains the necessary region for the selective entry feature.
[25] *Id*. at 1424.
[26] *Id*. at 1425.
[27] U.S. Patent Application No. 11/244,105, filed October 6, 2005, now U.S. Publication No. 20060149037. The inventors are K.T. Das Gupta, A. M. Chakrabarty, T. Yamada and A. Fialho.
[28] Application 11/244,105 at 32 (Claim 1).
[29] *Id.* (Claim 10). H.8 proteins are present upon the outer cell surface (membrane) of the bacterium *Neisseria meningitidis*. H8 and/or the azurin portion of the Laz gene are required for proteins which are cytotoxic to brain tumor cells.H.8 is potentially useful as a transport agent for therapeutic molecules and diagnostic agents into cancerous brain cells, in a manner similar to azurin entry into melanoma cells. Id. at 1, 13 and 18.
[30] *Id*. In another characterization of the invention, the linked compound is selected from the group containing: nanoparticles, toxins or exotoxin A, as well as detectable substances or a pharmaceutical composition. Id.
[31] *Id.* In one characterization of this method, cancer cells are re-introduced into a patient. These cancer cells originate from the following tumors within the patient: osteosarcoma, lung, colon carcinoma, leukemia, and breast cancer, as well as liver, bladder or prostate carcinoma. Id. at 2, 33.
[32] *Id*. at 33.
[33] *Id.* at 9. Relevant nanoparticles are described in more detail in U.S. Pat. No. 6,383,500 B1 (Wooley et al.). The cytotoxic activity of cupredoxins is described in more detail in U.S. patent applications 10/046, 710 and 10/720,603.
[34] *Id.* at 33. (Claims 32-36). Narrower characterizations of the invention rely upon polypeptides, proteins and nucleic acids from the application sequence listing. *See* 37 CFR section 1.181 et seq. (Application disclosures containing nucleotide and/or amino acid sequences).
[35] *Id.* at 13.
[36] *Id*. at 6.
[37] *Id.* at 7. The term "percent (%) amino acid sequence identity," between a cupredoxin entry domain and another amino acid sequence, is the percent amino acid residues in a cupredoxin entry domain which are identical with amino acid residues in another amino acid sequence candidate sequence (when the two sequence are congruently aligned by a well-known method). *Id.*
[38] *Id*. at 9. For example, a green florescent protein fuses to an entry domain sequence and then the combined compound moves across the cell surface into the cell interior. *Id.* Examples of nanoparticles with pharmaceutical utility are disclosed in U.S. Pat. No. 6,383,500.
[39] *Id.*
[40] *Id.* at 9,10
[41] *Id*. at 10. Cell cycle control proteins include cyclin-dependent kinase inhibitors such as p16, p21 and p27. 'Suicide' proteins include thymidine kinase, nitroreductase or cytokinase. Cytokines or other immune modulating proteins include interleukin, granulocyte macrophage colony stimulator factor, or bacterial toxins such as *Pseudomonas aeroginosa* exotoxin A. *Id.*
[42] *Id*.
[43] *Id*. Azurin-linked pharmaceuticals include previously existing cytotoxic chemotherapy compounds such as: methotrexate, tamoxifen, vincruistine. They also include alkylating agents such as nitrogen mustards, alkyl sulfonates, nitroureas, antimetabolites, purine analogues, pyrimidine analogues, antibodies, enzymes, hormones, and inhibitors. Other attached anti-cancer agents, which act by disrupting cancerous cells, include: microtubule disruptors, microtubule stabilizing agents, plant derived compounds, biological response modifiers, growth factors, immune modulators and monoclonal antibodies. *Id.*
[44] *Id.* at 13. Vectors are viruses or intracellular structures into which specific genes, either artificial or from other organisms, are inserted. *Id.*
[45] *Id*.
[46] *Id*. Cloning vectors are replicating plasmids (cell organelles, or cell components) or phages (viruses which invade bacteria) with nucleic acid (genes) into which the inventors insert foreign nucleic acid sequences. Thereafter the inserted nucleic acid replicates as an integral component of the original vector nucleic acid.

An expression vector, such as a plasmid, yeast or animal virus genetic material, introduces foreign nucleic acid into a host cell or tissue, as do other vectors. However there is the additional feature of "genetic directions" which transcribe and translate (interpret and produce) the foreign nucleic acid. In general, expression vectors contain signal sequences, origins of replication, marker genes, enhancer elements, promoters and transcription termination sequences as "genetic directions." For example, functionally linking a nucleic acid sequence for an entry domain to an indelible promoter can control expression for the naturally occurring azurin entry domain sequence.

[47] *Id.* Techniques for incorporating DNA into expression systems are well known to geneticists. *See* U.S. Pat. No 5,736,524.

[48] E-Mail communication of Dr. Craig Beattie, October 11, 2006.