

Microbial-based therapy of cancer

Current progress and future prospects

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The use of bacteria in the regression of certain forms of cancer has been recognized for more than a century. Much effort, therefore, has been spent over the years in developing wild-type or modified bacterial strains to treat cancer. However, their use at the dose required for therapeutic efficacy has always been associated with toxicity problems and other deleterious effects. Recently, the old idea of using bacteria in the treatment of cancer has attracted considerable interest and new genetically engineered attenuated strains as well as microbial compounds that might have specific anticancer activity without side effects are being evaluated for their ability to act as new anticancer agents. This involves the use of attenuated bacterial strains and expressing foreign genes that encode the ability to convert non-toxic prodrugs to cytotoxic drugs. Novel strategies also include the use of bacterial products such as proteins, enzymes, immunotoxins and secondary metabolites, which specifically target cancer cells and cause tumor regression through growth inhibition, cell cycle arrest or apoptosis induction. In this review we describe the current knowledge and discuss the future directions regarding the use of bacteria or their products, in cancer therapy.

Introduction

Each year, tens of millions of people are diagnosed worldwide with cancer, and more than half of these patients eventually die from this disease. In 2002, 10.9 million new cancer cases (excluding skin cancer) were diagnosed, and the number of deaths caused by this disease reached 6.7 million (GLOBOCAN).¹ Regarding the industrialized countries of the western world, cancer accounts for about one fifth of all deaths. Likewise, one person out of three will be treated for a severe cancer in their lifetime.² In Europe, cancer has become a major public health problem with an estimated prevalence of about 3%, increasing to 15% at old age.³ This rising burden is mainly due to a rapidly aging population, and demands a clear and coordinated response from oncologists, public health professionals, policy-makers and researchers.

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Conventional cancer treatments, such as surgery, chemotherapy and radiotherapy, often fail to achieve a complete cancer remission. Moreover, it has been widely recognized that radiotherapy and/or chemotherapy are likely to cause significant side effects. This has prompted the development of many new approaches for the treatment of cancer. One such example involves the use of live, attenuated bacteria or their purified products.

Live, Attenuated and Engineered Bacterial Strains in the Treatment of Cancer

In 1890, William B. Coley, a surgeon in the Memorial Hospital in New York, described for the first time bacteria as anticancer agents.⁴ He observed that several patients showed tumor regression after being infected with pathogenic bacteria. Later, in 1935, Connell used sterile filtrates from *Clostridium histolyticum* to treat advanced cancers and observed tumor regression, which he pointed to be the result of enzymes' production.⁵ Many scientists since then, and even today, know that certain live, attenuated and engineered microorganisms such as *Clostridium*, *Bifidobacterium*, *Salmonella*, *Mycobacterium*, *Bacillus* and *Listeria* have the ability to selectively target cancer cells and act as anticancer agents. They grow in the hypoxic core region of solid tumors, with very little oxygen and where most of the times radiation or chemotherapy are unsuccessful.⁶ Because of their selectivity for tumor micro-environment, these bacteria are also promising vectors for delivering therapeutic genes for anticancer therapies.

Clostridia are a group of anaerobes and, therefore, constitute an ideal tool to target solid tumors since in most cases these tumors show increased levels of hypoxia.⁷ In 1947, it was shown that the injection of spores of *Clostridium histolyticum* caused oncolysis, then referred to as "liquefaction," of a transplanted mouse sarcoma.⁸ *Clostridium tetani* spores were also used and tumor-bearing mice died 48 hours after the treatment in contrast to the healthy controls.⁹ This experience proved that a specific microenvironment is required to bacterial survival and development but also that toxicity remained a problem with live bacteria. Several approaches were used in an attempt to overcome this problem and increase the tolerance of this therapy. Using *Clostridium oncolyticum* M-55, Carey et al. showed the benign activity of this strain by injecting themselves without any dangerous effect.¹⁰ It was tested against several transplanted tumors in different animal models and the results indicated the need of

several conditions for the success: (1) a threshold in the tumor size (3 cm³ or 2 g of tumor weight); (2) a spore dose of 10⁶⁻⁹ and (3) intratumoral or intravenous injection modes.⁵ A clinical trial using *C. oncolyticum* M55 spores to treat glioblastomas resulted in oncolysis with almost all glioblastomas converted into brain abscesses one week after injection. However, in the 49 patients treated none exhibited an extension in their life span and the recurrence rate remained unaltered.^{11,12} In an effort to improve the efficiency of this therapy, combined chemotherapy agents plus the spores were tested. One of them consisted in the administration of 5-fluorodeoxyuridine, an antineoplastic antimetabolite that is metabolized to fluorouracil (5-FU) and alkylating agents of the ethyleneimino type, which induced sarcoma regression in mice.¹³ Raising the temperature inside the tumors up to 42–44°C (hyperthermia) in combination with radiotherapy was another approach developed for *C. oncolyticum* M55, creating a more favorable environment for the development of the microorganisms. Mice bearing three different types of neck tumors (Ehrlich adenocarcinoma, Harding-Passey-melanoma and fibrosarcoma) were treated with this strategy, and in all cases tumor lysis was increased due to the bacteria development.¹⁴ A third approach developed was by the modification on the level of oxygen given, reducing its content in the air inhaled by the animals to 11–12%. Oncolysis was observed and 30% of the animals were cured with this approach.⁵ Nevertheless, these approaches were not very successful in their clinical outcomes since there was tumor recurrence in the patients from the viable outer rim of the tumor.⁷

In the middle of the last decade, the use of engineered Clostridial strains to increase anti-tumor effects gathered more attention. Saccharolytic, non-pathogenic strains were reevaluated and it was seen that spores of *C. beijerinckii* NCIMB 8052 germinated in EMT6 tumor-bearing mice and spores of *C. beijerinckii* ATCC 17778, *C. limosum* DSM1400, *C. acetobutylicum* ATCC 824 and NI-4082 germinated in WAG/Rij rats with syngeneic rhabdomyosarcomas. In all cases, vegetative bacteria were only present in tumor regions but not in healthy tissues.^{15,16} *C. beijerinckii* NCIMB 8052 was also used to clone *Escherichia coli* cytosine deaminase (CD) and nitroreductase (NTR) genes, and in the presence of the correct prodrug, these enzymes were present in sufficient amount to induce EMT6 cells death. However, the studies showed that only intratumoral administration of the spores, but not intravenous, was able to produce a therapeutic effect.^{15,17} Genetically saccharolytic *C. acetobutylicum* strains containing cloned murine tumor necrosis factor α (mTNF α) or rat interleukin 2 (rIL2) were also constructed. The engineered strains gained the ability to produce significant amounts of these antitumor factors, but upon injection of the spores in rhabdomyosarcoma-bearing rats, tumor regression was not successful, even in combination with radiotherapy. This is likely to be due to the less ability of saccharolytic clostridia to colonize tumors when compared to proteolytic bacteria.¹⁸

Though proteolytic clostridia presented higher ability to colonize tumors, they were more difficult to manipulate. After a systemic administration of spores from both Clostridia types, tumor colonization from proteolytic Clostridia was about 1,000-fold higher than that of saccharolytic Clostridia.¹⁹ The proteolytic

strain *C. oncolyticum* M-55 was transformed with a plasmid containing the CD gene and injected intravenously together with the prodrug 5-fluorocytosine (5-FC). The results demonstrated higher antitumor activity than with other drugs tested, but failed to maintain the effect for more than one week, even though the bacteria remained in the tumor. More recently, this strain has been genetically engineered to overexpress the enzyme nitroreductase from *Haemophilus influenzae*, producing better antitumor results.²⁰

Volgstein et al. assessed the ability of several anaerobes in tumor therapy, using xenografted colorectal carcinoma, as model system. They compared 26 species belonging to the Clostridium, Lactobacillus and Bifidobacterium genera and found two with higher tumor colonization capacity, namely *Clostridium novyi* and *Clostridium sordelli*. In spite of presenting this higher capacity, more than one third of the tumor-bearing mice injected with these spores died within 16–18 hours due to a lethal toxin (α) produced by the strains. In *C. novyi*, the α -toxin was removed through heating (it was located in a phage) and the strain became non-toxic and was renamed as *C. novyi-NT*. This strain is very sensitive to oxygen and, thus, grows only in the core hypoxic region of the tumors. For this reason when it reached the well oxygenated rim area of the tumors, bacteria didn't survive thus tumor recurrence remained a concern.^{21,22} Resembling the other strains, alternative therapeutics were tested, combining the *C. novyi-NT* treatment with conventional chemotherapeutics (a strategy named COBALT—Combined bacteriological therapy). As example, the simultaneous use of the *C. novyi-NT* spores with a DNA-damaging drug and an anti-vascular agent, mitomycin C and dolastin-10, respectively, resulted in very satisfactory tumor shrinking, however it was always accompanied by severe toxicity.²³

Other drugs were or are being tested in combination with these spores: taxanes like docetaxel and MAC-321 which are microtubules stabilizers and have demonstrated decreased toxicity, although with slow tumor regression. In fact, with this combined strategy none of the animals died after treatment.

Also, combinations of *C. novyi-NT* with radiotherapy were evaluated which showed positive effects in several tumor models. Interestingly, this combination acts in distinct cell components. In solid tumors, radiation hits the rapidly proliferating and highly oxygen-exposed cells, and *C. novyi-NT* hits those cells resistant to radiation.⁵ The key advantage of this therapy is that the doses of radiotherapy can be much smaller than the ones used now, not damaging healthy tissues and with obvious advantages for patients' welfare.

Another genus with promising results in anticancer treatment is Bifidobacterium. These are anaerobic bacteria, non-motile, and, unlike the Clostridium genus, non-sporulating. This is the most abundant genus inhabiting human colon and it has been used as a probiotic for many years.⁵ In the 1980s, it was shown that Bifidobacteria selectively grow in the hypoxic areas of solid mouse tumors upon intravenous injection of 5 x 10⁶ cfu (colony forming units), and 96 hours after injection, virtually no bacteria were found in other tissues.²⁴ Bifidobacterium not only inhibit

cancer progression in mice but have also shown the ability to induce a powerful immune response, enhancing the killing activity of NK cells and recruiting the activity of IL-2, INF γ and INF α .²⁵

Several genes have been cloned into natural Bifidobacterium plasmids. pBLES100 was used to clone spectinomycin adenyltransferase, transformed into *B. longum* 105-A or 108-A and introduced to tumor-bearing mice where they were found to colonize specifically tumoral tissues.²⁶ The same thing happened when this strain was inoculated in rats with chemically induced tumors.²⁶ Cytosine deaminase (CD) of *E. coli* was also cloned in pBLES100 under the control of a strong promoter (HU gene) and the *B. longum* strain carrying the recombinant plasmid was able to convert the prodrug 5-FC into 5-FU, suggesting the strain as an effective enzyme/prodrug therapeutic agent.^{27,28} The CD gene, cloned into the pGEX-1 λ T plasmid, was also transformed in *B. infantis* and the positive results obtained in a mice melanoma model also supported the hypothesis of using this genus in cancer gene therapy.²⁹

Endostatin gene was cloned into the pBV220 plasmid and transformed into *B. adolescentis* and *B. longum* and both demonstrated the potential to induce tumor growth inhibition. *B. adolescentis* presented around 70% inhibition when compared to control mice after a systemic administration.³⁰ *B. longum* (orally administrated) selectively recognizes xenografted hepatocarcinoma cells.³¹ In addition, no biological effect or plasmid stability's lost occurred when selective antibiotic-resistance genes were removed, improving the ability of this system to act as a safe anticancer deliver gene therapy.³²

A number of other genes were also successfully expressed in Bifidobacterium. Very recently, an apoptosis-inducer gene, TRAIL (Tumor-necrosis factor-related apoptosis-inducing ligand) was introduced in the pBV22210 plasmid and transformed to *B. longum* (*B. longum*-TRAIL). Tumor-bearing mice were intravenously injected with this strain and sacrificed at several time points to analyze the tumor regression. Results indicated that almost all bacilli were specifically found in tumoral tissue region and only a few in apparently healthy tissues.³³ Comparisons in tumor weight and volume were made between *B. longum*-TRAIL and *B. longum*-Endostatin, alone or in combination, with the best results being obtained with the combination. Tumor was suppressed in weight by 79.6 and 73.6%, and in volume by 82.6 and 76.7% when compared to a dextrose-saline solution control and *B. longum* wild-type, respectively. *B. longum*-Endostatin was also combined with Adriamycin (5 mg/kg) and its antitumor effect was enhanced.³³

Despite the great interest of using these plasmids in anticancer therapies since they are all commercially available, they have a high molecular weight, making it somehow hard to engineer. Also, the oral administration of the strains can subject them to acid digestion in the stomach. However, once such obstacles are overcome, their activity can be of significant clinical impact. Bifidobacterium can inhibit cancer growth while it also enhances the immune system response, and growing in the host intestine, they can easily carry anticancer genes and become the reservoir of anticancer medicine.

Salmonella species are facultative anaerobes known to selectively colonize tumoral cells of solid tumors showing a replication ratio between tumor and healthy tissues of 1,000:1.³⁴ However, most strains are pathogenic, causing significant immunostimulation due to the presence of lipopolysaccharide (LPS) and other virulence factors.⁵ To solve this problem, *Salmonella typhimurium* was genetically modified in order to attenuate its virulence, disrupting the *msbB* gene, responsible for the terminal myristalization of lipid A, and *purI*, which introduced the need of an external adenine source. After showing the lower toxicity in several animal models, Pawelek and colleagues injected it into tumor-bearing mice and showed its specificity to tumors.³⁵ Additionally, Salmonella strains were engineered to produce several proteins and toxins with anticancer activity: CD, TNF α , mitomycin C, herpes simplex virus thymidine kinase (HSV-tk) and colicin E3. More recently, reports have appeared of attenuated strains with the ability to delay tumor growth, including metastasis, in several models.⁵ A vaccine strain of *Salmonella choleraesuis* was developed and used both as a single anticancer agent and in combination with low-dose cisplatin (a platinum-based chemotherapy drug) in lung tumor and hepatocarcinoma in mice models. This strain was capable of delaying tumor growth in subcutaneous tumors and metastasis models. In combination with cisplatin, this strain increased the rate of infiltrating neutrophils, CD8⁺ T cells, enhancing an antitumor immune response and inducing a higher level of tumor apoptosis, causing an extended life span of the mice.³⁶

Another developed strain has appeared based on *S. typhimurium*, termed A1. This strain is auxotrophic for leucine and arginine, but receives sufficient amounts of these amino acids from the tumor environment.³⁷ Inoculation of wild type *S. typhimurium* caused death of the mice after only 2 days, but those inoculated with the A1 strain survived as long as the control mice. The ability of this strain to induce tumor regression was proved on a xenografted human prostate cancer.³⁸ Although the results obtained with *S. typhimurium* A1 showed significant clinical potential, a new strain aiming at improving of the A1 tumor virulence was isolated. In order to isolate this variant, *S. typhimurium* A1 strain expressing GFP were injected in nude mice grafted with a colorectal derived cell line (HT-29). GFP expressing bacterial cells could then be isolated from the tumor tissue and cultured. Since it was a re-isolated A1 bacteria strain, the authors named it A1-R and both strains were compared in their adherence to tumor cells. Results showed that this re-isolated strain was capable of adhering to cancer cells, using HT-29 as model system, about 6 times more than the parental A1 strain. Using the prostate cancer cell line PC3 as model, it was also seen that A1-R strain killed cancer cells in about 2 hours, whereas the strain A1 showed the same result only after 24 hours.³⁹ The results obtained with A1-R were validated in other tumoral models. The same was verified in pancreatic tumors, where treatment with A1-R showed a clear reduction in the tumor size and in other primary tumoral models and metastases.⁴⁰ As an example, A1-R strain has demonstrated increased tumor regression, decreased metastasis number and enhanced animal survival in primary osteosarcoma and respective lung metastasis,⁴¹ spinal cord glioma,⁴² experimental lymph node metastasis, and in lung metastasis.⁴³

In the specific area of increased immune responses, *Listeria monocytogenes* is another example of bacteria that can be used to treat cancer. It is an intracellular bacterium, capable of infecting phagocytic and non-phagocytic cells.^{44,45} Upon infection with *Listeria*, a significant immune response is elicited to clear the organism.⁴⁶ A variety of live attenuated *L. monocytogenes* strains expressing viral and tumor antigens as fusion proteins have been produced during the last several years: HPV-16 E7,⁴⁷ Her-2/neu,^{48,49} HMW-MAA,⁵⁰ influenza NP⁵¹ and PSA.^{52,53} These recombinant strains cause specific CD4⁺ and CD8⁺ T cell responses in mice. In this bacterium, Listeriolysin O (LLO), a pore forming hemolysin, is the major virulence factor. Fusion of the antigens to a non-hemolytic truncated form of LLO resulted in enhanced immunogenicity and anti-tumor efficacy.^{47,49} It had the ability to induce inflammatory cytokines and an active strong immune response. Preclinical studies in several tumor models have proved the efficacy of this therapy. It also had the advantage of having already been found inside tumors and persisted in there for 10 days, while in the spleen and liver they only persisted for 3 days.⁵⁴ Clinical trials using this bacterium have already been performed. In 2002, an indication of safety was obtained through oral administration of an attenuated strain carrying no antigen to 20 healthy volunteers, without detection of any serious adverse effects or long-term sequels.⁵⁵ More recently, 15 patients with progressive cell carcinoma of the cervix, which previously failed to respond to chemotherapy, radiotherapy and surgery, were enrolled in a study with a live-attenuated vaccine strain of *Listeria monocytogenes*, carrying a vector expressing an LLO-HOV-16 E7 fusion protein.⁵⁶ Three different groups of five patients each were treated with escalating intravenous doses of bacteria, followed by ampicillin 5 days after the injection. The main toxicity problems associated with the treatment were flu-like symptoms, resembling those observed with interleukin-2, probably related to a strong immune response triggered by the bacteria. Though it was not the purpose of the study, patients' survival was analyzed and the results were very promising. Usually, patients diagnosed with this type of cancer present only a median survival time of 6–7 months, but the overall median survival time for those subjected to this clinical study was 2x fold higher (347 days), and three patients were still alive, over two years, at the time of the report (2009).⁵⁶ *L. monocytogenes* is not only a trigger of a potential immune response and an efficient antigen delivery system but mostly a new promising anticancer agent.

By far, the most successful case of live bacteria in the treatment of cancer is *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG). Its major application is in bladder cancer, where a complete response is given in approximately 80% of the patients.⁵⁷ BCG was originally developed as a vaccine against tuberculosis, but in 1950s BCG was studied as an anticancer agent. Despite the encouraging effects in some tumor models, further clinical trials showed delayed hypersensitivity type reactions when tumor implantations were attempted in the locals of infection.⁶ In 1976, Alvaro Morales established a therapeutic protocol for bladder cancer treatment which took in consideration the time intervals needed for the hypersensitivity reactions to occur and considered,

for the first time, intradermal BCG vaccination. The good results obtained with his first seven patients led to two larger clinical trials which confirmed the efficacy of this therapy. It was the beginning of the successful story of BCG in bladder cancer treatment. Today, the therapeutics protocols involve the intra-bladder administration of BCG bacteria using a urethral catheter, which is then removed leaving the bacteria inside.⁵⁸ The main action induced by bacteria is a massive immune response, with many different cytokines and immune cells involved in the process. BCG binds to FAP (fibronectin attachment protein) and is internalized by urothelial and inflammatory cells.⁵⁹ It is interesting to emphasize that BCG treatment is dependent on a functional immune system. CD4 and CD8 T cells,⁶⁰ Natural Killer cells⁶¹ and neutrophils⁶² have been shown to be necessary to achieve a full response, since their abrogation in murine models eliminated the BCG response. Monocytes and macrophages have also been linked to BCG response.⁶³ Several cytokines have been reported to be increased in patients treated with BCG: Interleukin-2, 6, 8, 10, 12, TNF and INF α , γ .⁶⁴ TRAIL is capable of inducing apoptosis in neoplastic cells but not in normal cells⁶⁵ and it appears to be of pivotal importance in BCG-response. Neutrophils are its primary producers and its levels are increased in the urine of BCG-responders compared to BCG non-responders.⁶⁶ Since high levels of cytokines appeared following BCG treatments, several attempts have been made to overcome the possible side effects on live bacteria injections. However, all the clinical trials produced no effective results, suggesting that a complicated cascade of immune responses is activated only when bacteria are present and that it is virtually impossible to replicate it only with cytokines treatment.⁶⁷ However, combination of BCG with cytokines were more effective, particularly when INF α was administered in addition to BCG.⁶⁸

Bacteria-Derived Anticancer Agents

Not only live bacteria have applications in cancer therapies, but also bacterially-derived products have been tested and some of them successfully used for this purpose. The mode of action of some strains is through the production of cytotoxic factors, enzymes, antibiotics and others secondary metabolites that can be used or adapted in a proper manner to specifically target cancer cells.

A major aspect in cancer, related to bad prognosis of the cancer patients, is its ability to metastasize. Cancer metastasis occurs during tumor progression and cause 90% of human cancer deaths. The homing of metastases is not a random process, is organ specific, and depends on the histotype of the primary tumor. In this process, tumor cells use chemokine-mediated mechanisms. Chemokines are molecules produced in order to direct the migration of cells toward their gradient, through binding to specific cell membrane-attached molecules: G-protein-coupled receptors, as example. This migratory behavior of cancer cells also occurs in embryonic development and immunity.⁶⁹ Interestingly, bacteria naturally produce several chemokine/adhesion receptor inhibitors, so this appears as a new appealing approach as an anticancer therapy.

Staphylococcal superantigens-like (SSL) are a class of bacterial proteins produced by *Staphylococcus aureus* capable of binding several eukaryotic receptors overexpressed in cancer cells. SSL10 binds CXCR4, a GPCR expressed on human T-ALL lymphoma and cervical carcinoma cells. The natural ligand for CXCR4 is CXCL12, but SSL10's action clearly inhibited the chemotactic response of HeLa (cervical carcinoma) cells towards the natural ligand.⁷⁰

The class of bacterial protein SSL5 has been demonstrated to bind to the receptor for P-selectin glycoprotein-1 (PSGL-1), inhibiting rolling of neutrophils on a surface by acting as a decoy and thus hampering the interaction with its natural ligand, the P-selectin. The inhibitory action of SSL5, without any observed toxicity, was demonstrated in HL-60 leukemia cells since these cells express the receptor P-selectin glycoprotein-1 (PSGL-1). Besides the novel therapeutic action towards hematological malignancies, the bacterial protein SSL5 also binds epithelial cells that circulate in the blood stream during metastasis and thus can be a very interesting therapeutic tool for metastatic carcinoma.⁷¹

Some bacterial enzymes are also candidate therapeutic agents for cancer treatment. This is the case of the amino acid-degrading enzyme arginine deiminase of *Mycoplasma arginini* (Ma-ADI), a tumor growth inhibitor and potentially a therapeutic agent for the treatment of in vitro and in vivo tumors, such as hepatocellular carcinoma, melanoma, leukemia, renal cell carcinoma and prostate cancer.⁷² Such inhibition of cancer cell growth by ADI is believed to be due to depletion of arginine.⁷³ Interestingly, Ma-ADI has not only anticancer activity, but also has been implicated in inhibiting the growth of viruses such as HIV-1 and hepatitis C.⁷⁴ These pleiotropic effects appear to suggest that the enzymatic activity of ADI, leading to arginine depletion, may not be the only contributing factor in the anticancer/antiviral activity of MA-ADI. Das et al. reported the crystal structure of ADI from *Mycoplasma arginini* where the N-terminal part of this enzyme harbored a putative CARD-like domain (caspase activation domain) with no significant amino acid sequence homology to any mammalian CARD proteins but with a low but discernable structural similarity to the CARD motif.⁷⁵ Likewise, *P. aeruginosa* also produces ADI (Pa-ADI) and its 5'-end of the sequence containing the CARD-like domain was cloned and expressed as a 17 kDa polypeptide (Pa-CARD). Unlike the full size (46 kDa) protein, the truncated form had no ADI enzymatic activity, but presented anticancer activity in fibrosarcoma, breast cancer and leukemia cells. Thus, it appears that ADI's anticancer activity via enzymatic depletion of arginine is not the only way but a second potent route exists, which is through the N-terminal putative CARD-like domain without ADI activity.⁷⁶

Two enzymes are required for the synthesis of arginine from citrulline: first, argininosuccinate synthetase (ASS) converts citrulline to argininosuccinate and then, this is converted to arginine by argininosuccinate lyase (ASL). Conversely, ADI converts arginine to citrulline and ammonia. While arginine is a non-essential amino acid in humans, certain cancers such as hepatocellular carcinoma, melanoma or renal cell carcinomas do not express ASS in vivo, making them sensitive to arginine deprivation due to ADI action.

ADI causes an immune response when injected in humans and therefore multiple injections are necessary to consume all arginine due to the enzyme's short half-life. To reduce immunogenicity, ADI was covalently bound to PEG 20,000 and renamed as ADI-PEG20 (Polaris, Inc.), maintaining approximately 50% of its specific activity and increasing half-life in the animal models.⁷⁷ It was shown that ADI-PEG20 inhibited in vivo and in vitro growth of human melanoma and hepatocellular carcinoma, which were found as auxotrophic for arginine due to a deficiency in ASS.^{78,79} Phase I and II clinical trials have been performed in patients with unresectable hepatocellular carcinoma and melanoma.⁸⁰⁻⁸⁶ The results of the hepatocellular carcinoma showed only a moderate antitumor activity with tolerable toxicity, and in some cases, some patients couldn't complete the therapy as a result of allergic diseases. In the malignant melanoma clinical trials a higher anti-tumor activity was observed.⁸⁶ Recent studies appear to indicate that a major advantage of ADI seems to be as a part of combinatorial therapies with interleukin 2, reducing the hypotension cause by nitric oxide, a common feature when high doses of IL-2 are given to patients, and its ability to cause nutritional stresses in cancer cells may be positive for the action of chemotherapeutic agents.

Recombinant immunotoxins based on *Pseudomonas aeruginosa* exotoxin A (PE) are also promising anticancer agents. These chimeric proteins contain the Exotoxin A (a toxic domain for cytotoxicity) fused either to monoclonal antibodies or antibody fragments or physiologically important ligands like cytokines and growth factors. Exotoxin A from *P. aeruginosa* is a potent virulence factor that catalyzes the ADP-ribosylation of the eukaryotic elongation factor 2 (eEF-2) in host cells, affecting protein synthesis and cell viability.⁸⁷ It shares an A-B structure with other bacterial toxins. The B-domain is responsible for the interaction with eukaryotic host cell receptors, after which the A-domain translocates to the cytoplasm and exerts its action.^{88,89} Recombinant Exotoxin A-immunotoxins kill cancer cells by binding specifically to overexpressed cell-surface receptors, which carries them into the cell interior, where they arrest protein synthesis and induce apoptosis.⁸⁷

Clinical trials with different Exotoxin A-immunotoxins have already been performed with positive results in leukemia and bladder cancer.⁸⁸⁻⁹³ However, it was frequently observed that these immunotoxins missed the ability to exert a strong cytotoxic activity against solid tumors, with better results obtained with hematologic tumors.⁹⁴ These cells are more accessible for immunotoxins than solid tumors because these proteins need to overcome several natural barriers, which leads to the loss of their efficacy. Protein engineering has recently been developed to increase affinity towards cancer cells, increasing specificity, cytotoxicity and maximum tolerated doses in the treated mice and also PEGylated forms of immunotoxins were tested.⁹⁵ Previous studies showed cytotoxicities were similar to the native form, but half-lives were prolonged and animal toxicity was considerably reduced.⁹⁶

Microbial secondary metabolites, such as antibiotics and pigments, are also a source of new drugs for cancer treatment. Pharmaceutical industries have invested in the identification of

their biological activities, but in the beginning of the 1990s the appearance of new techniques favoring chemical synthetic compounds downsized the research funding of new natural compounds. Nowadays, research in this area is re-gaining strength and new natural or natural-derived products are emerging.⁹⁷ Farnesyltransferase inhibitors, prodiginines and epothilones are three examples of these products.

Activating K-ras mutations represents the most common type of abnormality of a dominant oncogene in human cancer, with specificity and type of mutation varying in relation to tumour type.⁹⁸ Among the critical transforming alterations occurring in cancer it is widely accepted that mutations in K-ras are very frequent genetic somatic events, occurring in about 30% of all tumour types. Further, Ras activation is crucial to activate signaling pathways involved in cancer progression, namely proliferation, differentiation, cell survival and migration. Ras is a GTP-binding protein and in normal cells it switches between an active form, GTP-bound, and an inactive form, GDP-bound. Ras mutations cause a constitutively active form resulting in the constant activation of its downstream signaling pathways, MAP-kinase as example.⁹⁹

In order to be functional proteins, Ras needs to be anchored in the inner side of plasma membrane which requires farnesylation. Farnesyltransferase inhibitors (FTI) are metabolites capable of blocking this step and thus impair Ras activation, even in the presence of a mutant Ras. Several FTIs of microbial origin have been discovered in the past few years, namely Manumycin A and Gliotoxin. Manumycin A is an antibiotic produced by *Streptomyces parvulus* and is one of the most studied, which has shown both in vitro cytotoxic activity against several cell lines (human pancreatic tumor, thyroid carcinoma, leukemias, myeloma and hepatocellular carcinoma) and in vivo against human cancer xenografted models (pancreatic cancer and thyroid cancer).¹⁰⁰⁻¹⁰⁷ A combined treatment with Manumycin and Paclitaxel (a mitotic inhibitor used in cancer chemotherapy), had a synergistic cytotoxic effect on anaplastic thyroid carcinoma cells.¹⁰⁷ Angiogenesis and Vascular Endothelial Growth Factor are also inhibited by this combination, showing higher cytotoxic effects than when used singly.

TAN-1813, a farnesyltransferase inhibitor isolated from a fungus strain, *Phoma* sp. FL-4151, was shown to arrest a mouse embryonic fibroblast cell line NIH3T3 at G₁ and the G₂/M phases impairing cell proliferation. In this cancer model, xenografted tumors in nude mice have also been inhibited by this metabolite.¹⁰⁸

Overall, several farnesyltransferase inhibitors of microbial origin can induce apoptosis in cancer cells through the disruption of farnesylation or the farnesylated proteins, but more insights in the mechanisms are needed to optimize their use alone or combined with other chemotherapeutic agents.

Prodiginines are bacterial secondary metabolites with immunosuppressive and anticancer properties, characterized by a pyrrolydipyrromethene skeleton. The anticancer activity has been shown in several cancer derived cell lines (liver, spleen, blood, colon, gastric, lung, breast and chronic myeloid leukemia) and also in vivo in mice with a xenografted liver cancer. Moreover,

these metabolites show little or no activity at all in normal cells.¹⁰⁹ Prodiginines are capable of inducing apoptosis and act through different modes targeting multiple cellular pathways: (1) inducing or correcting DNA damage, (2) by cell cycle arrest; (3) changes of intracellular pH and (4) showing resistance to multi-drug pumps that are often responsible for inducing resistance to other anticancer agents. Prodigiosin, a red pigment produced by many strains of the bacterium *Serratia marcescens*, is a prodiginine already tested against dozens of cancer cell lines exhibiting an average inhibitory concentration of 2.1 μ M.¹¹⁰

Obatoclax (GX15-070) is a synthetic derivative of natural prodiginines developed at Gemin X Pharmaceuticals.¹¹¹ This compound was designed to antagonize the effects of Bcl-2 anti-apoptotic proteins family, through binding and sequestering Bcl-2 proteins, releasing the pro-apoptotic Bax and Bak proteins. Obatoclax is the leading prodiginine candidate to be applied in clinics. This new drug is now undergoing multiple phase I and phase II clinical trials against several forms of cancer both as a single agent and in combined therapies.^{109,111-113}

Epothilones were discovered as cytotoxic metabolites in the myxobacterium *Sorangium cellulosum*. They show anti-tumor activity in cancer cell lines, many of those multidrug-resistant or paclitaxel-resistant (Taxol[®], Bristol-Myers Squibb).¹¹⁴ Epothilones bind to the α , β -tubulin dimer of microtubules, inducing its polymerization and stabilization which causes cell cycle arrest at the G₂/M transition and apoptosis. The first studies on the mechanism of binding seemed to indicate that these natural drugs shared pharmacophores with taxanes; however, recent crystallography results with epothilones indicated a unique tubulin-binding pocket that is not common to taxanes.¹¹⁵

Natural epothilones B and D and five derivatives (ixabepilone, BMS-310705, ZK-EPO, ABJ879, KOS-1584) are under investigation in several pre-clinical, and phase I and II clinical trials (reviewed in ref. 116). The modifications in the derivatives are made in order to improve specific properties that hamper the biological activity like water and plasma solubility, toxicity and binding ability to the microtubules.¹¹⁴ Unlike paclitaxel, ZK-EPO, one of epothilone B derivatives, is able to cross the blood-brain barrier and, therefore, is a potential agent for penetration into the central nervous system. Pre-clinical studies have demonstrated in vitro activity in breast, lung and colon cancer cell lines, amongst others. These natural drugs have shown an average activity one order of magnitude more potent than taxanes in cancer cell lines with an inhibitory concentration (IC₅₀) in the sub or low nanomolar range.^{117,118} In murine xenograft tumor models with both intravenous and oral administration of ixabepilone, a second generation epothilone B,¹¹⁸ successful tests have been conducted, particularly for multidrug resistant models, such as MCF7/ADR and 16C/ADR breast, Pat-7 ovarian and HCT116/VM46 human colon carcinoma.¹¹⁹ Breast cancer models of metastasis have striking tumor growth inhibition when treated with ZK-EPO.¹²⁰ Different schedules for drug administrations have been reported for different cancer models in order to determine the maximum tolerated doses and the dose-limiting toxicities (DLT). The most common DLT encountered was diarrhea for epothilone B and its derivatives and sensory neuropathy

for epothilone D.¹¹⁴ More ongoing clinical trials will bring better insights regarding the precise effects of epothilones whilst more research exploring new derivatives or new natural epothilones will allow extensive studies on the clinical and pharmacological use of this new class of drugs.

Bacterial Azurin as a Novel and Promising Anticancer Agent

It has now been demonstrated that *Pseudomonas aeruginosa* produces at least two cytotoxic proteins against cancer cells. One protein, interestingly, is not known as a virulence factor or an enzyme but a water soluble low molecular weight copper-containing redox protein named azurin (128 aa–14 kDa) involved in the electron transport chain. Zaborina et al. showed that azurin has cytotoxic activity against murine macrophage cell line J774 when its release was observed in the presence of eukaryotic proteins in the growth media.¹²¹ Besides azurin, cytochrome *c*₅₅₁, believed to be a partner of azurin in electron transfer, also demonstrated somewhat reduced cytotoxic activity towards the macrophage cells. Since J774 is a tumor cell line, it was of interest to see if azurin would demonstrate similar cytotoxicity towards human cancer cells. Melanoma (UISO-Mel-2)¹²² and breast cancer (MCF-7)^{123,124} were two models tested, where the efficacy of azurin to induce apoptosis and allow significant regression of these two cancers was demonstrated. In addition, intraperitoneal administration of azurin in nude mice xenografted with the two human cancer cells led to statistically significant tumor regression in vivo, with no apparent toxic effects to the animals.^{122,124}

A variant of azurin termed Laz has also been characterized from gonococci and meningococci such as *Neisseria meningitidis*, which can cause meningitis, an inflammation of the brain meninges. Contrasting with other bacterial azurins, this Neisserial azurin has an extra epitope (39 amino acids) in the N-terminal called H.8. This epitope is responsible for entry in glioblastoma cells, an ability that azurin does not possess. Brain is a highly protected organ exhibiting blood-brain barrier (BBB) which restricts the uptake of various compounds including drugs into the brain. This epitope was cloned in the N-terminal part of *P. aeruginosa* azurin, allowing its entry and cytotoxic activity in glioblastoma cells. This H.8 epitope may as well be useful to allow entry of other drugs in the brain.¹²⁵

Recently, azurin and Laz have been demonstrated to exhibit cytotoxic activity against liquid cancers, in addition to the solid tumours already described. Using HL60, an acute myeloid cell line, and K562, a chronic myeloid cell line, Kwan et al. demonstrated the high cytotoxicity of Laz towards both HL60 and K562 cells at modest concentrations (10 μ M induced a loss in cell viability of about 90%).¹²⁶

So far, three different modes of azurin's action towards cancer cells have been proposed. Azurin has been shown to bind avidly to the intracellular tumour suppressor p53, stabilizing it and, thus, leading to increased expression of pro-apoptotic protein Bax and Bax-dependent apoptosis in cancer cells;^{122,123,127} it also binds to several Eph receptor tyrosine kinases, a family of extracellular receptor proteins known to be upregulated in many tumours,¹²⁸

and this binding with EphB2 interferes in its phosphorylation at the tyrosine residue, resulting in inhibition of cell signalling and cancer growth (Fig. 1).⁶

With regard to azurin-p53 interaction and induction of apoptosis,¹²⁹⁻¹³¹ several recent studies have been conducted to understand the nature of such interactions. p53 is a four-chain, 393 residue protein, organized into four functional domains: an N-terminal domain (NTD), a sequence-specific DNA-binding domain (DBD), a tetramerization domain (TD) and a C-terminal regulatory domain (CTD) (Fig. 2). The DBD is the largest domain and its structure has been solved by NMR. This p53 domain is relatively unstable and often suffers mutations capable of altering its stability. The TD has also been characterized by NMR. In contrast, the NTD and CTD are predominantly unstructured under normal conditions.¹³²⁻¹³⁵ The first evidence of complex formation between p53 and azurin came from glycerol gradient and column binding, co-elution experiments.¹³⁰ Subsequent experiments with isothermal calorimetry demonstrated that four azurin molecules bind per 1 monomer of p53 with a dissociation constant of 33 ± 12 nM, presumably in the p53 NTD, according to the observed quenching of tryptophan residues (Fig. 2).¹²⁷ Regarding azurin, a specific region has been implicated in the complex formation by site-directed mutagenesis: amino acids Met-44 and Met-64, located in a hydrophobic patch of the protein, have been shown to be important for the interactions with p53 and their substitutions resulted in altered complex formation with p53.^{123,129,131} Further, more insight in the interaction between these two molecules at the single molecule level has been obtained by atomic force microscopy (AFM).¹³⁶ The p53 protein was immobilized in a gold substrate and azurin was tethered in the AFM tip. The results confirmed the interaction between both proteins with an estimated dissociation constant of 6 μ M (Fig. 2), lower than that estimated before in bulk, and establishing a strong affinity in the range of others like the transient complex azurin-cytochrome *c*₅₅₁ (1–100 μ M)¹³⁷ and close to that of an antigen-antibody pair (0.1–10⁻⁵ μ M).¹³⁶ Through protein docking, the nature of the complex formed between azurin and DBD and NTD of p53 was investigated. p53 DBD acquires a β -sandwich fold (PDB entry 1TUP_B), formed by two antiparallel β -sheets, S_I and S_{II}, with 4 and 5 strands, respectively (s₁, s₂, s₃, s₈ and s₄, s₆, s₇, s₉ and s₁₀). This structure is a scaffold for two large loops, L₂ and L₃, and a loop-sheet-helix (L₁-S_{III}-H₂).^{138,139} The Zdock docking program, was used to model the complex structure (p53 DBD domain_Azurin) generating more than 2,000 predicted structures.¹⁴⁰ Only the models where Met-44 and Met-64 were within a reasonable distance to be involved in the binding process were accepted, reducing the number to 194 models. Moreover, the models were grouped in clusters according to their similarities and the 3D structures were compared to eliminate geometric similar structures, obtaining a final number of ten candidates.¹⁴⁰ Physical properties of each model was calculated like the ASA, the accessible surface area, and all of them presented values between 690–800 \AA^2 before molecular dynamic (MD) simulations, comparable to the values for transient complexes (400–1,000 \AA^2).¹⁴¹ MD simulations and estimation of the free energy of binding were performed to

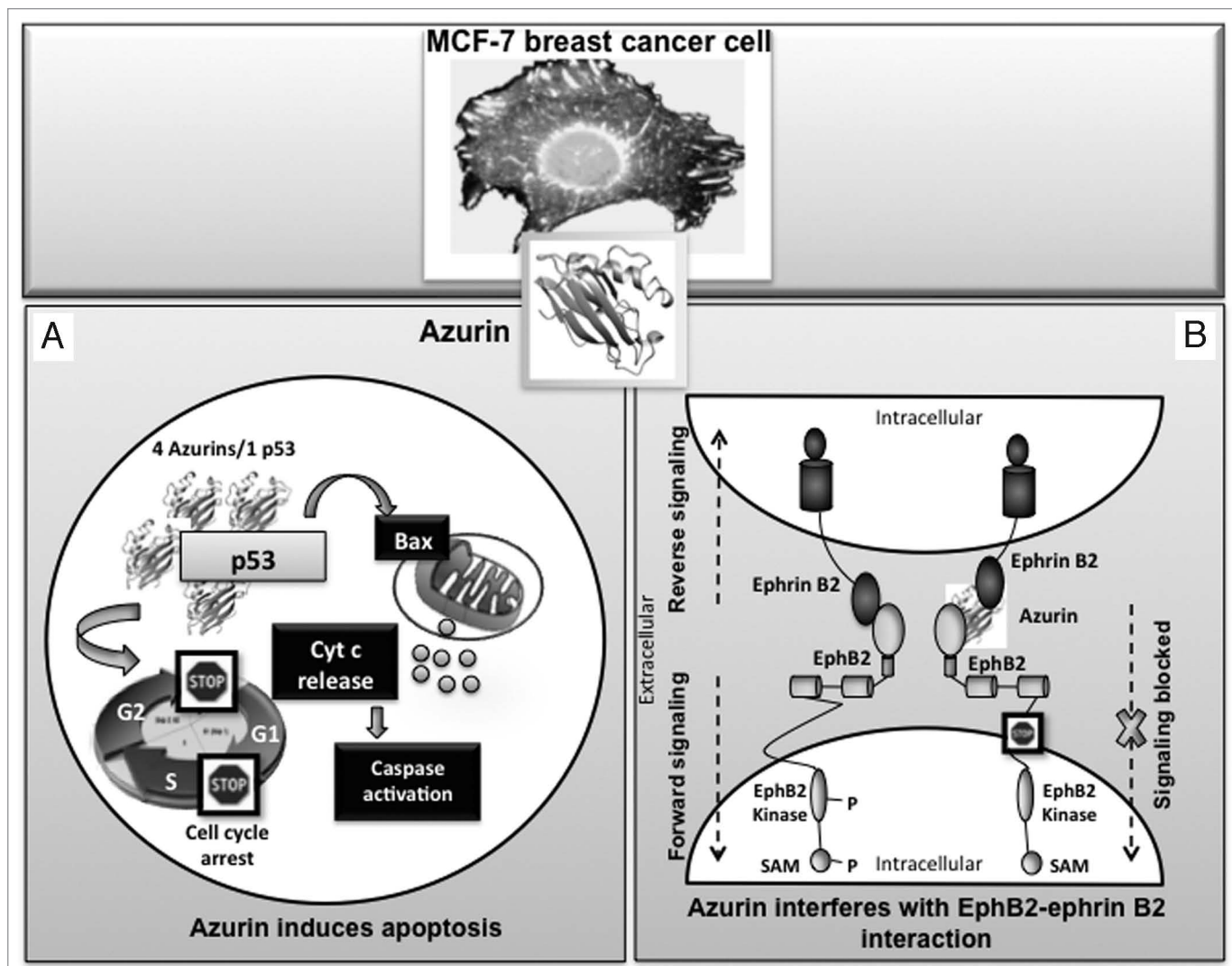


Figure 1. The mode of action of azurin in the induction of apoptosis and growth inhibition of a MCF-7 breast cancer cell is shown schematically. Azurin can enter the cancer cell to form a complex with tumor suppressor protein p53 (4 azurin molecules per 1 p53 molecule), stabilizing it and enhancing its intracellular level, which leads to apoptosis via caspase-mediated mitochondrial cytochrome c release pathways (A). Azurin can also bind avidly to the surface—exposed receptor tyrosine kinase EphB2, interfering in its binding with the ligand ephrinB2, and thereby preventing cell signaling that promotes cancer cell growth (B).

refine the models. The best model with the lowest binding energy ($-403.5 \text{ kJ mol}^{-1}$), was found to involve the L_1 loop and strands s_7 and s_8 in the p53 DBD binding interface and the residues in the hydrophobic patch of azurin (Fig. 2). The authors proposed that the loops in p53 belong to the most flexible region of the domain, thus capable of a strong structural adaptation, observed in the MD simulations, which lead to a tighter packing of both proteins and resulted in constructive Van der Waals contacts. Also, since the p53 DBD regions involved in the binding are in a peripheral position, this flexibility restriction can be one of the reasons for the azurin-induced stability of p53.¹⁴⁰ The NT domain of p53 has also been implicated in the binding to azurin and as for the DBD, Zdock, MD and free energy calculations have been applied to determine a good model for the complex.¹³⁸ The NTD structure is predicted to be organized in an α -helix, H_1 , and two turns assuming another α -helix structure, H_{II} and H_{III} , linked

by a fragment of 30–32 residues. As for the DBD, 2,000 models were generated and clustered by analysis of the 3D structures into five groups. The calculated ASA values were again in the range of the transient complexes. After the MD simulations, the most probable complex conformation is characterized by a free energy of $-527.4 \text{ kJ mol}^{-1}$ (Fig. 2). The regions involved in the binding are the helices H_{II} and H_{III} of p53, and the simulation showed a strong adaptation of this region to the azurin shape, increasing packing between both proteins, through numerous and favourable Van der Waals interactions. Apiyo and Wittung-Stafstedt¹²⁷ had already suggested this region as the one involved in the binding due to the quenching of the tryptophan emission which was corroborated by this model because Trp-23 is located at the protein interface and exposed to be quenched by azurin binding.¹³⁸ When one compares the two models for azurin and p53 binding in the two different p53 domains, the best result is obtained by

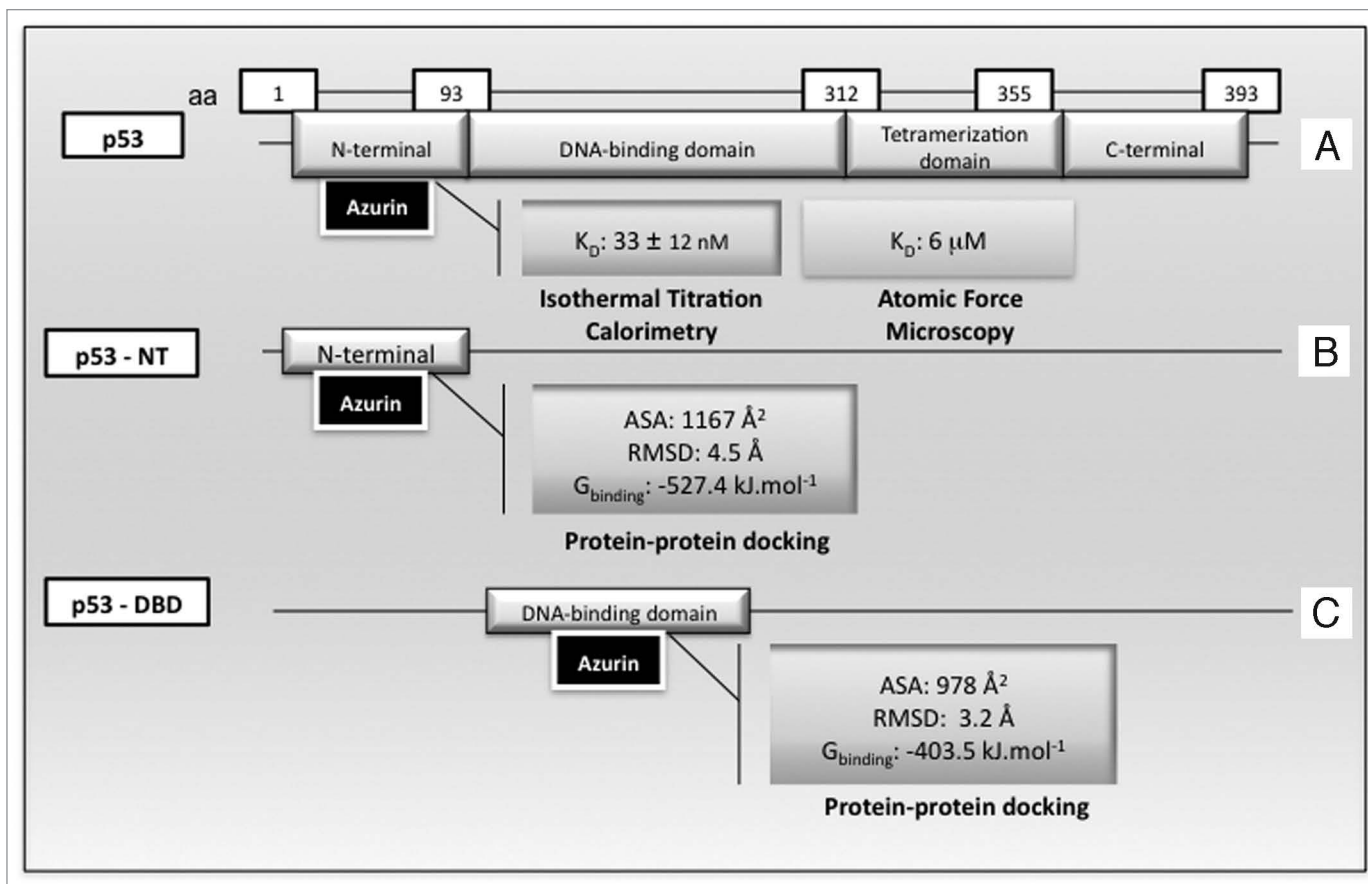


Figure 2. Studies of complex formation between the bacterial protein azurin and the transcription factor p53. (A) Binding studies using Isothermal Titration Calorimetry¹²⁶ and Atomic Force Microscopy.¹³⁵ (B and C) Docking studies and free energy simulation for the interaction between azurin and p53 N-terminal (p53-NT)¹³⁸ and azurin and p53 DNA binding domain (p53-DBD).¹³⁹

the latter, with a lower free energy. Since it was also shown by the same authors that 4 azurin monomers bind to each p53 monomer, one cannot exclude the fact that more than one configuration is possible, as highlighted by the authors of this study.¹³⁸

In order to interact with p53, azurin must come out of the periplasmic space of *P. aeruginosa* and enter mammalian cells. Indeed, azurin has been shown to be secreted to the outside medium when *P. aeruginosa* cells are exposed to human cancer cells, but less so when exposed to normal cells.¹⁴² Azurin was proficient in entering the human cancer cells but was deficient in entering the normal cells.¹⁴³ The entry of azurin in cancer cells has been studied by Yamada et al.¹⁴³ through a series of GST fusions of truncated forms of azurin at the central, N- and C-terminal regions. Azurin was internalized but GST alone was not and several fragments of azurin were tested to uncover the entry domain of this bacterial protein. The preferential entry of azurin in cancer cells has been identified to be a domain between amino acids 50 and 77 of azurin, termed p28, which can be used as a vehicle to transport cargo proteins.¹⁴³ p28 forms an extended amphipathic helix with both a hydrophobic (amino acids 50 to 66) and a hydrophilic (amino acids 67 to 77) domain with antiangiogenic activity.¹⁴⁴

A chemically-synthesized version of the p28 peptide has not only entry specificity in cancer cells, but similar to azurin, has

been shown to interfere with angiogenesis, inhibiting the formation of capillary tube formation of the human umbilical vein endothelial cells (HUVEC) in a dose dependent mode.^{6,145,146} Using a scratch wound migration assay, it was shown that p28 inhibited the migration of these cells in Matrigel, proving that this peptide has broad range effects in the morphological characteristic of angiogenesis. Interestingly, in addition to its ability to kill cancer cells, azurin and p28 can also interfere in oncogenic transformation to prevent precancerous lesion formation in mouse alveolar and ductal mammary glands exposed to a carcinogen Dimethyl-benz-anthracene (DMBA).¹⁴⁷

Taylor et al.¹⁴⁶ provided new information regarding the entry of the chemically-synthesized peptide p28 in human cancer cells. They refined the protein transduction domain (PTD) of 28 amino acid (p28) to only 18 (p18) in the N-terminal region, from amino acids 50 to 67. They showed that the entry of p28 and p18 occurs mainly via a nonendocytic rather than an endocytic membrane receptor mediated process. The results also suggested that the entry occurs without loss of membrane integrity. Recently, a start-up company CDG Therapeutics has initiated phase I human clinical trials of p28 for its anticancer activity (<http://clinicaltrials.gov>; clinical trials.gov identifier: NCT00914914).

Another important feature of azurin is its ability to recognize and bind to certain cellular receptors exposed at the cells' surface. Eph receptor tyrosine kinases are a family of 14 extracellular receptor proteins which bind to their ligands, the ephrins. These receptors are generally divided in two classes (A and B): A1 to A8 are cell-membrane linked proteins while B1 to B6 have highly conserved intracellular domains as well as a transmembrane domain.⁶ After the receptor-ligand interaction, a series of cellular signalling processes are initiated resulting in the proliferation, migration, invasion and angiogenesis of many types of human tumors. Different Eph receptors and ephrin ligands are present in distinct locations and some have been shown to be upregulated in some tumors, as it was for the EphB2 which is upregulated in glioblastoma, hepatocellular carcinoma, gastrointestinal and renal carcinomas, and prostate, lung and ovarian cancers.¹²⁸ Chaudari et al.¹²⁸ tested three different cupredoxin proteins which exhibited structural similarities with the ephrinB2 ectodomain and found high values of interaction between azurin and EphB2 and also EphA6, using Surface Plasmon Resonance (SPR). However, since both EphB2 and azurin have been involved in cancer cell signalling or cancer cell death, the authors proposed that this interaction could be a way of blocking the signalling process, antagonizing the Eph-ephrin mediated tumor progression (Fig. 1). In particular, a fragment of azurin, Azu 88-113, coincident with the G-H loop of ephrinB2-Fc, had a high affinity for binding with EphB2 (12 nM). Moreover, pre-incubation of EphB2-Fc with azurin diminished protein binding to an immobilized surface with ephrinB2 by up to 99% at stoichiometric or excess concentrations of the bacterial protein. Azurin and a fragment of it comprising amino acids 88 to 113 (Azu 88-113) showed the highest inhibition abilities. Altogether, the binding and the inhibition data indicate an affinity of azurin to interfere with the EphB2-ephrinB2 binding and the signalling process that can lead to tumor progression.¹²⁸

Unlike the rationally designed drugs that target a specific step in the signaling pathway involved in cancer, azurin intervenes in multiple pathways of cancer growth, thus exhibiting promiscuity. This is probably due to the fact that azurin has some interesting 3D-structural features. Azurin has structural similarity with variable domains of various immunoglobulins, thereby clearly demonstrating its single antibody-like structure as represented by ribbon drawing of eight antiparallel strands stabilized by a disulphide bridge-giving rise to a β -sandwich core, an immunoglobulin fold.^{147,148} It has a 28 amino acid (p28) extended α -helix PTD in the middle part that allows its entry specificity in cancer cells. It also has four exposed loop regions that are believed to be involved in its bindings with other proteins. By far the most interesting characteristic of azurin is its ability to bind various unrelated mammalian proteins relevant in cancer, conferring on it the property of a natural scaffold protein.¹⁴⁸ The scaffold proteins are non-antibody recognition proteins belonging to different and divergent families but that demonstrate immunoglobulin-like binding characteristics.¹⁴⁹ The ability of the azurin peptide p28 to act as a vehicle to carry cargo proteins inside cancer cells and azurin's ability to bind many different proteins because of its unique structural features makes azurin a potentially important natural scaffold protein for therapeutic purposes. A further development

in the concept of "scaffold" implies the generation of azurin variants displaying multiple affinities to pre-defined targets relevant in cancers. This strategy can be reached by randomized substitution of amino acids or substitutions of only a few selected residues within preexisting binding sites, specifically the exposed α -helix (p28) and the four surface exposed loops.

Future Perspectives

In the field of cancer drug discovery, recent developments are often related to rationally designed drugs, targeting a specific molecule or a signaling pathway. The use of high-throughput assays and combinatorial chemistry has supported the development of new synthetic chemical compounds with specific properties and structures directed to a specific receptor or protein that blocks a reaction or signaling cascade promoting cancer cell growth, survival and/or invasion. Different targets are used in anticancer drugs development, including those in growth signaling cascades and in DNA replication, cell division, angiogenesis and apoptosis. Overall different therapeutic modalities are now commercially available and in clinical practice, including monoclonal antibodies, drugs and prodrugs combinations and small natural or synthetic molecules.¹⁶ However, cancer cells, as with other disease agents, have the ability to mutate rapidly, causing a significant loss of therapeutic efficacy. Combinations of different drugs are often used to overcome this problem but this can cause a considerable increase in the costs and poses a problem to acquired resistance to multiple drugs. This has prompted the development of novel and alternative approaches for the treatment of cancer.

Microbial-based therapy of cancer is one of the emerging cancer treatment modalities. Over the past few years, important advances have been made to study and develop live bacteria, with or without additional cloned genes that encode toxins targeted specifically to cancer cells, or bacterial products with cancer killing ability. Various types of obligate anaerobic and facultative anaerobic bacteria can preferentially target cancer cells for growth in the hypoxic regions of the solid tumour. Attention has also recently been directed not only to the use of live bacteria, but bacterial proteins that can enter preferentially to cancer cells and disrupt their growth or kill them by multiple mechanisms.

During the past decade, cancer drug discovery or development is based on the single-target, single-compound paradigm. This concept contrasts with a more recent drug design approach, where a single drug can target with high efficacy multiple steps in cancer progression. Such second-generation drugs are often much more efficacious with little side effects. Of particular interest in this respect is azurin, a protein produced by the pathogenic bacteria *P. aeruginosa*. Azurin induces apoptosis through complex formation with tumor suppressor protein p53 or inhibits growth of cancer cells by interfering in receptor tyrosine kinase-mediated cell signaling or preventing angiogenesis.¹⁵⁰ In vivo cancer regression by azurin has also been demonstrated. The ability of a single bacterial protein, azurin, to interfere in the growth of cancer, is an interesting example of a potential drug candidate that can target multiple unrelated targets, interfering in multiple steps in the disease progression.

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