Combinatorial Genomics™: New tools to access microbial chemical diversity

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ABSTRACT

Natural products, primarily of microbial origin, have accounted for one-third of the $250 billion worldwide pharmaceutical market and are an important source of specialty chemical, agrochemical, and food or industrial processing products. Recent evidence suggests, however, that only about 5% of microorganisms from environmental samples have been previously accessible for discovery and development programs. The remaining 95%, termed "non-culturables", do not respond to traditional culture isolation methods and represent a rich, untapped source of chemical diversity for discovery programs. We have developed novel methods for accessing this diversity by isolating genomic DNA directly from environmental samples, preparing that DNA in a form that can be combined with the DNA of culturable host microorganisms, and creating through either protoplast fusions or liposome:protoplast fusions hybrid transformants that are capable of expressing biosynthetic pathways encoded in DNA obtained from "non-culturable" microbial sources.

Discussion

Sales of pharmaceuticals in the United States now exceed $100 billion with world-wide sales in excess of $250 billion. Despite the enormous size of this market and the attractive profit margins enjoyed by companies for novel pharmacophore-based patented drugs introduced into this market, the pharmaceutical industry has been in a difficult transitional period during recent years. Pricing and therefore profit margins on older drugs have been under attack, intensifying the pressure on drug companies to discover and develop truly novel drugs having greater pricing flexibility and therefore higher profit potential. Furthermore, many important diseases still lack adequate therapeutic solutions, offering substantial revenue potential for new breakthrough medicines. As a result, spending on research & development in the pharmaceutical industry continues to grow. More than $20 billion is spent annually by US-based drug companies on product development -- roughly 20 times the R&D spending in the early 1970's. Despite this phenomenal investment, the productivity of drug discovery research has been declining as measured by total R&D spending compared with the number of new chemical entities (NCE), or truly novel drugs, brought to market. For example, on a worldwide basis an estimated 40 NCE's were introduced in the mid-1990's, compared with about 60-70 NCE's annually during the early 1970's. One central reason for this decline in the productivity of drug research is the diminishing returns from existing natural product based libraries employed to screen new drugs.

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Current barriers to efficiently discovering and commercially exploiting novel pharmaceutical agents from microbial sources include both: i) difficulty in accessing and culturing the majority of micro-organisms, and ii) inadequate methods to rapidly screen these sources for multiple product opportunities with significant marketplace potential. Oceanix Biosciences Corporation has developed a powerful set of innovative, biotechnology-based tools which enable the discovery, development and production of new pharmaceuticals from formerly recalcitrant, non-culturable microorganisms. The vast majority (>95%) of microorganisms present in an environmental sample cannot be cultured. Consequently, the secondary metabolites or pharmacophores these organisms produce have never been evaluated for their pharmaceutical activity. Oceanix' Combinatorial Genomics technology is designed to circumvent this barrier to drug discovery by isolating non-culturable microbes or their DNA directly from crude environmental samples, by genetically inserting large blocks of that DNA into readily cultured microorganisms, and by eliciting the expression of the multiple genes encoded in that DNA resulting in the production of novel natural products. Through its NovaScreen Division, Oceanix routinely performs approximately 150 antibiotic and receptor-based drug assays that are available to screen for bioactive molecules produced through Combinatorial Genomics.

Bacteria are a prime source of natural products and are abundant in the environment, reaching for example, densities of up to 1×10^6 cells/ml in seawater samples, and 5×10^{10} cells/g dry wt in sediment. Although quite numerous, it has been demonstrated that less than 5% of the total species of microorganisms can be cultured. Accordingly, the numerous examples of microbial compounds which have been commercialized as pharmaceuticals represent only a small fraction of the potential range of bioactive molecules from these sources. This implies that the majority of the potential bioactive metabolite producers from environmental samples would be missed in drug discovery screening programs, since they rely on growing cultures. A technology that could allow the recovery of compounds from the 95% of bacteria that are otherwise not accessible is the fusion of protoplasts or liposomes containing environmental DNA of the non-culturable bacterial strains with protoplasts of a culturable recipient strain. By definition, hybrid microorganisms produced by these manipulations should be a source of truly novel and diverse chemicals, useful for drug discovery.

Oceanix Biosciences Corporation has been granted an initial patent by the US Patent and Trademark Office covering its Combinatorial Genomics technology for generating new molecules from natural sources for drug discovery and other applications. US Patent No. 5,773,221 was issued to Oceanix on June 30, 1998. Combinatorial Genomics is a direct, rapid, and powerful set of manipulations which allow large regions of random genetic materials or entire genomes from non-culturable or "donor" microbes to be transferred and expressed in easily cultured "host" microbes. As a result of these Combinatorial Genomics methods, the natural products present in a previously unexplored population of microbes can be potentially produced in fermentable organisms. Furthermore, these technologies may be used for production of novel chemical structures, or "unnatural" natural products, through recombination of the sequence of genes coding for biosynthetic pathways of the donor and the host microbes. Combinatorial Genomics is a biological process that could be considered some-what analogous to combinatorial chemistry methods for producing wide arrays of synthetic chemicals in test tubes.

Essentially, the Company's Combinatorial Genomics technology entails isolating non-culturable microorganisms or their high molecular weight DNA directly from environmental samples followed by the integration and expression of that genetic material in well characterized microbial host species. This Oceanix technology is not based on the precise tools of molecular biology which result in known and predictable results but is a more random and phenomenological genetic survey of unknown genetic materials. The Company cannot predict what genetic materials or natural products its technology will reveal.

For use in the Company's technology environmental DNA may be isolated either in a "naked" form and subsequently encapsulated in liposomes prior to use, or may be contained in non-culturable microbial cells which are converted into spheroplasts or protoplasts prior to use. Liposomes, spheroplasts, or protoplasts containing environmental DNA are then fused, employing standard cell fusion techniques such as polyethylene glycol (PEG) mediated fusion or electrofusion, with spheroplasts or protoplasts of well characterized and easily cultured host micro-organisms. A battery of well characterized host microbes can be employed as recipient organisms including gram-positive (including actinomycete species) and gram-negative prokaryotic species as well as certain fungal and archaebacterial host species. The selected host microbe species include strains which contain non-reverting auxotrophic mutations and thus require specific nutritional supplementation for growth and cellular proliferation. Following a fusion event between a host microbe protoplast or spheroplast (auxotrophic) cell and a prepared environmental DNA sample containing liposomes, protoplasts, or spheroplasts the viable, colony-forming cells will be those in which the delivered environmental DNA is expressed and has masked the auxotrophic phenotype of the host cell. These colony forming cells have incorporated and are expressing environmental DNA and thus present an enriched population which may have incorporated and may be expressing additional environmental DNA materials. Certain of these additional environmental DNA materials may encode for enzymes or enzyme pathways which result in the production of novel natural products. The genetically segregating populations of growing microbes which result from these fusion events are rapidly screened for the presence of bioactive compounds not present in the well characterized host microbial strains.

Protoplast and liposome fusion is a versatile and well explored technique to induce genetic recombination in a variety of prokaryotic and eukaryotic microorganisms. It does not require transducing phages, plasmid sex factors, or competency development; however, it does require identification of procedures to form stable protoplasts, to fuse the protoplasts, and to regenerate viable cells from fused protoplasts. Protoplasts are prepared by removing the cell wall by treating cells with lytic enzymes in the presence of osmotic stabilizers. In the presence of a fusogenic agent, such as PEG, or by treatment in electrofusion chambers, protoplasts and liposomes are induced to fuse and form transient hybrids or diploids. During the hybrid state, the genomes reassort and extensive genetic recombination can occur. The final, crucial step is the regeneration of viable cells from the fused protoplasts, without which no viable recombinants can be obtained.

For initial discovery efforts we have chosen to employ traditional Petri plate based antimicrobial screening methods, which are designed to rapidly detect antibacterial and antifungal agents being produced by the genetically segregating microbial populations that result from the Combinatorial Genomics manipulations. Essentially, segregating hybrid

populations are plated at an appropriate dilution over a lawn of indicator bacterial or fungal species. Microbial colonies elaborating antibiotic agents can be rapidly identified by areas where the indicator bacteria or fungi are not proliferating, as exemplified by halos of no growth after a period of incubation. Antibiotic producing colonies can then be isolated and cultured individually to isolate and more fully examine the bioactive agents they are elaborating. Further analysis of the recovered bioactive agents include: i) their further characterization in additional antibiotic tests employing a wider range of indicator microbial species, ii) their anti-cancer activity in a battery of malignant cell lines, and iii) their agonist or antagonist activity in a relevant central nervous system (CNS) focused battery of NovaScreen receptor binding assays or other appropriate NovaScreen assays. Those bioactive agents which display promising activity as antibiotic agents, as anti-cancer agents or as pharmacologically relevant materials are then purified via assay-guided fractionation and subject to chemical structural analysis. Novel bioactive agents are examined for their safety and toxicology properties both in a broad profile of NovaScreen assays and in cell culture-based assays.

Use of only anti-infective activity as a screen for initial bioactive material detection limits the range of pharmacological materials we are able to recover. Other screening methods are under development. However, this approach appears most cost- and time-effective since: i) Petri plate based antibiotic screens can accommodate the large genetically segregating populations which result from the Combinatorial Genomics fusion events, ii) Oceanix has determined from its past work that antimicrobial natural product agents which are active against gram positive bacteria and fungal species but are inactive against gram negative bacterial species often display specific anti-cancer properties, and iii) many bioactive agents which display antibiotic activities often have additional pharmacological activities. For example, many antibiotic compounds are used commercially as drugs for activities other than their antibiotic action. These include notable examples of antitumor agents (e.g., mitomycin, bleomycin, daunorubicin, and doxorubicin), immunosuppressive agents (e.g., cyclosporin, FK-506, rapamycin, and mycophenolic acid), hypocholesterolemic agents (e.g., lovastatin and pravastatin) and antimigraine agents (e.g., ergot alkaloids) among other pharmacological activities.

The Company's Combinatorial Genomics technology described here (US Patent # 5,773,221) differs substantially from the combinatorial biology approaches now being initiated by others to exploit the genetic diversity present in microbes in both the tools we employ as well as the technical approach we have chosen. Other laboratories practicing combinatorial biology primarily use restriction endonuclease enzymes as gene splicing tools, plasmids as genetic vectors, and often employ known microbes in their search for specific modifications to characterized natural product families (Service, 1997). Combinatorial biology, as practiced by others, generally employs a precise set of molecular tools directed towards manipulation of a sometimes known metabolic pathway. In contrast, the Company's Combinatorial Genomics technology employs the blunt tool of cell or liposome fusion, followed by largely random and extensive genetic segregation to recombine and express unknown metabolic pathways. The power of our approach lies in the use of rapid screening systems to identify bioactive materials followed by extensive pharmacology assays available through the Company's NovaScreen Division which are able to sort through the bioactive materials which result from Combinatorial Genomics manipulations.

References

Service, R. F., Hijacking a Cell's Chemical Paths to Make New Antibiotics, Science 227:378.

US Patent Number 5,773,221, Method of Recovering a Biological Molocule from a Recombinant Microorganism.