

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 May 2002 (23.05.2002)

PCT

(10) International Publication Number  
**WO 02/40717 A2**

(51) International Patent Classification<sup>7</sup>: **C12Q 1/68**

(21) International Application Number: PCT/US01/43726

(22) International Filing Date:  
14 November 2001 (14.11.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/248,410 14 November 2000 (14.11.2000) US  
10/008,107 13 November 2001 (13.11.2001) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

US	60/248,410 (CIP)
Filed on	14 November 2000 (14.11.2000)
US	Not furnished (CIP)
Filed on	13 November 2001 (13.11.2001)

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: *IN VITRO* CELL-BASED METHODS FOR BIOLOGICAL VALIDATION AND PHARMACOLOGICAL SCREENING OF CHEMICAL ENTITIES AND BIOLOGICALS

(57) Abstract: This patent describes a novel *in vitro* cell-based method for biological validation and pharmacological screening of drugs, new chemical entities (NCEs) and biologics, which is predictive of *in vivo* testing for efficacy and adverse events in patients, as occurs in clinical trials. The same method can be used to create an *in vitro* cell-based assay to identify the 'right marketed medication for the right patient' (personalized medicine), and to identify responders/non-responders in ongoing clinical trials with NCEs. In addition this approach can be used to identify new indications for existing medicines and new indications for NCEs that were unsuccessful in their intended uses.



WO 02/40717 A2

# ***IN VITRO* CELL-BASED METHODS FOR BIOLOGICAL VALIDATION AND PHARMACOLOGICAL SCREENING OF CHEMICAL ENTITIES AND BIOLOGICALS**

## **BACKGROUND OF THE INVENTION**

5           The pharmaceutical industry spends more than \$40 billion worldwide on research and development of new drugs, but only 5 to 10% of drugs entering the clinical phase of drug development will be approved for marketing. Currently the average cost per successful drug development program is between \$500 and \$900 million, and its duration is on average 8 to 12 years (1,2,3). This average cost figure is  
10 this high because 75% of that \$500-900 million the pharmaceutical companies spend per drug is related to drug failures along the way (2).

          An important reason for the high failure rate in clinical trials is the poor predictive value of currently used screening technologies for biological validation, pharmacological testing, and screening for success or failure of chemical entities and  
15 biologicals in clinical trials involving human subjects. These screening technologies are based on *in vitro* cell-based screening models and *in vivo* animal models, which often lack or inadequately represent the clinical disease phenotype of the patients in which the tested chemical entities or biologicals are intended to be used in the future. Therefore, success of these chemical entities or biologicals in these models does not  
20 necessarily translate into clinical success in patients. Hence, the majority of chemical entities or biologicals, while successful in these preceding screening and animal models, fail in clinical trials, particularly in late phase II and phase III trials (38). It has been estimated that more than 90% of new chemical entities (NCEs) fail in clinical trials, of which approximately two third fail for pharmacodynamic reasons (lack of  
25 efficacy and/or an unacceptable adverse event profile); the remaining third fail for pharmacokinetic reasons (3).

          According to a Lehman Brothers report, the problem of poorly predictive models will become increasingly worse in the genomic era because a higher number of inadequately biologically validated NCEs will enter clinical trials (1). This will  
30 decrease the overall success rate of clinical trials even further. This report predicts that the average R&D budget needed to develop an NCE will have to increase from a

current value of \$500-900 million to \$1.5 billion in the next five years, unless significant improvements are made.

The lack of available predictive technologies for success or failure in clinical trials leads to the current situation. Long and expensive preceding studies (in general more than five years and upfront investments of tens to hundreds of million dollars) are needed both in animals and humans before success or failure of NCEs can be established in phase II or phase III studies. Until better models are developed, the majority of NCEs will fail in phase II and III trials, either due to lack of efficacy or an unfavorable side effect profile. A cell-based method that could better predict success or failure in phase II and III trials, without the need for large up front investments, would represent a tremendous advantage from a pharmaco-economic perspective, as it would eliminate drug candidates or biologicals likely to fail early on, without the requirement of large upfront investments. Eventually, such a method would allow the production of medicines that are safer and more effective, at a much-reduced cost. In addition, such a model would reduce the need for *in vivo* animal testing.

Furthermore, most drugs show significant inter-individual variation in therapeutic efficacy and adverse event outcome (4,5,6,7,8). Evaluation of effectiveness and adverse event profile is still based on the average response of a study group. Inspection of the data from individual subjects, however, usually reveals significant numbers of patients with little or no response, as well as those who have dramatic responses. In cases of complex diseases, this 'one-drug-fits-all' attitude subjects patients to empirical trial-and-error periods before acceptable treatment regimens are found (4,8).

Assays for the personalized medicine application and the identification of responders/ non-responders in clinical trials are currently based on single nucleotide polymorphisms (SNPs) or haplotypes (4,8). Despite major investments made to develop the SNP approach for these applications, the numbers of successfully developed assays are small and their predictive value is often only modest. The trial-and-error nature of current clinical practice is a significant economic burden on the health care system and keeps many patients effectively untreated for sustained periods of time. A test tool that could predict whether a registered medication would be effective in a specific patient in a timely manner would offer tremendous benefit for patients and healthcare economics.

Moreover, the same principle could also be used to identify responders/non-responders in clinical trials with not yet registered NCEs. A large number of patients has to be recruited for each individual clinical phase II and III trials, in order to demonstrate efficacy and safety in a statistically meaningful manner.

- 5 Typically 50 to 200 patients are recruited in phase II and hundreds to thousands of patients in phase III. An important reason for the large numbers of patients are needed is the strong inter-individual variation in therapeutic efficacy and adverse event outcome in a randomly recruited patient population. The elimination of non-responders in these clinical trials would reduce the variability in trial outcome. This, in turn, would reduce the need for a large sample size of patients dramatically. Therefore, a test tool that avoids inclusion of patients likely to be non-responders in a clinical trial would lead to cost reduction on the order of hundreds of millions up to billions of dollars.

- 15 The development of predictive cell-based models has been hampered for various reasons, including the availability of human cells and tissues, in particular with the right genotype and disease phenotype, and the identification of validated cellular endpoints that have proven to predict *in vivo* responses after drug exposure. An ideal cell-based model should be using target cells or target tissues from patients who would be ultimately treated with the tested drugs. The availability of human cells for drug testing is limited, and often from questionable quality due to limitations in the preservation and the homogeneity of excised human tissues. Embryonic stem cell-based technologies are currently considered, but have inherent restrictions due to ethical considerations, and limitations in defining disease phenotype in these embryos that do not have manifestations of disease to be treated by investigational drugs or biologicals. Therefore the value of embryonic stem cells to predict pharmac-responses in specific patient populations with a well-defined disease phenotype is restricted. The identification of cellular pharmac-response that reliably predicts pharmac-responses in real patients with defined disease phenotype is another important obstacle. Ideally, this would require an experimental setting in which both cellular endpoints and *in vivo* patient endpoints after exposure to the same drug can be obtained to allow for a within-subject comparison, and to establish a strong *in vitro/in vivo* correlation.

Accordingly, a need remains in the art for a cell-based assay that can better predict success or failure of NCEs in phase II and III trials. A need also remains in the art for an assay that can identify patients likely to be non-responders in a clinical trial. Finally, a need remains in the art for an assay that can predict whether a medication or a  
5 chemical entity will be effective in a specific patient.

## SUMMARY OF THE INVENTION

The present invention provides an *in vitro* method of predicting an *in vivo* response in a patient to a chemical entity. Such a method generally comprises creating a reference set of cellular responses in peripheral blood mononuclear cells ("PBMCs"),  
10 which are extracted from groups of subjects, each group exposed to a different chemical entity, approved for treatment of a certain disease indication. These cellular responses are classified by the clinical indication of the subjects from whom the PBMCs are extracted. The reference set can also include *in vivo* responses for efficacy and/or safety (adverse events profile) of the same subjects to the group of chemical  
15 entities to which the cellular responses are correlated, in order to create cellular response profiles. The method further comprises drawing PBMCs from a patient suffering from the same disease indication for whom the predicted *in vivo* response is desired, detecting the cellular response of the patient's PBMCs to a chemical entity, then finding the *in vivo* response to which the cellular response corresponds in the  
20 reference set by using the cellular response profiles. The patient, when exposed to the chemical entity *in vivo*, is predicted to have the *in vivo* response that corresponds with the cellular response in the reference set. The clinical indication may pertain to efficacy, adverse effect, or safety of the chemical entity. Examples of a chemical entity include a registered chemical entity, a novel chemical entity, an environmental reagent,  
25 or a biological. Examples of cellular response include gene expression, increased motility, chemotaxis, contraction, relaxation, biosynthesis, secretion of signaling molecules, depolarization, repolarization, degranulation, adhesion, aggregation, change in metabolic rate, or immediate cellular responses. Examples of PBMCs include T-lymphocytes, B-lymphocytes, monocytes, natural killer cells, or peripheral blood stem  
30 cells. The PBMCs obtained from the patient can be divided into two or more portions and each portion is tested on a different chemical entity.

In one embodiment, the detection of the cellular response of the patient's PBMCs to a chemical entity is accomplished by transducing the patient's peripheral blood mononuclear cells with a zinc finger protein that specifically expresses or upregulates the target for the chemical entity; exposing the transfected peripheral blood mononuclear cells to the chemical entity; performing mRNA extraction on the exposed peripheral blood mononuclear cells; constructing a cDNA library from the extracted RNA; performing a cDNA subtraction with another cDNA library; and detecting resultant cellular responses of the patient's peripheral blood mononuclear cells' to the chemical entity.

10 The present invention also provides a method of screening chemical entities for their efficacy in treating a disease. Such a method generally comprises drawing PBMCs from subjects diagnosed with a particular disease who have been treated with known chemical entities, then performing gene expression analyses on the subjects' PBMCs. These expression analyses results are then compared within the reference set  
15 and correlated to an *in vivo* response of the known chemical entities against the disease to create cellular response profiles. These cellular response profiles can then be used as markers for other chemical entities to predict their efficacy and/or adverse event profile in treating the disease. Again, the clinical indication may pertain to efficacy, adverse effect, or safety of the chemical entity. Examples of a chemical entity include a  
20 registered chemical entity, a novel chemical entity, an environmental reagent, or a biological. Examples of cellular response include gene expression, increased motility, chemotaxis, contraction, relaxation, biosynthesis, secretion of signaling molecules, depolarization, repolarization, degranulation, adhesion, aggregation, change in metabolic rate, or immediate cellular responses. Examples of PBMCs include T-  
25 lymphocytes, B-lymphocytes, monocytes, natural killer cells, or peripheral blood stem cells. The PBMCs obtained from the patient can be divided into two or more portions and each portion is tested on a different chemical entity.

In one embodiment, the gene expression analysis of the patient's PBMCs is obtained by drawing peripheral blood mononuclear cells from the patient diagnosed  
30 with the specific disease; performing gene expression analysis on the peripheral blood mononuclear cells of the patients; comparing the results of the gene expression analysis to a reference set of gene expression analysis results that are correlated with an *in vivo*

response to the specific disease; and determining the *in vivo* response which correlates with the cellular response in the reference set.

The present invention further provides a method for detecting changes in gene expression in PBMCs in response to a chemical entity. Such a method generally comprises transfecting the PBMCs with zinc finger proteins ("ZFPs") that turn on genes encoding known targets for an effector, then exposing the PBMCs to the effector. The mRNA is then isolated from the PBMCs. Subtraction hybridization is used to eliminate all cDNAs expressed in unexposed PBMCs and/or expressed in clinical non-responders. The PBMCs are then assayed for the remaining over- and under-expressed cDNAs that indicate a difference in gene expression in response to the chemical entity. The detection of a remaining over- or under-expressed cDNA indicates a change in gene expression in the PBMCs in response to the chemical entity. Examples of a chemical entity include a registered chemical entity, a novel chemical entity, an environmental reagent, or a biological. Examples of PBMCs include T-lymphocytes, B-lymphocytes, monocytes, natural killer cells, or peripheral blood stem cells. The PBMCs obtained from the patient can be divided into two or more portions and each portion is tested on a different chemical entity.

Finally, the present invention provides a method for rapidly assessing whether a new patient diagnosed with a particular disease is likely to respond to a particular chemical entity. Such a method generally comprises creating a reference set of cellular responses to a group of chemical entities in PBMCs extracted from subjects who are classified by clinical indication and correlating them to *in vivo* responses from the same subjects in order to create cellular response profiles. These cellular response profiles are then captured on a suitable assay. PBMCs are drawn from the new patient and the cellular response to the chemical entity is detected. The new patient's cellular response is then compared to the cellular response profiles in the reference set to predict the chemical entity's *in vivo* response in the new patient. A patient having a cellular response that correlates with the cellular response in the reference set is likely to respond positively to the chemical entity. In one embodiment, the suitable assay is a microarray. In an alternative embodiment, the suitable assay is microbeads. The clinical indication may pertain to efficacy, adverse effect, or safety of the chemical entity. Examples of a chemical entity include a registered chemical entity, a novel chemical entity, an environmental reagent, or a biological. Examples of cellular

response include gene expression, increased motility, chemotaxis, contraction, relaxation, biosynthesis, secretion of signaling molecules, depolarization, repolarization, degranulation, adhesion, aggregation, change in metabolic rate, or immediate cellular responses. Examples of PBMCs include T-lymphocytes, B-lymphocytes, monocytes, natural killer cells, or peripheral blood stem cells. The PBMCs obtained from the patient can be divided into two or more portions and each portion is tested on a different chemical entity.

In one embodiment, the detection of the cellular response of the patient's PBMCs is accomplished by transfecting the patient's PBMCs with a zinc finger protein that specifically expresses or upregulates the target for the chemical entity; exposing the transfected PBMCs to the chemical entity; performing mRNA extraction on the exposed PBMCs; constructing a cDNA library from the extracted RNA; performing a cDNA subtraction with another cDNA library; and detecting resultant cellular responses of the patient's PBMCs to the chemical entity.

15

## DETAILED DESCRIPTION OF THE INVENTION

### DEFINITIONS

In this invention, the following terms will be used as defined:

**Zinc Finger Proteins (ZFP):** Proteins that act as modulators of transcription for specific sequences of nucleic acids. One zinc finger domain makes base-specific contact with three base pairs. In one embodiment of the invention, a polydactyl protein of a 6-zinc finger domain equivalent, which makes contact with a 18 base pair address, a sequence long enough to specify a unique site in the human genome is used. A 6-zinc finger domain equivalent transcription factor functions as a highly specific and potent transcriptional regulator.

**Peripheral Blood Mononuclear Cells (PBMCs):** The mononuclear cell population used in the invention is obtained from patients in which the drug is intended to be used. PBMCs are isolated with the Ficoll-Hypaque gradient method, and the population thus obtained from a healthy donor consists of a mixture of about 5% B lymphocytes, 5-15% monocytes, 60-70% lymphocytes, and 5-15% natural killer cells



(43, 44). The obtained cell population consists of the subsets B-lymphocytes, monocytes, T-lymphocytes, natural killer cells and a number of peripheral stem cells. One subset or a mixture of subsets of PBMCs are be used in this invention.

**Reference set:** A set of cellular responses correlated with *in vivo* responses.

5       **In vivo response:** Clinical result in a patient due to exposure to a chemical entity. For example, an *in vivo* response to a chemical entity in asthma could be changes in forced expiratory volume in one second (FEV1), as measured through a patient's use of a spirometer (26), or in depression as measured with HAM-D scores.

10       **Cellular responses:** Measurable *in vitro* responses in a cell type to the exposure of a chemical entity to the cell. These responses can include gene expression, increased motility, chemotaxis, contraction, relaxation, biosynthesis, secretion of signaling molecules, depolarization, repolarization, degranulation, adhesion, aggregation, change in metabolic rate, and immediate cellular responses.

15       **Cellular response profiles:** A set of cellular responses predictive for *in vivo* responses.

**Chemical entity:** Any substance tested to evaluate an *in vivo* response using a reference set of cellular responses. Examples of these are registered chemical entities, novel chemical entities, environmental reagents, and biologicals.

**Efficacy:** As defined in clinical studies (37, 38).

20       **Adverse events:** As defined in clinical studies (37,38).

**Safety:** As defined in clinical studies (37,38,39).

**Patient:** Individual with a clinical condition whose clinical outcome following exposure to chemical entities is being predicted.

25       **Subject:** Individual who is screened to acquire *in vivo* response versus cellular response correlations for reference sets.

      This application describes a method of providing improved predictability for the efficacy and/or adverse event profile of chemical entities or effectors (novel chemical entities, registered chemical entities, environmental reagents, or biologicals) especially in the context of treating disease. The invention is a method that comprises finding  
30       correlations between *in vitro* cellular and *in vivo* responses to chemical entities, to

create a new or utilize an already existing reference set of *in vitro* cellular responses to predict the effect the *in vivo* effect the chemical entity will have on a certain patient.

The reference set is a cellular response profile obtained from a separate subject population with a disease phenotype comparable to the target patients. The *in vitro*

5 cellular responses in PBMCs transformed into a bioassay using a specific ZFP are correlated with traditional *in vivo* clinical responses in terms of efficacy and adverse event profile. The reference *in vitro* data is then used to ascertain the relative efficacy and/or adverse event profile of novel chemical and biological entities to treat a disease in a similar group of patients.

10 Reference sets can be pre-existing or produced using the invention. The reference set is obtained by correlating the *in vitro* cellular response data gathered from a patient to an *in vivo* response data. The reference set contains clinical outcomes to a certain medical condition, each outcome accompanied by cellular response data gathered for the same subject. As described above, cellular responses can be one of  
15 many measurable responses a cell could have to the presence of a chemical entity.

Generally, these *in vitro* responses are detected by extracting blood from a subject or patient, and separating the peripheral blood mononuclear cells (PBMCs) from other parts of the blood (43, 44). These PBMCs are described above as a group of nucleated cells that are in the blood. Potentially, any type of cell could be used that  
20 could express a target of a chemical entity and produce a discernible cellular response that could be correlated with an *in vivo* response. Cell types that already express a target for the chemical entity being studied can simply be exposed to the chemical entity and their cellular response can be measured. PBMCs have targets for some chemical entities, but not for all chemical entities. In situations where the chemical  
25 entity being studied does not, or insufficiently has a target expressed in the cell type being exposed to it, the cells can be transduced using various techniques with a transcription factor such as a zinc finger protein (ZFP) that causes these targets to be expressed in the cells, as defined above. Other transcription factors could also be transduced into the cells as long as they somehow caused a target of the given chemical  
30 entity to be expressed in the cell.

Nucleic acids could also be introduced into the cells that encode transcription factors, or other proteins that could effect the expression of target molecules. The

protein would not have to directly interact with the gene that it was modulating, it need only upregulate the expression of a target for a chemical entity.

After the cell is transduced and exposed to the chemical entity, some cellular response is measured. This measurement is then compared to reference measurements in a subject population from which a relevant subject set has been made. What makes up a relevant subject set depends on the condition being studied, but generally, it is a set of subjects that have the same or a similar clinical condition as the patient. If the patient had asthma, for example, subject sets from subjects with asthma being treated by different chemical entities with different clinical outcomes could be relevant.

The relevant reference set depends upon the disease or condition, and upon the markers for efficacy or adverse effects being measured. In this manner, certain cellular responses are correlated with certain *in vivo* responses. This gives some basis for prediction of a patient response to a drug when the patient displays a certain clinical condition. The methods of the present invention can also be used to study how certain novel drugs might affect a patient with a certain condition. The methods can also be used to choose which drug would be the most effective in treating a patient. The methods can also be used to study how certain chemical entities might interact with each other in the context of a patient's *in vivo* response. The methods of the present invention can also be used to ascertain certain adverse event or safety concern a chemical entity might induce when people are exposed to it. Finally, the methods can also be used to screen patients to ascertain which of them would respond to certain chemical entities under certain conditions, and which would not respond. Any clinical indication, in any therapeutic area could be studied with this method.

The *in vitro/in vivo* correlation can be obtained from a prospective study, in which both the *in vitro* and *in vivo* data has yet to be collected. The correlation can also be obtained from a retrospective study, in which *in vivo* data is already available, but *in vitro* data yet needs to be created. An example of the latter is when exposure to a chemical entity has caused life-threatening adverse events (such as valvular fibrosis in subjects who were treated with Fenfluramine) and, therefore, it would be unethical to administer this chemical entity to other subjects. In this case, subjects with, and subjects without the adverse events (*in vivo data*) after exposure to the chemical entity would be recruited, and *in vitro* data would be measured from the PBMCs of these subjects, in order to identify a cellular response profile to predict the adverse event *in*

*vivo response*. This profile can be used to test new chemical entities or biologicals for their potential to cause these unacceptable adverse events.

In one embodiment of the invention, PBMCs are extracted from a patient with a certain disease (43, 44). The cells are transformed with a ZFP via retroviral infection. This ZFP is responsible for the expression of a target for a chemical entity that is to be screened for efficacy and/or adverse events in treating the disease. The PBMCs are then exposed to the chemical entity. RNA extraction is then performed as known in the art (41). This RNA is converted into a DNA library (34), and this is subtracted from another DNA library (35) made in a similar manner with untreated PBMCs, resulting in a number of genes being measured up or down regulated by the chemical entity. These up and down regulated genes are then compared to cellular response profiles associated with a positive outcome for the disease and/or with fewest adverse events. If the chemical entity's response in this patient's PBMCs correlates with a positive response in the reference set of cellular response profiles for this disease, then the drug is more likely to cause a positive outcome in the patient.

In another embodiment of the invention, the PBMCs from the above patient are split into a number of portions. Each portion is transduced with a different ZFP for a different chemical entity as above, and gene expression changes are detected for each portion as described above. The changes in gene expression of each of the chemical entities could be compared with a distinct cellular response profile in the cellular response profile, which profile is predictive for *in vivo* responses. The chemical entity that displays the most desirable profile could be used to treat the patient.

In another embodiment of the invention, the chemical entities in the above invention are novel chemical entities that had not been used before in the context of that disease state. They are screened for their ability to produce a cellular response that correlates with a positive clinical outcome.

In another embodiment, the chemical entities are known chemical entities that are used in a different disease state, but had not been used in the disease state that the patient above is displaying. They are screened for their ability to produce a cellular response that correlates with a positive clinical outcome.

In yet another embodiment of the invention, PBMCs are extracted from subjects with a certain disease being treated with a certain chemical entity. Differential gene

expression is measured as described above. This is repeated for many subjects, collecting what genes are induced and repressed using a certain chemical entity in a certain disease state, and what the *in vivo* response to the treatment was in the subject. This data is collected using different chemical entities with the same disease to create a  
5 reference set of chemical response profiles correlated with *in vivo* responses. This reference set can be used, used, as described above, to create a cellular response profile predictive for *in vivo* results in patients with a certain disease.

In another embodiment, PBMCs are extracted from subjects that have been exposed to a chemical entity in their environment. The cellular responses of their  
10 PBMCs could then be compared with the cellular responses obtained from subjects in whom exposure resulted or did not result in some clinical condition, to identify a cellular response profile predictive for *in vivo* response.

In yet another embodiment, genes that are up or down regulated in the case of a desired *in vivo* response, could be attached to a microarray on a chip (42) or  
15 microbeads. In this manner, subtracted cDNA from a patient could be quickly tested to see if it correlates with a certain *in vivo* response.

## EXAMPLES

### EXAMPLE 1 USE OF THE INVENTION TO MIMIC PHASE III CLINICAL TRIALS

This invention carries key characteristics of a traditional phase III trial (38);  
20 except that drug testing is performed on cells of patients, instead of the patients themselves and the use of cellular responses, which are validated against *in vivo* responses, to predict *in vivo* traditional responses. The key characteristics of a traditional clinical trial which are addressed in the invention include:

- Recruitment of patients with the desired disease phenotype.
- 25 • The use of inclusion/exclusion criteria to select patients.
- The use of efficacy endpoints which are validated and predictive for efficacy in the target population.
- The use of safety (adverse event) endpoints which are validated and predictive for safety in the target patient population.

- The calculation of a sample size sufficient to allow for the required statistical power.
- The definition of success or failure of the test medicine on the basis of the choice of, and magnitude of desired change in endpoints validated against and predictive for success or failure in the target patient population.
- Ethical review requirements such as study review and approval through an IRB.
- Informed consent of the patients participating in the studies.

10           The same invention can also be used to predict which registered medication in a certain disease indication is likely to be effective and/or will have an acceptable adverse event profile in a given patient diagnosed with that disease (the concept of personalized medicine).

15           The same invention can also be used to predict, prior to participation, in phase II and phase III trials (38), which patients are likely to classify as a non-responder or responder to a NCE or biological intended to be tested in those clinical trials. Exclusion of non-responders allows for a smaller sample size needed in the study to ensure acceptable statistical power.

20           The same invention can also be used to identify alternative clinical indications for existing drugs, registered for use in a certain clinical indication.

          The same invention can also be used to identify alternative clinical indications for novel NCEs or biologicals that failed in terms of efficacy during clinical trial testing in a certain indication.

#### **EXAMPLE 2    USE OF GENE EXPRESSION PROFILES AS AN *IN VIVO* RESPONSE**

25           The principle of pharmacotherapy is that a pharmacological response is initiated by a drug at its site of action on its so-called target. Several thousands of molecular targets have been cloned and are available as potential drug targets. These targets include more than 750 GTP-binding protein coupled receptors (GPCRs), over 100 ligand-gated ion channels, more than 60 nuclear receptors and 50 cytokines, and  
30   approximately 20 reuptake/transport proteins and a number of enzymes (15,16).

Signal transduction pathways involve a series or cascade of events that occur after a drug binds to its receptor, and culminate in the activation of effector mechanisms that result in a cellular response. Following binding of a drug to its receptor, immediate or late effects may occur. Immediate effects are due to modulation of cellular effector molecules that are already expressed by the target cell, and examples are increased motility, chemotaxis, contraction or relaxation, biosynthesis and secretion of other signaling molecules, depolarization or repolarization, degranulation, adhesion and aggregation, or a change in metabolic rate. Late effects are due to activation of nuclear transcription factors that either stimulate or inhibit gene expression. The cell response occurs later, following gene transcription (mRNA production), translation, protein synthesis and expression of newly synthesized proteins.

Gene expression profiles are a powerful tool to help dissect the mechanism of action of drugs and drug candidates. They will also increasingly contribute to the analysis of metabolic pathways for drugs, the understanding and prediction of adverse events *in vitro* and *in vivo*, as well a tool to predict the right dose and efficacy of a drug in the clinical setting.

Subsets of gene expression profiles can be used as a unique fingerprint of a specific drug action, and presumably, in cases in which the patients' disease condition improved, also as a fingerprint for clinical response. This principle was demonstrated in studying gene expression changes induced by toxic agents (toxicogenomics). Gene expression profiles are either causally linked to the toxic outcome or are downstream sequelae of the toxic exposure. Monitoring gene expression profiles, induced directly or indirectly by different classes of toxicants should eventually allow recognition of signature patterns that are representative of specific toxicities. Once recognized, these patterns could be used to evaluate new compounds (pharmaceutical candidates) possessing undefined toxicities (17,18,19,20,21).

The same principle is used as demonstrated to study toxicology on a molecular biology basis, to study efficacy and adverse events related gene expression profiles. Treatment with drugs with known mechanisms of action can be used to define a reference of response to which new drugs can be compared (17).

Genes can be upregulated or downregulated as a result of the drug action on the cell. Typically hundreds of genes are involved, and publications report a range of 100-1000 genes (17,18), however the number of the same genes over/under-expressed in a repeated manner is only a small fraction of that number. Certain gene induction events occur consistently, while others are highly variable. A study showed that in HepG2 cells exposed to cis-platinum, 200 or more genes were differentially expressed, but only 14 genes were consistently differentially expressed (17).

The approach of using ZFPs to upregulate genes in order to express drug targets in PBMCs offers a unique opportunity to study downstream metabolic pathways in clinical responders and to differentiate these from clinical non-responders. This in turn may increase understanding of the underlying diseases, as it increases understanding of pharmacological drug actions at a molecular level.

Subsets of gene expression profiles can be used as a unique fingerprint of a specific drug action, and, in cases in which the patient's disease condition improved, also as a fingerprint for clinical response.

### **EXAMPLE 3    PREDICTING DRUG EFFICACY AND ADVERSE EVENTS AGAINST A DISEASE**

In one embodiment of the invention a reference set of *in vitro* gene expression profiles predictive for *in vivo* pharmaco-responses (in terms of efficacy and adverse event profiles) are identified using a known drug (either drug A, B or C etc) registered for use in a certain clinical indication, for example disease X, to treat patients diagnosed with disease X. PBMCs are obtained from patients who are diagnosed with disease X, and these cells are used to create a bioassay, by expressing the drug target using specific ZFP in these cells. Drug responses induced by exposing drugs to the PBMC-based bioassay are obtained in parallel with clinical *in vivo* drug responses in these patients. Both cellular responses and *in vivo* patient responses are used to create a reference set. In this embodiment, the cellular *in vitro* responses are compared with the subject *in vivo* responses, both obtained within the same subject (within-subject comparison). A number of subjects diagnosed with disease X are treated with drug A, B or C, etc. If a given subject is treated with drug A, then the drug target for treatment A is expressed in the PBMCs of that subject, using a ZFP to express the target for drug A. This results in a PBMC-based bioassay, which is exposed to drug A to induce



cellular *in vitro* responses. This procedure is also performed with subjects treated with other drugs B and C on PBMCs that were treated with ZFPs specific for drugs B and C.

Amongst the subjects treated with drug A, a number of subjects are selected who classify as *in vivo* responders as defined by standard traditional definitions for clinical response. A number of subjects are also selected who classify as non-responders according to the same definition. Cellular gene expression profiles obtained from the clinical responders are grouped, as are the cellular gene expression profiles of clinical non-responders. In the group of the clinical *in vivo* responders to drug A, profiles of *in vitro* gene expression that all or the majority individual responders have in common are identified, along with which profiles are not or hardly present in the non-responders to drug A. This common *in vitro* cellular response gene expression profile therefore has a high predictive value for *in vivo* subject response to drug A (predictive expression profile A). Similarly, profiles can be constructed for subjects treated with drug B or C.

The gene expression profiles predictive for response to drugs A, B or C are used for the creation of drug screening assays to test novel drugs with a pharmacological action similar to drug A, B, or C respectively. The gene expression predictive profiles predictive for response to drugs A, B or C are also used to develop the reference for the personalized medicine diagnostic product.

In addition, a gene expression profile that all or majority *in vivo* responders to drug A, B, C, etc have in common is identified, along with which profile is not or hardly present in the *in vivo* non-responders to drugs A, B, C, etc. This gene expression profile reflects gene expression patterns, which are associated with *in vivo* clinical responses ('getting better') irrespective of the mechanism of action of the drug used, as all three drugs, with a each a different mechanism of action to induce them. Therefore, this gene expression profile can be used to screen NCEs and biologicals with novel mechanisms to treat disease X.

In addition to identifying gene expression profiles predictive for efficacy of drugs A, B, C, etc in patients, gene expression profiles can also be identified, predictive for adverse events of these drugs in these patients. Patients experiencing a certain adverse event are separated from the patients who do not experience that adverse event. The gene expression profiles in the PBMCs of these patients (after transformation into

a bioassay and exposed to the appropriate drug, as described for efficacy testing above) are analyzed to identify a gene expression profile that predominantly occurs in patients experienced with that adverse event, and not, or hardly, in patients who do not experience that adverse event. Differentially expressed genes are used to create  
5 customized microarrays or a different suitable assay, like microbeads, to allow a higher level of throughput testing.

#### EXAMPLE 4 SCREENING NCEs FOR EFFICACY AND/OR ADVERSE EVENT PROFILE

In another embodiment of the invention, the method is used to screen new chemical entities (NCEs) or biologicals for their efficacy and/or adverse event profile.  
10 By using a cellular response profile of validated gene expression profiles predictive for *in vivo* responses (translated into customized microarrays) by disease state, *in vitro* predictions about the *in vivo* efficacy of a novel NCE Y intended to treat disease X can be made, using the invention comprising the following components:

1. PBMCs from patients with disease X in which a future phase III study  
15 conduct is intended.
2. ZFP specifically expressing the target for NCE Y.
3. PBMC-based bioassay to be exposed to NCE Y.
4. mRNA extraction from these PBMCs.
5. Hybridization of mRNA to customized microarrays composed from  
20 gene expression profiles differentially expressed in clinical *in vivo* responders (in terms of efficacy and/or adverse event profile) compared to non-responders as described in example 3.
6. Analysis of hybridization pattern determines success or failure of the NCE Y in terms of efficacy and/or adverse event profile.

25 If NCE Y has a pharmacological mechanism of action similar to one of the registered drugs (for example drug A) used in the cellular response profile study, then the customized microarrays used in step 5 are composed from the differentially expressed gene data predictive for *in vivo* response (efficacy and/or adverse events) to drug A.

If NCE Y has a novel pharmacological mechanism of action intended for the treatment of disease X, then the customized microarrays used in step 5 are composed from the differentially expressed gene data predictive for *in vivo* response (efficacy and/or adverse events) that two or more registered drugs (each with a different pharmacological mechanism of action) have in common as described in Example 3. These commonly occurring gene expression data are assumed to predict disease modification, and are independent of the pharmacological mechanism of action of the individual drugs used. This is applicable for NCEs and biologicals.

#### **EXAMPLE 5 SCREENING DRUGS FOR RELATIVE EFFICACY**

In another embodiment of the invention, the method is used prior to drug administration, to ascertain which out of several candidate medications registered for a certain disease indication (X) is likely to be effective and/or has an acceptable adverse event profile in a given patient diagnosed with that disease X (the concept of personalized medicine). Gene expression profiles that specifically predict *in vivo* response to an individual drug A or B or C etc, indicated and marketed to treat a certain disease X are used to create a customized microarray, or other suitable technology like microbeads.

This *in vitro* diagnostic tool rapidly assesses whether a given patient diagnosed with disease X should be prescribed drug A, or B, or C etc. to improve the condition of disease X. PBMCs, of these patients are obtained, transformed into a bioassay, and exposed to drugs A, B, or C, etc, respectively, and the drug-induced cellular response (gene expression profile) is compared with a reference set of cellular responses correlated with *in vivo* responses. This cellular responses are obtained from a different but comparable set of subjects with disease X (as described in example 3), through *in vitro/in vivo* correlation. The cellular response *in vitro* gene expression profile predictive for *in vivo* response to drug A, B or C is used to create a microarray, or different suitable assay, for drug A, B or C, respectively (see below). This diagnostic test is comprised of the following components:

- Obtain PBMCs from the patient with disease X, and divide into three fractions (if three drugs are intended to be tested).
- Transfect one group of PBMCs with a ZFP specifically expressing target for drug A.

- Expose drug A to this PBMC-based assay.
  - Extract mRNA from these PBMCs.
  - Hybridize mRNA to predictive gene expression array for drug A.
  - Analysis of hybridization pattern determines likely response or non-response to drug A in the new patient.
- 5
- Transfect one group of PBMCs with a ZFP specifically expressing target for drug B.
  - Expose drug B to this PBMC-based assay.
  - Extract mRNA from these PBMCs.
- 10
- Hybridize mRNA to predictive gene expression array for drug B.
  - Analysis of hybridization pattern determines likely response or non-response to drug B in the new patient.
  - Transfect one group of PBMCs with a ZFP specifically expressing target for drug C.
- 15
- Expose drug C to this PBMC-based assay.
  - Extract mRNA from these PBMCs.
  - Hybridize mRNA to predictive gene expression array for drug C.
  - Analysis of hybridization pattern determines likely response or non-response to drug C in the new patient.
- 20
- The *in vitro* response predictions of new patients to drugs A, B or C, etc allow selection of the drug treatment that is most likely to result in the best clinical outcomes in terms of efficacy and/or adverse event profile in these patients.

**EXAMPLE 6 SCREENING PATIENTS FOR BEING RESPONDERS OR NON-RESPONDERS TO NCEs**

- 25
- In yet another embodiment, the method of the invention could be used to ascertain which patients diagnosed with disease X are likely to classify as a non-responder or responder to a NCE or biological intended to be tested in phase II and phase III clinical trials.

By using a cellular response profile of validated gene expression profiles predictive for *in vivo* responses translated into customized microarrays by disease state as described above, it is rapidly predictable *in vitro* whether a novel NCE (Z) intended to treat disease X will be effective *in vivo* in a clinical trial, using the invention

5 comprising of the following components:

- Isolate PBMCs from each of the patients with disease X, recruited to participate in the clinical trial
- ZFP specifically expressing the target for NCE-Z.
- PBMC-based bioassay to be exposed to NCE-Z.
- 10 • mRNA extraction from these PBMCs.
- Hybridization of mRNA to customized microarrays. The microarray used depends on the pharmacological mechanism of action of NCE-Z (see below).
- Analysis of hybridization pattern from patient to patient predicts which
- 15 patient is likely to become a responder or a non-responder to NCE-Z.

If NCE Z has a pharmacological mechanism of action similar to one of the registered drugs (for example drug A) found in the cellular response profile, then the customized microarrays used in step 5 can be composed on the basis of differentially expressed gene data predictive for *in vivo* response (efficacy and/or adverse events) to

20 drug A.

If NCE Z has a novel pharmacological mechanism of action, intended for the treatment of disease X, than the customized microarrays used in step 5 can be composed on the basis of the differentially expressed gene data predictive for *in vivo* response (efficacy and/or adverse events) that two or more registered drugs (each with

25 a different pharmacological mechanism of action) have in common as described in example 3. These commonly occurring gene expression data are assumed to predict disease modification and are independent of the pharmacological mechanism of action of the individual drugs used. This is applicable for NCEs and biologicals.

**EXAMPLE 7 FINDING NEW USES FOR A DRUG**

In yet another embodiment of the invention, the method is used to ascertain new uses for already existing chemical entities.

5 This test tool identifies an alternative clinical indication for already marketed drugs that proved to be effective in a different clinical indication. This test tool allows to assessment of whether a drug (drug H) that is effectively used to treat disease X, is also effective in the treatment of disease Y in patients as follows:

- Isolate PBMCs from patients diagnosed with disease Y.
- Transfect the PBMC with a ZFP specifically expressing the target for  
10 drug H.
- PBMC-based bioassay to be exposed to drug H.
- mRNA extraction from these PBMCs.
- Hybridization of mRNA to customized microarrays composed on the  
15 basis of cellular response gene expression profiles differentially  
expressed in clinical *in vivo* responders, and induced commonly by at  
least two different drug with different mechanisms of action in disease  
Y.
- Analysis of hybridization pattern predicts whether drug H is likely to be  
effective in disease Y.

20 In the cellular response *in vivo*, responses are obtained in patients diagnosed with disease Y and treated with already marketed drugs, in parallel with *in vitro* gene expression analysis in PBMC-based assays from the same patients, exposed to the same drugs, to allow *in vivo/in vitro* comparison of endpoints.

*In vivo* response data from patients who respond to a given drug are correlated  
25 with the unique gene expression fingerprint derived from their PBMC-based assay. These gene expression studies in parallel with *in vivo* response monitoring are conducted with at least two drugs from a different pharmacological class, but effective in the same indication Y. Analysis across all responders in all drug categories identifies the subset of expressed genes that these two or more drugs have in common,  
30 and thus correlates with disease state improvement, regardless of the drug used. These

predictive profiles are used to create customized microarrays, or other suitable technology. *In vivo* response can be obtained for drug efficacy and adverse event profile. This is applicable for chemical entities and biologicals.

5       **EXAMPLE 8     SCREENING NCEs THAT WERE NOT EFFECTIVE AGAINST ONE  
                          DISEASE TO ASCERTAIN THEIR EFFICACY AGAINST ANOTHER  
                          DISEASE**

10       In yet another embodiment of the invention, the method is used to identify new disease indications for an NCE (HF) that fail to be effective in an initially targeted disease. This test tool allows assessment of whether an NCE (drug HF) that was tested but failed to be effective in a certain disease X, would be effective in treating disease Y as follows:

- Isolate PBMCs from patients diagnosed with disease Y.
- Transfect PBMCs with a ZFP specifically expressing the target for NCE HF.
- 15       • PBMC-based bioassay to be exposed to NCE HF.
- mRNA extraction from these PBMCs.
- Hybridization of mRNA to customized microarrays composed from cellular response gene expression profiles differentially expressed in clinical *in vivo* responders, and induced commonly by at least two different drug with different mechanisms of action and registered in the treatment of in disease Y.
- 20       • Analysis of hybridization pattern predicts whether NCE HF is likely to be effective in disease Y.

25       In the reference study, *in vivo* responses are obtained in patients diagnosed with disease Y expression analysis in PBMC-based assays from the same patients, exposed to the same drugs, to allow *in vivo/in vitro* comparison of endpoints.

30       *In vivo* response data from patients who respond to a given drug are correlated with the unique gene expression fingerprint derived from their PBMC-based assay. These gene expression studies in parallel with *in vivo* response monitoring are conducted with at least two drugs from a different pharmacological class, but effective in the same indication Y. Analysis across all responders in all drug categories

identifies the subset of expressed genes that these two or more drugs have in common, and thus correlates with disease state improvement, regardless of the drug used. These predictive profiles are used to create customized microarrays, or other suitable technology. *In vivo* responses are obtained for drug efficacy and adverse event profile.

5 This is applicable for new chemical entities and biologicals.

#### **EXAMPLE 9 ZFP TRANSDUCTION OF PBMCs**

PBMCs are isolated from a patient using the Ficoll-Hypaque gradient method (43,44). T-lymphocytes are a cell type in the subset of PBMCs used in ZFP transduction. T-lymphocytes are isolated from a patient's PBMC mixture using FACS

10 sorting (27, 28), or by sorting with magnetic beads (29). T-lymphocytes can then be transduced with a ZFP through a retroviral technique (30), following pre-activation steps (with anti-CD3, IL-2 and/or phytohaemagglutinin (PHA), as used widely (31)) and co-localization of retroviral particles and target cells on a template.

When transduction efficiency is insufficient, it is necessary to enrich the cell

15 mixture, by separating out the non-transduced cells. To distinguish between transduced and non-transduced cells, the presence of a marker gene on the retrovirus may be used (30). This marker gene can encode either an antibiotic resistance protein (30), or for an easily detectable marker protein such as GFP (30). Enrichment in regard to successfully transduced cells can be obtained by exposing cells to an appropriate

20 antibiotic or, if a GFP was used, by fluorescence-activated cell sorting.

If it is impossible to use a marker gene, the efficiency of transduction can be determined by analyzing expression of the transgene (*i.e.*, zinc finger protein) or target gene (upregulated by zinc finger protein) using flow cytometry. In case the target gene product following transcription and translation is a cell surface protein, a primary

25 antibody specific for this protein can be used, combined with a fluorescent-labeled secondary antibody, followed by FACS sorting. If the target gene product is an intracellular protein, a cell-permeabilizing step is needed prior to the FACS sorting (32) following addition of the primary and secondary fluorescent-labeled antibody.

In addition to T-lymphocytes, B-lymphocytes can be used to create cell lines

30 (for example using Epstein-Barr Virus infection to stimulate the B-cells (40)) expressing the drug target of interest. Also subsequently, peripheral blood stem cells can be used with or without mobilization steps, which can be stimulated to proliferate



through specific cytokines (21,22). Because these stem cells are not terminally differentiated, they may offer a better alternative than T-lymphocytes to ensure that the expressed drug targets are functionally integrated in these cells.

Among the various viral vectors, retroviral vectors are preferred because they are easy to use, fast to prepare and induce long term expression of the target following integration in the host genome (30). However, retroviruses only infect dividing cells. If this is a significant disadvantage, alternatives such as lentiviral vectors (33), which can infect resting cells, are used.

In a more specific example, a ZFP for Albuterol, a  $\beta$ 2-adrenergic receptor agonists used as a cAMP mediated bronchodilator in Asthma (23), is constructed and tested using the above methods. The technical feasibility of the use of the Albuterol ZFP is considered successful, if:

1. This ZFP is capable of upregulating expression of the  $\beta$ 2-adrenergic receptor in the T-lymphocytes.
2. The expressed  $\beta$ 2-adrenergic receptor is successfully integrated in the T-lymphocytes.

The proof of technical feasibility experiment includes the following steps:

- Selection of a ZFP protein for this drug target.
- Configuration of the ZFP using proprietary linkers.
- Confirmation of the specificity of the ZFP.
- Attachment of a regulatory domain to configure ZFP.
- The transfer of ZFP to retrovirus for cell delivery.
- Test ZFP for ability to up-regulate target receptor in PBMCs, using specific antibodies.
- Exposing the successfully modified T-lymphocytes with Albuterol, and measuring intracellular cAMP production.

Increased intracellular levels of cAMP following expose of these T-lymphocytes with Albuterol, as compared to T-lymphocytes, not transduced with the ZFP, and exposed to Albuterol, is regarded as proof of technical feasibility for this ZFP.

**EXAMPLE 10 ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES**

cDNA library construction (34) and subtraction (35) can be used to identify differentially expressed genes in clinical responders and non-responders. In addition, available microarrays can also be used for this purpose (36). Response and  
 5 non-response can be obtained *in vivo* from the target patient population, in which the NCE/drug is intended to be used, and is defined according to standard clinical definitions (37, 38, 39).

PBMCs in which the drug target is expressed by means of ZFP transduction are selected by flow cytometry or magnetic beads, as detailed above. PBMCs in which the  
 10 target for a drug is expressed are incubated with that drug. Incubation is with two separate doses (intermediate and high), for three time durations (0 (predose), 8 hour, and 24 hour).

To enable the cDNA library construction and subtraction, mRNA is extracted (41) from a minimum of 10 million successfully transduced cells that are treated with  
 15 drug as described above. The following libraries were pooled:

- I. Postdose responders: cDNA from cells from a selected number of responders (R) are pooled, including the two doses for both postdose time points.
- II. Postdose non-responders: cDNA from cells from a selected number of  
 20 non-responders (NR) are pooled, including the two doses for both postdose time points.
- III. Predose responders: cDNA from cells from responders (R), as selected under I, are pooled from the predose samples.
- IV. Predose non-responders: cDNA from cells from non-responders (NR)  
 25 as selected under II, are pooled from the predose samples.

The following subtracted libraries are considered from pooled material:

- I. Postdose (R)–Postdose (NR)
- II. Postdose (NR)–Postdose (R)
- III. Predose (R)–Predose (NR)
- IV. Predose (NR)–Predose (R)

- V. Postdose (R)–Predose(R)
- VI. Predose (R)–Postdose(R)
- VII. Postdose (NR)–Predose (NR)
- VIII. Predose (NR) Postdose (NR)

5 This is followed by 3' sequencing of clones obtained from the subtractions. Each 3'-terminal sequence is searched on-line using the BLAST program at the NCBI site for possible matches in the non-redundant and EST public databases. Accession numbers from the BLAST search are used to search the UNIGene database to identify non-redundant Unigene clusters. A Unigene search is also performed on the public  
10 EST sequence libraries for selected Stratagene libraries. If the 3'-terminal sequence does not allow the identification, this clone undergoes 5'-terminal sequencing.

Hybridization patterns of probes prepared from the subtracted libraries I-VI are used to create our own customized microarrays displaying subtracted sequences. This is followed by hybridization of probes of all individual patient samples onto the  
15 microarrays displaying the subtracted libraries. This step is needed to demonstrate that differentially expressed genes as obtained from subtractions of pooled samples, are differentially expressed in all or the majority of the individual responders.

Commercially available microarrays can also be used to examine differentially expressed genes, using a hybridization reaction between the sequences on the  
20 microarray and a fluorescent sample (36). After hybridization, the microarrays are read with high-speed fluorescent detectors and the intensity of each spot is quantified. The location and intensity of each spot reveal the identity and amount of each sequence present in the sample. The data are then mined and modeled using the tools of computational biology. Thousands, or tens of thousands of gene fragments can be  
25 present on a single microarray.

This technique can be used on multiple drugs with the same batch of isolated PBMCs. The batch is split into as many groups as there are drugs to be tested, and the same procedure is run on each group with their own ZFPs and drugs.

**EXAMPLE II ASCERTAINING DRUG EFFICACY IN CONJUNCTION WITH ALBUTEROL  
IN ASTHMA**

Screening of drug efficacy can be conducted in asthma (24,25). For many patients, the disease has its roots in infancy, and both genetic factors and environmental factors contribute to its inception and evolution. Of the chronic diseases of childhood, asthma is the most common, with reported prevalence in children ranging from 3% to 27% across different countries. In the U.S. alone, about 17 to 20 million individuals have asthma, and the total sales of respiratory therapies exceed \$17 billion a year. There are five major classes of asthma drugs, including  $\beta$ 2-adrenergic receptor agonists, leukotriene antagonists, inhaled corticosteroids, phosphodiesterase inhibitors and anticholinergic drugs. The targets of these drugs are extensively studied and well defined, and there are currently 28 NCEs being developed for respiratory and lung diseases.

The primary objective is to identify a set of gene expression profiles in T-lymphocytes (with expressed  $\beta$ 2-adrenergic receptor using specific ZFP) in asthma patients treated effectively with Albuterol. Albuterol is a  $\beta$ 2-adrenergic receptor agonists used as a bronchodilator in asthma.  $\beta$ 2-adrenergic receptor agonists relax bronchial smooth muscle through cyclic AMP (cAMP)-mediated pathways (23). An additional objective is to identify a set of gene expression profiles predictive for most frequently occurring adverse events following a single dose of Albuterol in our study.

Twenty to forty patients diagnosed with moderate to severe asthma, with defined clinical phenotype, are recruited and asked to interrupt corticosteroid treatment for 24 hours and treatment with  $\beta$ 2- adrenergic receptor treatment for 12 hours prior to participation in this study. The patients are treated with the bronchodilator Albuterol, a  $\beta$ 2-adrenergic receptor. They are monitored for *in vivo* efficacy for bronchodilation, using the forced expiratory volume in one second (FEV1), and adverse events. Simultaneously, *in vitro* gene expression data is obtained from T-lymphocytes from the same patients. FEV1 and adverse event are recorded prior and at regular intervals after Albuterol treatment.

The FEV1 is the primary measurement assessed with spirometry for evaluating asthma severity and assessing change in the degree of airway obstruction (26). Spirometry is an excellent procedure for documenting changes in Asthma, because it is

reliable, reproducible, and standardized (26). The reported positive clinical response rate in terms of bronchodilation after Albuterol treatment varies from 26.3% (7) to 54% (23).

Adverse events are collected for at least 8 hours after administration of  
5 Albuterol. Prior to administering Albuterol to the patients, T-lymphocytes are isolated from these patients, and transformed into bioassays using specific ZFPs to express  $\beta$ 2-adrenergic receptors, followed by drug exposure and mRNA analysis and sequencing.

Although  $\beta$ 2-adrenergic receptors are expressed in T lymphocytes of healthy  
10 people, in drug-free asthmatic patients,  $\beta$ 2-adrenergic receptor density (and hence cAMP response) is significantly reduced, without changes in affinity of these receptors to the ligand (45,46,47). Therefore T-lymphocytes of asthmatic patients would offer an ideal cell type to assess the potential of a specific ZFP to increase expression of  $\beta$ 2-adrenergic receptors in these cells.

15 Gene expression data of clinical responders is separated from clinical non-responders and a gene expression profile is identified that occurs in all or the majority of responders, but absent or hardly present in non-responders. Similarly, a gene expression profile is identified that occurs in all or the majority of adverse responders, but absent or hardly present in non-adverse responders.

20 Predictive cellular response gene expression profiles are used to design customized microarrays or microbeads. These microarrays or microbeads can either be used to develop screening tools or to predict whether a patient responds to a specific Albuterol (diagnostic).

25 The embodiments explained above are for explanatory purposes only. In no way should they limit the invention from other embodiments not listed here.

All references included above are incorporated in their entirety.

## References

1. Lehman Brothers Report. The Fruits of Genomics. January 2001.
2. Boston Consulting Group. The Revolution in R&D: The Impact of Genomics. June 2001.
- 5 3. Houlihan, Lokey, Howard & Zukin. Advanced Valuation Techniques in Life Sciences; February 2001.
4. Liggett SB. Pharmacogenetic applications of the human genome project. *Nature Medicine* 2001;7:281-283.
- 10 5. Drysdale CM, McGraw DW, Stack CB, Stephens JC, Judson RS, Nandabalan K, Arnold K, Ruano G, Liggett SB. Complex promoter and coding region  $\beta$ 2-adrenergic receptor haplotypes alter receptor expression and predict *in vivo* responsiveness. *PNAS* 2000;97:10483-10488.
- 15 6. Drazen JF, Yandava CN, Dube L, Szczerback N, Hippensteel R, Pillari A, Israel E, Schork N, Silverman ES, Katz DA, Drajesk J. Pharmacogenetic association between ALOX5 promoter genotype and the response to anti-asthma treatment. *Nature Genetics* 1999;22:168-170.
7. Martinez FD, Graves PE, Baldini M, Solomon S, Erickson R. Association between genetic polymorphisms of the  $\beta$ 2-adrenoceptor and response to albuterol in children with and without a history of wheezing. *J Clin Invest* 1997; 20 100:3184-3188.
8. Poste G. The right treatment for the right patient. *Scrip Magazine*, January 2000: 11-14.
9. Kang JS, Kim JS. Zinc Finger Proteins as designer transcription factors. *J Biol Chem* 2000;275:8742-8748.
- 25 10. Kim JS, Pabo CO. Getting a handhold on DNA: Design of poly-zinc finger proteins with femtomolar dissociation constants. *PNAS* 1998;95:2812-2817
11. Beerli RR, Segal DJ, Dreier B, Barbas CF III. Toward controlling gene expression at will: specific regulation of the *erbB2/HER-3* promoter by using polydactyl zinc finger proteins constructed from modular building blocks. 30 *PNAS* 1998;95:14628-14633.
12. Beerli RR, Dreier B, Barbas CF III. Positive and negative regulation of endogenous genes by designed transcription factors. *PNAS* 2000;97:1495-1500.
13. Pierce JH, Ruggiero M, Fleming TP, Di Fiore PR, Greenberger JS, Varticovski L, Schlessinger J, Rovera G, Aaronson SA. Signal transduction through the ECF receptor transfected in IL-3-dependent hematopoietic cells. 35 *Science* 1988;239:628-631.
14. Riese DJ II, van Raaij TM, Plowman GD, Andrews GC, Stern DF. The cellular response to neurogulins is governed by complex interactions of the *erbB* receptor Family. *Molecular and Cellular Biology* 1995;15:5770-5776.
- 40 15. Ohlstein EH, Ruffolo RR Jr, Elliott JD. Drug discovery in the next millennium. *Annual Rev Pharmacol Toxicol* 2000;40:177-191
16. Drews J. Drug Discovery: A Historical Perspective. *Science* 2000; 287:1960-1964.

17. Burczynski ME, McMillian M, Ciervo J, Li L, Parker JB, Dunn RT II, Hicken S, Farr S, Johnson MD. Toxicogenomics-based discrimination of toxic mechanism in HepG2 human hepatoma cells. *Toxicological sciences* 2000; 58:399-415.
- 5 18. Afshari CA, Nuwaysir EF, Barret JC. Application of complementary DNA microarray technology to carcinogen identification, toxicology, and drug safety evaluation. *Cancer Res* 1999;59:4759-4760.
19. Braxton S, and Bedillion T. The integration of microarray information in the drug development process. *Curr Opin Biotech* 1998;9:643-649
- 10 20. Nuwaysir EF, Bittner M, Trent J, Barrett JC, Afshari CA. Microarrays and toxicology: The advent of toxicogenomics. *Mol Carcinog* 1999;24:153-159.
21. Aurran-Schleinitz T, Imbert A, Humeau L, Bardin F, Charbord P, Chabannon C. Early progenitor cells from human mobilized peripheral blood express low levels of the flts receptor, but exhibit various biological responses to flts3-L. *Br J Haematol* 1999;106:357-367
- 15 22. Pollard Y, Watts MJ, Grant D, Chavda N, Linch DC, Machin SJ. Use of the haemopoietic progenitor cell count of the Sysmex SE-9500 to refine apheresis timing of peripheral blood stem cells. *Br J Haematol* 1999;106:538-544
23. Wilber ST, Wilson JE, Blanda M, Gerson LW, Meerbaum SH, Janas G. The bronchodilator effects of intravenous glucagon in asthma exacerbation: A randomized, controlled trial. *Annals of Emergency Medicine* 2000;36:427-431
- 20 24. Busse WW, Lemanske RF Jr. Asthma. *NEJM*, 2001;344:350-362.
25. Barnes PJ. Inhaled glucocorticoids for asthma. *NEJM* 1995;332:868-875.
26. Blaiss MS. Outcomes analysis in asthma. *JAMA* 1997; 278: 1874-1879.
- 25 27. Galboraith, D. W., et al., Flow cytometric analysis and FACS sorting of cells based on GFP accumulation. *Methods in Cellular Biology* 1999; 58:315-41.
28. Nielsen, M.B., et al., Status of activation of circulating vaccine-elicited CD8+ T cells. *Journal of Immunology* August 15, 2000; 165(4):2287-96.
29. Luxembourg, A.T. et al., Biomagnetic isolation of antigen-specific CD8+ T cells usable in immunotherapy. *Nature Biotechnology* Mar, 1998; 16(3):281-5.
- 30 30. Current Protocols in Molecular Biology Ed. Ausubel, F.M., et al. 1998 Chapter 9.
31. Nanki, T., et al., Cutting edge: stromal cell-derived factor-1 is a costimulator for CD4+ T cell activation. *Journal of Immunology* May 15, 2000; 164(10):5010-4.
- 35 32. Berki, T., et al. Production and flow cytometric application of a monoclonal anti-glucocorticoid receptor antibody. *Journal of Immunology Methods* May 1, 1998; 214(1-2):19-27.
33. Naldini, L. et al. Lentiviral vectors. *Advances in Virus Research* 2000; 55:599-609.
- 40 34. Current Protocols in Molecular Biology Ed. Ausubel, F.M., et al. 1998 Chapter 5.1-5.7

35. Current Protocols in Molecular Biology Ed. Ausubel, F.M., et al. 1998 Chapter 5.8-5.9
36. Winzeler, E.A., et al. Fluorescence-based expression monitoring using microarrays. *Methods in Enzymology* 1999; 306:3-18.
- 5 37. The Textbook of Pharmaceutical Medicine. 2<sup>nd</sup> edition. Eds. Griffin JP, O'Grady J, Wells FO. Executive Editor: D'Arcy PF. The Queen's University of Belfast, publishers.
38. New Drug Development: A Regulatory Overview. Revised 5<sup>th</sup> edition.. Ed. Mathieu M. Parexel, publishers
- 10 39. Drug Safety. A Shared responsibility. Glaxo Group Research. ISBN 0443046557. Churchill Livingstone, publishers.
40. Bejarano, M.T., et al. Interleukin-10 abrogates the inhibition of Epstein-Barr virus-induced B-cell transformation by memory T-cell responses. *Blood* Dec. 1, 1998; 92(11):4256-62.
- 15 41. Current Protocols in Molecular Biology Ed. Ausubel, F.M., et al. 1998 Chapter 4.
42. Hess, K.R. et al., Microarrays: handling the deluge of data and extracting reliable information. *Trends Biotechnol.* Nov. 2001, 19(11):463-8.
43. Reichert T, et al. Lymphocyte subset reference ranges in adult Caucasians. *Clin Immunol Immunopathol* 1991;60:190-208.
- 20 44. Boyum A. Separation of white blood cells. *Nature* 1964;204:793-794.
45. Gillespie E, Valentine MD, Lichtenstein LM. Cyclic AMP metabolism in asthma: studies with leukocytes and lymphocytes. *J Allergy Clin Immunol* 1974;53:27-33
- 25 46. Parker CW, Smith JW. Alternations in cyclic adenosine monophosphate metabolism in human bronchial asthma. Leukocyte responsiveness to beta-adrenergic agents. *J Clin Invest* 1973;52:48-59.
47. Motojima S, Fukada T, Makino S. Measurement of  $\beta$ 2-adrenergic receptors on lymphocytes in normal subject and asthmatics in relation to  $\beta$ 2-adrenergic hyperglycaemic response and bronchial responsiveness. *Allergy* 1983; 38:331-337.
- 30 48. Jenne JW. Whither beta-adrenergic tachyphylaxis? *J Allergy Clin Immunol* 1982; 70:413-416.
49. Green SA, Turki J, Bejarano P, Hall IP, Liggett SB. Influence of  $\beta$ 2-adrenergic receptor genotypes on signal transduction in human airway smooth muscle cells. *Am J Respir Cell Mol Biol* 1995; 13:25-33.
- 35



We claim:

1. A method of predicting a patient's *in vivo* response to a chemical entity, comprising:
  - (i) creating a set of reference cellular responses to the chemical entity in peripheral blood mononuclear cells that are correlated with *in vivo* responses for a clinical indication in a population of subjects;
  - (ii) drawing peripheral blood mononuclear cells from the patient;
  - (iii) detecting the cellular response of the patient's peripheral blood mononuclear cells to the chemical entity;
  - (iv) identifying the cellular response in the reference set of step (i) that corresponds to the patient's cellular response of step (iii); and
  - (v) determining the *in vivo* response which correlates with the cellular response in the reference set identified in step (iv);wherein the patient is predicted to have the *in vivo* response determined in step (v) when exposed to the chemical entity *in vivo*.
2. The method of claim 1, wherein the clinical indication is a member of the group consisting of efficacy, adverse effect, and safety.
3. The method of claim 1, wherein the chemical entity is a member of the group consisting of a registered chemical entity, a novel chemical entity, an environmental reagent, and a biological.
4. The method of claim 1, wherein step (iii) comprises:
  - (a) transducing the patient's peripheral blood mononuclear cells with a zinc finger protein that specifically expresses or upregulates the target for the chemical entity;
  - (b) exposing the transfected peripheral blood mononuclear cells to the chemical entity;
  - (c) performing mRNA extraction on the exposed peripheral blood mononuclear cells of step (b);
  - (d) constructing a cDNA library from the extracted RNA;
  - (e) performing a cDNA subtraction with another cDNA library; and
  - (f) detecting resultant cellular responses of the patient's peripheral blood mononuclear cells to the chemical entity.

5. The method of claim 1, wherein the patient's peripheral blood mononuclear cells are divided into two or more portions and each portion is tested on a different chemical entity.
6. The method of claim 1, wherein the cellular response is a member of the group consisting of gene expression, increased motility, chemotaxis, contraction, relaxation, biosynthesis, secretion of signaling molecules, depolarization, repolarization, - degranulation, adhesion, aggregation, change in metabolic rate, and immediate cellular responses.
7. The method of claim 1, wherein peripheral blood mononuclear cells are members of the group consisting of T-lymphocytes, B-lymphocytes, monocytes, natural killer cells, and peripheral blood stem cells.
8. A method for predicting an *in vivo* response for clinical indications that a chemical entity will cause in a patient afflicted with a specific disease, comprising:
  - (i) drawing peripheral blood mononuclear cells from the patient diagnosed with the specific disease;
  - (ii) performing gene expression analysis on the peripheral blood mononuclear cells of the patients;
  - (iii) comparing the results of the gene expression analysis of step (ii) to a reference set of gene expression analysis results that are correlated with an *in vivo* response to the specific disease; and
  - (iv) determining the *in vivo* response which correlates with the cellular response in the reference set;wherein the chemical entity is predicted to cause the *in vivo* response for a clinical indication determined in step (iv).
9. The method of claim 8, wherein the clinical indication is a member of the group consisting of efficacy, adverse effect, and safety.
10. The method of claim 8, wherein step (ii) comprises:
  - (a) transducing the patient's peripheral blood mononuclear cells with a zinc finger protein that specifically expresses or upregulates the target for the chemical entity;

- (b) exposing the transfected peripheral blood mononuclear cells to the chemical entity;
  - (c) performing mRNA extraction on the exposed peripheral blood mononuclear cells of step (ii);
  - (d) constructing a cDNA library from the extracted RNA;
  - (e) performing a cDNA subtraction with another cDNA library; and
  - (f) detecting resultant cellular responses of the patient's peripheral blood mononuclear cells' to the chemical entity.
11. The method of claim 8, wherein the chemical entity is a member of the group consisting of a registered chemical entity, a novel chemical entity, an environmental reagent, and a biological.
12. The method of claim 8, wherein the patient's cells are divided into two or more portions and each portion is tested on a different chemical entity.
13. The method of claim 8, wherein the cellular response is a member of the group consisting of gene expression, increased motility, chemotaxis, contraction, relaxation, biosynthesis, secretion of signaling molecules, depolarization, repolarization, degranulation, adhesion, aggregation, change in metabolic rate, and immediate cellular responses.
14. The method of claim 8, wherein peripheral blood mononuclear cells are members of the group consisting of T-lymphocytes, B-lymphocytes, monocytes, natural killer cells, and peripheral blood stem cells.
15. A method for detecting changes in gene expression in peripheral blood mononuclear cells, comprising:
- (i) transducing the peripheral blood mononuclear cells with a zinc finger protein that will turn on genes encoding known targets for a chemical entity;
  - (ii) exposing the peripheral blood mononuclear cells to the chemical entity;
  - (iii) isolating RNA from the peripheral blood mononuclear cells;
  - (iv) constructing a cDNA library from the isolated RNA;
  - (v) using subtraction hybridization to eliminate all cDNAs expressed in unexposed peripheral blood mononuclear cells; and

- (vi) assaying for the remaining over- and under-expressed cDNAs, wherein the detection of a remaining over- or under-expressed cDNA indicates a change in gene expression in the peripheral blood mononuclear cells.
16. The method of claim 15, wherein step (v) further comprises using subtraction hybridization to eliminate all cDNAs expressed in clinical non-responders.
17. The method of claim 15, wherein peripheral blood mononuclear cells are members of the group consisting of T-lymphocytes, B-lymphocytes, monocytes, natural killer cells, and peripheral blood stem cells.
18. The method of claim 15, wherein the patient's cells are divided into two or more portions and each portion is tested on a different chemical entity.
19. The method of claim 15, wherein the chemical entity is a member of the group consisting of a registered chemical entity, a novel chemical entity, an environmental reagent, and a biological.
20. A method for detecting changes in gene expression in peripheral blood mononuclear cells, comprising:
- (i) transducing the peripheral blood mononuclear cells with a zinc finger protein that will turn on genes encoding known targets for a chemical entity;
  - (ii) exposing the peripheral blood mononuclear cells to the chemical entity;
  - (iii) isolating RNA from the peripheral blood mononuclear cells;
  - (iv) constructing a cDNA library from the isolated RNA;
  - (v) using subtraction hybridization to eliminate all cDNAs expressed in clinical non-responders; and
  - (vi) assaying for the remaining over- and under-expressed cDNAs, wherein the detection of a remaining over- or under-expressed cDNA indicates a change in gene expression in the peripheral blood mononuclear cells.
21. The method of claim 20, wherein step (v) further comprises using subtraction hybridization to eliminate all cDNAs expressed in unexposed peripheral blood mononuclear cells.

22. The method of claim 20, wherein peripheral blood mononuclear cells are members of the group consisting of T-lymphocytes, B-lymphocytes, monocytes, natural killer cells, and peripheral blood stem cells.
23. The method of claim 20, wherein the patient's cells are divided into two or more portions and each portion is tested on a different chemical entity.
24. The method of claim 20, wherein the chemical entity is a member of the group consisting of a registered chemical entity, a novel chemical entity, an environmental reagent, and a biological.
25. A method for predicting whether a patient diagnosed with a specific disease is likely to respond positively to a chemical entity, comprising:
- (i) creating a set of reference cellular responses to the chemical entity in peripheral blood mononuclear cells that are correlated with *in vivo* responses for a clinical indication in a population of subjects afflicted with the specific disease that have responded positively to the chemical entity;
  - (ii) capturing these reference cellular responses on a suitable assay;
  - (iii) drawing peripheral blood mononuclear cells from the patient;
  - (iv) detecting the cellular response of the patient's peripheral blood mononuclear cells to the chemical entity;
  - (v) identifying the cellular response in the reference set of step (i) that corresponds to the patient's cellular response of step (iv); and
  - (vi) determining the *in vivo* response which correlates with the cellular response in the reference set;
- wherein the patient having a cellular response that correlates with the cellular response in the reference set is predicted to be likely to respond positively to the chemical entity.
26. The method of claim 25, wherein the patient's cells are divided into two or more portions and each portion is tested on a different chemical entity.
27. The method of claim 25, wherein the cellular response is a member of the group consisting of gene expression increased motility, chemotaxis, contraction, relaxation, biosynthesis, secretion of signaling molecules, depolarization, repolarization,

degranulation, adhesion, aggregation, change in metabolic rate, and immediate cellular responses.

28. The method of claim 25, wherein peripheral blood mononuclear cells are members of the group consisting of T-lymphocytes, B-lymphocytes, monocytes, natural killer cells, and peripheral blood stem cells.
29. The method of claim 25, wherein step (iii) comprises:
  - (a) transducing the patient's peripheral blood mononuclear cells with a zinc finger protein that specifically expresses or upregulates the target for the chemical entity;
  - (b) exposing the transfected peripheral blood mononuclear cells to the chemical entity;
  - (c) performing mRNA extraction on the exposed peripheral blood mononuclear cells of step (b);
  - (d) constructing a cDNA library from the extracted RNA;
  - (e) performing a cDNA subtraction with another cDNA library; and
  - (f) detecting resultant cellular responses of the patient's peripheral blood mononuclear cells to the chemical entity.
30. The method of claim 25, wherein the suitable assay is a microarray.
31. The method of claim 25, wherein the suitable assay is microbeads.
32. The method of claim 25, wherein the positive response is a member of the group consisting of efficacy, lack of adverse effect, and safety.