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(54) Title: METHODS AND COMPOSITIONS FOR INFLUENCING THE TRANSPORT OF MOLECULAR SPECIES THROUGH BIOLOGICAL BARRIERS

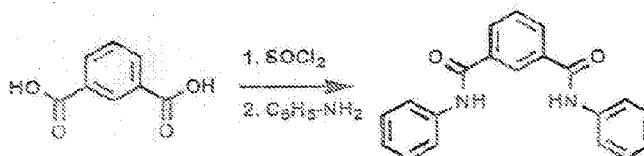


FIG. 1

(57) Abstract: A process is described for influencing the effectiveness of transport, transformation, and/or transfection of molecular species (such as nucleic acids, peptides and proteins, and charged compounds/pharmaceuticals) through a variety of membranes and/or other biological barriers into a variety of cellular systems by using anion complexing agents. The main anion complexing agents are anion binding agents having amidic hydrogen bond donor(s) such as found in bis(amide)s.

Methods and Compositions for Influencing the Transport of Molecular Species Through Biological Barriers

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. patent application serial number 61/741,001, filed July 9, 2012, the entire disclosure of which is incorporated herein by reference.

GRANT STATEMENT

[0002] This invention was made with Government support under CHE-0957535 awarded by the National Science Foundation. The Government has certain rights in the invention.

FIELD OF INVENTION

[0003] The present invention relates to the fields of complexation and transport of molecular species such as ionic species through biological barriers such as cell walls and membranes. Certain aspects of the invention are drawn to utilizing the interaction of ion binding agents with nucleic acids in such a way that transformation or transfection of cells is enhanced or repressed.

BACKGROUND OF INVENTION

[0004] It is known in the art that several methods are available to assist in the transport of nucleic acids through biological barriers such as cell membranes, cell walls, etc., so that the nucleic acids may influence the cell. For example, insertion of a DNA plasmid into a cell that remains viable may produce a gene product and alter the cell's phenotype and genotype.

[0005] It has now been more than three decades since diethylaminoethyl dextran (diethylaminoethyl cellulose; DEAE-dextran) was introduced as a transfection agent. In addition, a number of other transformation or transfection agents or methods are well-known. These include viral techniques such as those described in *Proceedings of the National Academy of Sciences U S A* **1972**, 69, 3423-3427 and *Journal of Biochemistry* **1972**, 71, 513-518; "poration" as described in *Journal of*

Photochemistry and Photobiology B **1996**, 36, 41-46; the use of cationic lipids as described in *Current Protocols in Immunology* **2001**, Chapter 10, Unit 10 16; the use of anionic lipids as described in the *Biophysical Journal* **2004**, 87, 1054-1064 and *Bioconjugate Chemistry* **2009**, 20, 1765-1772; and the use of various polymer systems, and other approaches as described in *Biophysical Journal* **2004**, 87, 1054-1064 and in *Bioconjugate Chemistry* **2009**, 20, 1765-1772.

[0006] The most common transformation or transfection chemical agents are calcium phosphate, DEAE-dextran, polyimines, cationic lipids, and cationic polymers as described in *Journal of Virology* **1967**, 1, 891-897. Cationic lipids such as Lipofectamine® are commercially available and broadly used, primarily in eukaryotic applications. The essential operational feature of each of these agents is that each permits the boundary barrier — bilayer membrane or cell wall or both — of a cell to be penetrated by DNA, although in many cases the mechanism or mechanisms by which this occurs remains obscure.

[0007] A broad description of gene transfer techniques is presented in the monograph edited by W. C. Heiser, titled *Gene Delivery to Mammalian Cells Volume 1: Nonviral Gene Transfer Techniques*; Humana Press: Totowa, NJ, 2004; Vol. 245. Another general reference may be found in the monograph co-edited by W. Bielke and W. Erbacher, titled *Nucleic Acid Transfection*; Springer Verlag: Heidelberg, 2010; Vol. 296.

[0008] It is reported in the *New Journal of Chemistry*, **2012**, 36, 1231-1245, that certain anthracene-labeled bispyridinium amides show interactions with DNA. This work is directed to the development of chemosensors.

[0009] Several methods are known in the art that describe processes for facilitating or enhancing transformation or transfection. For example, several related United States Patents (5,736,392; 6,051,429; 6,376,248 B1; and 8,058,068 B2) disclose compositions claimed to be useful for transfecting eukaryotic cells comprising nucleic acid complexes with peptides. In these patents, the peptides may be linked by covalent bonding to a nucleic acid-binding group and to cationic lipids or to dendrimers.

[0010] United States Patent 5,589,368 discloses a process for transfecting a mammalian cell culture which involves incubating a cell culture in the presence of a transfection medium that includes a growth medium that preferably includes fetal

bovine serum and a transfection medium that includes serum from a higher mammal. This transfection medium may also include a sterol.

[0011] United States Patent 5,633,156 discloses a method for transfection of eukaryotic cells in which calcium phosphate particles and a specified nucleic acid are grown to a desirable size and subsequently brought together under conditions appropriate to increase the host cell's exposure to optimally-sized particles.

[0012] United States Patent 6,881,577 B2 discloses a method for improving transfection efficiency by using peptides called K16-peptides. The patent discloses several peptide sequences that can be used in the claimed process.

[0013] United States Patent 6,733,777 B2 discloses the use of compounds called cationic cytofectins and liposomes. The compounds and their aggregates are said to be useful for both *in vivo* and *in vitro* delivery of exogenous compounds into cells. The liposomes may be formed from such amphiphiles as the common membrane monomer dioleoylphosphatidylethanolamine (DOPE) or similar related compounds. The cytofectins are also lipids and have the general structure $R^1R^2R^3N-(CH_2)_n-NR^4R^5R^6$. The patent provides both methods of delivery and transfection kits.

[0014] United States Patent 6,074,667 discloses a method for transfecting a cell with a nucleic acid in which the cell is contacted with a liposomal transfection composition. This composition comprises the nucleic acid, sphingosine or a sphingosine derivative that can be protonated in the sphingosine group, and an additional lipid referred to therein as a "helper lipid." United States Patent 7,732,420 is titled "Combinations of transfection lipids exhibiting increased transfection efficiencies."

[0015] Nonetheless, though many of the compounds or methods that have been devised or used for transporting DNA into a cell, almost all conventional compounds or methods have a harsh or damaging effect on the cell *per se* or on surrounding cells or require complex and expensive reagents to effect the transport. Thus, currently there is no effective method for transporting large size DNA plasmids (such as the DNA plasmids having 15 or more kilobases) into cellular systems.

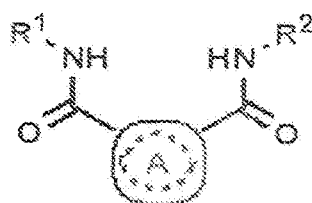
[0016] Furthermore, highly sensitive and difficult to maintain mammalian cell cultures pose a different challenge compared to other systems. Different mammalian cells vary in cell membrane composition and require special attention for growth and maintaining the cell culture (Singer, S.J.; Nicolson, G.L., The structure and chemistry of mammalian cell membrane; Amer. J. Pathol. 1971, 2, 427-439). The success of

gene delivery depends on the type of cell line and optimal growth conditions (Gibson, G.; Muse, S.V. *A Primer of Genome Science*; Sinauer Associates; Sunderland, MA, 2009, 370 pp.).

[0017] Therefore, there is a need to provide a method for assisting the transport, transformation, or transfection of nucleic acids or other large molecular species through membranes or into cell systems with increased efficiency. There is also a need to provide a method to assist the transport, transformation, or transfection of DNA plasmids having sizes of 15 kilobases or more through membranes or into cell systems.

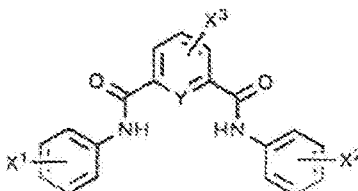
SUMMARY OF INVENTION

[0018] The present invention provides a method for assisting the transport of nucleic acids and / or other large molecules through membranes and / or into cellular systems in the presence of an anion complexing agent. Various compounds having structures that permit binding with anions such as phosphate could be employed as the aforesaid anion complexing agent. According to one embodiment of the invention, the aforesaid anion complexing agent may be, but is not limited to, a *bis*(amide) compound with a chemical structure illustrated schematically as Formula 1



Formula 1

[0019] More specifically, the aforesaid *bis*(amide) compound may have a chemical structure such as Formula 2

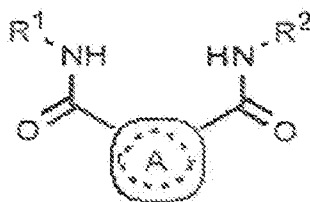


Formula 2

with unsubstituted or substituted aromatic groups in which X^1 and X^2 indicate the substitution patterns on the arenes, Y indicates the presence or absence of a heteroatom in the linker arene and the further substitution of the linker arene is indicated by X^3 . Most specifically, the aforesaid anion complexing agent may be substituted or unsubstituted dianilides of isophthalic acid and 2,6-dipicolinic acid.

[0020] The inventive method may be employed to assist the transport of various large molecules through membranes (or various biological barriers). Molecules being transported may include nucleic acids, peptides, proteins and / or proteins fragments, and charged compounds / pharmaceuticals. The aforesaid nucleic acids may include DNA plasmid, mRNA, siRNA, miRNA, shRNA, ssDNA or dsDNA and related species; while the aforesaid charged compounds / pharmaceuticals may include NTPs, NDPs, NMPs, AZT, and the like. The aforesaid cellular systems may further comprise various types of biological cells, such as bacterial cells, yeast cells, animal cells, and plant cells.

[0021] Certain embodiments of the invention are drawn to method for transporting a molecular species through a cell wall or cell membrane. Such methods first comprise combining a cellular system, a media conducive to maintaining the integrity of the cellular system, the molecular species, and an anion complexing agent, wherein the anion complexing agent is a *bis*(amide) compound that comprises a chemical structure of Formula 1.



Formula 1

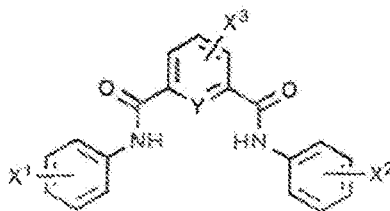
The methods then comprise incubating the molecular species and anion complexing agent in the media with the cellular system to allow for transport of the molecular species through the cell wall or cell membrane of the cellular system. In certain embodiments, the molecular species is transported through the cell wall or cell membrane into the cellular system. In certain embodiments, the molecular species is transported through the cell wall or cell membrane out of the cellular system.

[0022] In certain embodiments of the invention, the molecular species comprises a nucleic acid molecule. In certain embodiments, the molecular species comprises

DNA. In certain embodiments, the molecular species comprises a DNA plasmid, such as a DNA plasmid of up to about 50 kilobases in size, a DNA plasmid of up to about 25 kilobases in size, or a DNA plasmid of from about 5 kilobases to about 25 kilobases in size. In certain embodiments, the molecular species comprises a polypeptide. In certain embodiments, the molecular species comprises a charged compound.

[0023] In certain embodiments of the invention, the cellular system may be bacterial cells, yeast cells, animal cells, or plant cells. In certain embodiments of the invention wherein the cellular system comprises animal cells, the animal cells are mammalian cells.

[0024] In certain embodiments, the *bis*(amide) compound comprises a chemical structure of Formula 2:



Formula 2

[0025] In certain embodiments of the invention, the *bis*(amide) compound is a derivative of substituted or unsubstituted dianilides of either isophthalic acid or 2,6-dipicolinic acid. In certain embodiments of the invention wherein the anion complexing agent is a *bis*(amide) compound that comprises a chemical structure of Formula 1, A is benzene, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group. In certain embodiments of the invention, substituents are present at other positions within the structure or there may be more than one substituent present. In certain embodiments of the invention wherein the anion complexing agent is a *bis*(amide) compound that comprises a chemical structure of Formula 1, A is pyridine, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group. In certain embodiments of the invention wherein the anion complexing agent is a *bis*(amide) compound that comprises a chemical structure of Formula 1, (a) A is benzene, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy,

chloro, or cyano group and wherein the cellular system comprises bacterial cells; or (b) A is pyridine, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group and wherein the cellular system comprises bacterial cells. In certain embodiments of the invention wherein the anion complexing agent is a *bis*(amide) compound that comprises a chemical structure of Formula 1(a) A is benzene, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group and wherein the cellular system comprises yeast cells; or (b) A is pyridine, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group and wherein the cellular system comprises yeast cells.

[0026] In certain embodiments of the invention, the cellular system comprises yeast cells and the method does not comprise the addition of ssDNA. In certain embodiments of the invention, the anion complexing agent comprises the compound *N,N'*-*bis*-(4-methoxyphenyl)isophthalamide.

[0027] Certain embodiments of the invention are drawn to kits having reagents suitable for transporting a molecular species through a cell wall or cell membrane. Such kits may comprise two or more of the following: a cellular system as described elsewhere herein, a media conducive to maintaining the integrity of the cellular system, a molecular species as described elsewhere herein, and an anion complexing agent, wherein the anion complexing agent is a *bis*(amide) compound that comprises a chemical structure of Formula 1 and as described in more detail elsewhere herein.

[0028] In certain embodiments of the methods of transporting a molecular species through a cell wall or cell membrane described herein, such transport causes a phenotypic change to the cellular system. In certain embodiments, the cellular system is transformed or transfected.

BRIEF DESCRIPTION OF DRAWINGS

[0029] Figure 1. An exemplary synthesis scheme for the *bis*(amide)s compounds.

[0030] Figure 2. Transformation of competent *E. coli* cells using a ~2.6 kb DNA plasmid mediated by isophthalic acid dianilide derivatives having a hydrogen

substituent or substituents in the 4-position of the aniline including 4-methoxy, 4-chloro, 4-nitro, and 4-cyano.

[0031] Figure 3. Transformation of competent *E. coli* cells using a ~2.6 kb DNA plasmid mediated by 2,6-dipicnic acid dianilide derivatives having substituents in the 4-position of the aniline including 4-methoxy, 4-chloro, and 4-nitro.

[0032] Figure 4. Transformation efficiencies plot. CFU after *E. coli* was exposed to a 2 μ L aqueous solution containing 0.1 μ g $\cdot$ mL⁻¹ plasmid (pVIB) and 2 μ L isophthalic acid and dipicolinamide derivative and plated on ampicillin. Bar: 1, R = OCH₃, CFU = ~43; 2, R = H CFU = ~33; 5, R = OCH₃, CFU = ~48; 6, R = H, CFU = ~25; Control: 2 μ L DMSO, CFU = ~4; 2 μ L Milli-Q (18 M Ω) H₂O, CFU = ~3.

[0033] Figure 5. Transformation of *Saccharomyces cerevisiae* with a ~9.4 kb DNA plasmid in the absence of added single stranded (ss) DNA. Iso refers to isophthalic acid dianilides. Dipic refers to dianilides of 2,6-dipicnic acid. DH₂O refers to distilled Milli-Q water.

[0034] Figure 6. Transformation of *Saccharomyces cerevisiae* with a ~9.4 kb DNA plasmid in the presence of added single stranded (ss) DNA. Iso refers to isophthalic acid dianilides. Dipic refers to dianilides of 2,6-dipicnic acid. DH₂O refers to distilled Milli-Q water.

[0035] Figure 7. Comparison of transformation of *Saccharomyces cerevisiae* with a ~9.4 kb DNA plasmid in the presence and in the absence of added single stranded (ss) DNA. DH₂O refers to distilled Milli-Q water. Iso OCH₃ refers to the compound of Formula 1 in which A is benzene and R¹ and R² are 4-methoxyanilides. The overall structure is isophthalic acid 4,4'-dimethoxydianilide.

[0036] Figure 8. The structure of bromide anion complex of isophthalic acid dianilide. The structure was reported in the *Journal of the American Chemical Society* 1997, 119, 2325-2326. This structure is recorded in the Cambridge Structural Database (CSD) under the index code RINGOK.

[0037] Figure 9. Results of MTT Assay. X axis represents different ratios of TA:phosphate DNA and other controls. The Y axis shows the absorbance measured at $\lambda = 540$ nm.

[0038] Figure 10. Transfection efficiency of tris-arenes in HEK cells.

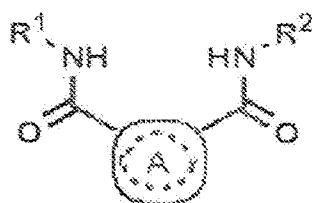
DETAILED DESCRIPTION OF THE INVENTION

[0039] The term phenotype, as used herein, refers to an organism's overall, observable qualities. These qualities can include, but are not limited to, appearance (morphology), developmental characteristics, and biochemical function. A phenotype results from a combination of genetic and environmental influences. In experiments such as those described herein, the phenotype referred to is typically due to a genetic alteration that is observed after new genetic material has been inserted into an organism.

[0040] Certain aspects of the invention provide for methods for enhancing the effectiveness of transport of molecular species (such as large molecular species including, but not limited to, nucleic acid molecules, peptides and proteins, and charged compounds/pharmaceuticals) through various biological barriers (such as, but not limited to, cells walls and cell membranes) into a variety of cellular systems by using anion complexing agents. It is understood that transport through or across biological barriers can occur in more than one direction. Thus, although transformation or transfection generally involves the transport of an exogenous nucleic acid molecule across a cell wall or membrane into the interior of a cellular system, transport could also occur in the opposite direction, i.e., from the interior through the cell wall or membrane to the exterior of the cellular system. Therefore, certain embodiments of the invention are drawn to methods wherein transport is through a biological membrane from the interior of a cellular system to its exterior. In certain embodiments, the molecular species is a nucleic acid molecule that comprises DNA, such as a linear or circular DNA plasmid. In certain embodiments, the transport results in transformation or transfection of the molecular species into a cellular system. Particularly, certain inventive methods for transporting molecular species through a biological barrier into a cellular system comprise treating said cellular system with media containing said molecular species and an anion complexing agent. In certain embodiments, this is accomplished by forming a combination that comprises the cellular system, a media conducive to maintaining the integrity of the cellular system, the molecular species, and an anion complexing agent, and then incubating the molecular species and anion complexing agent in the media with the cellular system to allow for transport of the molecular species across the cell wall or cell membrane of the cellular system. The combination can be formed by combining the components. The components may be combined by

adding each stepwise or adding two or more simultaneously, or adding all the components simultaneously. One of skill in the art will recognize the different options and orders of combining that may be used. For example, in one embodiment, the molecular species and anion complexing agent may be combined together as the cellular system is maintained separately in the media, and then the combined molecular species and anion complexing agent are added to the media containing the cellular system to combine all the components together so that they may be incubated to allow the molecular species to be transported across the cell wall or cell membrane.

[0041] According to one embodiment of the invention, the anion complexing agents may be a *bis*(amide) compound having the chemical structure of Formula 1:



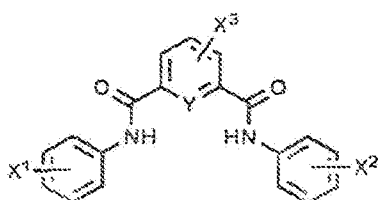
Formula 1

[0042] Such *bis*(amide) compounds of Formula 1 have not been suggested for the application of enhancing transport of molecular species, including large molecular species such as large DNA plasmids, into a cellular system. An example of anion complexation is found in isophthalamides, which was reported by Crabtree and coworkers in the *Journal of Organic Chemistry* **1999**, *64*, 1675-1683. These compounds may be prepared by methods known in the art and they are known to be complexing agents for such anions as Cl⁻ and Br⁻ and other common anions. Structural variants of N¹,N³-dimesitylisophthalamide are known in the art. Many of these compounds are known to bind an anion or an array of anions including F⁻, Cl⁻, Br⁻, CH₃COO⁻, and the like. A general review of anion binding and binders may be found in monographs such as *Anion Receptor Chemistry*; Royal Society of Chemistry: Cambridge, 2006, and *Anion Coordination Chemistry*; Wiley VCH: New York, 2012.

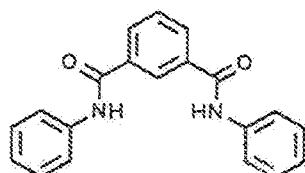
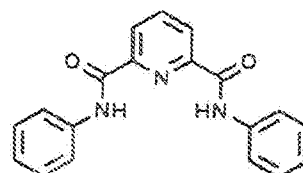
[0043] The meaning of the term *bis*(amide) in the context of the present invention is an organic chemical entity that has at least two amide NH bonds situated so that each NH can contact and facilitate binding to an anion. One or more NH groups may

be involved in binding the same anion. This is the plain and ordinary meaning of the term within the art. Beyond the plain and ordinary meaning, we define *bis*(amide) to include compounds in which one or more amide NH bonds may be present and equivalent structures are those having H-bond donors suitably situated to facilitate anion binding, even if they are not simple amides or diamides. Such would include carbamate and urea residues that have acidic H-bond donors, alcohols, and, for example, the acidic NH of pyrrole.

[0044] Two types of *bis*(amide)s are isophthalic acid dianilide and pyridine-2,6-dicarboxylic acid dianilide. These compounds are illustrative of certain embodiments of the present invention and examples of their function are included herein. The compounds labeled *N,N'*-diphenylisophthalamide and pyridine-2,6-dicarboxylic acid *bis*(phenylamide) are illustrative examples of the structure labeled Formula 2. The compound *N,N'*-diphenylisophthalamide is represented by Formula 1 in which $R^1 = R^2 = \text{phenyl}$ and A is 1,3-benzo. The same compound may be represented by Formula 2 in which $X^1 = X^2 = X^3 = \text{H}$ and Y = CH. Likewise, the compound pyridine-2,6-dicarboxylic acid *bis*(phenylamide) can be represented by Formula 1 in which $R^1 = R^2 = \text{phenyl}$ and A is 2,6-pyrido. The same compound can be represented by Formula 2 in which $X^1 = X^2 = X^3 = \text{H}$ and Y = N.



Formula 2

*N,N'*-DiphenylisophthalamidePyridine-2,6-dicarboxylic acid *bis*(phenylamide)

[0045] The substituents X^1 , X^2 , and X^3 may represent a variety of substituents and they may be the same or different. The substituents may be normal or branched alkyl having 1-5 carbons, aryl, aralkyl, halogen such as fluoro, chloro, bromo, or iodo, and such other substituents as normal or branched alkoxy, aryloxy, thioalkyl, thioaryl, ester, amide, carbamate, urea, cyano, nitro, and the like.

[0046] Illustrative, non-limiting examples representative of the aromatic residue / linker given by "A" in Formula 1 may be benzene, naphthalene, pyridine, furan, pyrrole, thiophene, or other 5- or 6- membered ring heterocycles and the like. The aromatic residue / linker given by "A" in Formula 1 may also be organic ring systems such as cyclobutane, cyclopentane, cyclohexane, cycloheptane, and the like, and

appropriately substituted derivatives thereof in which the carboxylic acid residues are situated so that the corresponding diamides are positioned to interact with an anion such as a phosphate group on DNA.

[0047] The synthesis of compounds such as *N,N'*-diphenylisophthalamide and pyridine-2,6-dicarboxylic acid *bis*(phenylamide) may readily be accomplished by methods known in the art. An example is to treat 1,3-dicarboxybenzene (isophthalic acid) with a chlorinating agent such as thionyl chloride (SOCl_2) to convert the diacid into the diacid chloride. Subsequent addition of the appropriate amine such as aniline is known to convert a diacyl chloride such as isophthaloyl chloride into the corresponding diamide, in this case a dianilide. Likewise, the reaction of pyridine-2,6-dicarboxylic acid with thionyl chloride followed by treatment with aniline will produce pyridine-2,6-dicarboxylic acid *bis*(phenylamide). A typical reaction is illustrated in Figure 1. It is meant to exemplify the synthetic approach and process but not to be in any way limiting.

[0048] The arenes that are part of the amide and are attached to nitrogen may be mono- di-, tri- tetra- or pentasubstituted. Thus, X^1 and X^2 may represent multiple substituents and they may represent the same or different substituents and combinations of substituents. In addition, X^1 and X^2 may represent additional carbocyclic substituents such as a fused saturated or unsaturated aliphatic 4-membered ring, a fused saturated or unsaturated aliphatic 5-membered ring, a fused saturated or unsaturated 6-membered ring and the like, or an aromatic or heteroaromatic ring.

[0049] Illustrative examples of a cellular system of the invention include, but are not limited to, bacterial cells, yeast cells, plant cells, and animal cells.

[0050] In one example of an embodiment of the invention, a plasmid delivered through the cell wall or cell membrane of a bacteria transforms the microbe by imparting to it resistance to the antibiotic ampicillin. In all experiments, the results were gauged by counting the number of colonies that grow on a medium containing ampicillin. Typically, growth will be strongly impeded by the presence of the antibiotic. The colonies that grow after the transformation experiment or the control experiment are counted and the data are shown on the ordinate axis of the graph as colony forming units or CFUs. The larger the number of CFUs observed compared to controls, the more effective the transformation agent is judged to be.

[0051] In another illustrative example of an embodiment of the invention, the yeast (*Saccharomyces cerevisiae*) strain used lacked the ability to synthesize endogenous amino acids uracil (*S. cerevisiae* uracil auxotroph). The delivery of a plasmid through the cell wall or cell membrane transformed the yeast and imparted to it the ability to synthesize uracil. Typically, growth of yeast cells will be strongly impeded due to the absence of uracil in the media. The colonies of yeast cells that grow after the transformation or control experiment are counted and the data are shown on the ordinate axis of the graph as colony forming units or CFUs. The larger the number of CFUs observed compared to controls, the more effective the transformation agent is judged to be.

[0052] In another illustrative example of an embodiment of the invention, mammalian cells (HEK-293) were transfected. A plasmid was delivered through the cell membrane, transfecting the cells by imparting to them resistance to the antibiotic neomycin (aminoglycoside). In all experiments, the results were gauged by measuring the absorbance of the cells that survive in the medium containing G418 (aminoglycoside). Typically, growth will be strongly impeded by the presence of the antibiotic. The cells that survive and grow after the transfection experiment or the control experiment are measured by determining the absorbance of viable cells (MTT assay) and the results were compared to number of cells originally used for the experiment. The data are shown on the ordinate axis of the graph as percentage of the cells transfected. The larger the percentage of cells transfected compared to controls, the more effective the transfection agent is judged to be.

[0053] Fetal Bovine Serum is a component of growth media generally required for the healthy growth of mammalian cells. Mammalian cell transfection utilizing previously known transfection reagents requires either, DNA to be treated with transfection reagent in fetal bovine serum (FBS) free media, or the cells to be prepared for transfection in a FBS free media. In certain embodiments, the method utilizing tris-arenes is conducted by mixing DNA and transfection reagent (tris-arene) without any media where the cells were grown in media containing FBS. Hence, there is no requirement of either preparing the cells or the DNA-reagent mixture in a 'Serum-Free' media, when using tris-arenes.

[0054] In another illustrative example of an embodiment of the invention, plant cells (*Arabidopsis thaliana*) used for the experiment lacked the ability to express the enhanced yellow fluorescent protein (EYFP). The plasmid delivered through the cell

wall or cell membrane transformed the plant cells by imparting to them the ability to synthesize EYFP. The plant cells that synthesize and express the EYFP after the transformation or control experiment are counted under the confocal microscope. The data are shown as number of cells expressing the EYFP. The larger the number of cells expressing the EYFP observed compared to controls, the more effective the transformation agent is judged to be.

[0055] In certain embodiments of the invention, Gram negative bacterium *Escherichia coli*, and the primitive eukaryote *Saccharomyces cerevisiae*, are transformed. In certain other embodiments, mammalian cells such as Human embryonic kidney cells (HEK 293) and plants such as *Arabidopsis thaliana* are transformed or transfected. Transfection of mammalian cell lines in particular is significant. Genetically modified mammalian cell cultures are used for production of various enzymes, hormones, proteins and vaccines that can be produced only by mammalian cells. Mammalian cell lines are used by biomedical researchers to conduct *in vitro* studies for biomedical/biotechnology research.

[0056] Transfection efficiency has been observed to be highest in bacterial and mammalian cells, when ratio of tris-arene molecule to the phosphates in the DNA backbone was at 1:1. Such dependence of transfection efficiency on the ratio of tris-arene molecule to the phosphates in DNA backbone has not been seen with the previously known transfection reagents.

[0057] Illustrative studies were done initially with competent JM 109 (Promega) *E. coli* cells. Generally similar results were obtained when wild type *E. coli* was used that had been treated by standard protocols to convert them into competent cells. The experiments were done by using a ~2.6 kilobase (kb) DNA plasmid in the presence and absence of anionic compounds.

[0058] Figure 2 shows the results of the transformation of competent *E. coli* cells using a ~2.6 kb DNA plasmid mediated by isophthalic acid dianilide derivatives. The *bis*(amide) compounds have the structure of Formula 1 in which A is benzene (an isophthalic acid derivative) R¹ and R² are identical aniline derivatives, unsubstituted in the 4-positions or substituted in the 4-positions by methoxy, chloro, or cyano groups. The compounds represented in Figure 2 are all isophthalic acid dianilides. In this study, when the substituents were both chloro, no enhancement was observed in transformation compared to controls. Other substituents are contemplated in any of the aromatic rings and in any location within the structure that

does not prohibit the interactions described hereinabove. Such additional substituents include, but are not limited to, the types of functional groups illustrated and exemplified as well as linear, branched, or cyclic alkyl groups, aralkyl groups, aromatic and heteroaromatic groups and the like.

[0059] The 4-cyano-substituted compound showed a transformation efficacy less than controls. In certain cases, compounds of the instant invention may have an effect such that the apparent transformation or transfection results are less than that observed for controls although transport through the cell wall or membrane is increased over controls. Compounds of the instant invention that have this effect are considered to be within its scope. *Tris*-arenes having substituents such as CN and NO₂ have been found to prevent the transformation of bacterial plasmid DNA that encodes antibiotic resistance. Such an activity profile would also prevent the transformation of plasmid DNA encoding virulence factors. Bacteria can transfer (horizontal gene transfer) (plasmid) genes that contain antimicrobial resistance or virulence factors between the same species or among different species. Bacteria can acquire plasmid DNA from the surrounding environment that encodes for antibacterial resistance or virulence factors. Horizontal gene transfer, often conducted via plasmid, is the primary reason for bacterial antibiotic resistance and the emergence of new, virulent strains of bacteria. *Tris*-arenes having CN and NO₂ substituents inhibit/prevent the horizontal gene transfer of plasmid DNA that contains bacterial resistant genes.

[0060] The present invention is further demonstrated by the use of 2,6-dipicolinic acid diamides. Figure 3 illustrates the results of the transformation of competent *E. coli* cells using a ~2.6 kb DNA plasmid mediated by 2,6-dipicolinic acid dianilide derivatives. In this case, A in Formula 1 is pyridine and R¹ and R² are identical aniline derivatives that are unsubstituted or substituted in the 4-positions by methoxy, chloro, or cyano. Transformation of *E. coli* using a ~2.6 kb DNA plasmid and the indicated *bis*(amide)s is demonstrated by the enhancements when the *bis*(amide) is unsubstituted or substituted by 4-methoxy. The dipicolinanilide having 4-chloro or cyano substituents were not effective transformation agents. However, when such substituents are positioned in *bis*(amide)s in such a way that transformation is more effective than in their absence, the compounds will fall within the scope of the invention.

[0061] Figure 4 shows transformation efficiencies plot. A further surprising and unexpected aspect of the present invention is demonstrated by the transformation efficiencies plot. In these replicated experiments, *E. coli* was transformed using the *bis*(amide) compounds of the present invention and a DNA plasmid of up to about 25 kb. This is a size generally regarded as too large to be used in a transformation experiment such as this. The data of Figure 4 show that both isophthalic acid dianilides and dipicolinanilides are not only effective transformation agents, they succeed where other methodologies are limited. For both the isophthalic acid and dipicolinic acid anilides, the unsubstituted and 4-methoxy-substituted *bis*(amide) compounds of Formula 1 are highly effective compared to controls.

[0062] Figure 5 and Figure 6 show representative results of transformations of *Saccharomyces cerevisiae* in the absence of added single stranded (ss) DNA. Still another surprising and unexpected result of the present invention is demonstrated by the experiments whose results are disclosed in the graphs of Figures 5 and 6. In the experiments that used the yeast *S. cerevisiae*, transformation of a ~9.4 kb DNA plasmid was attempted by using the compounds of Formula 1 in the presence (Figure 5) and absence (Figure 6) of single strand DNA (ssDNA). Single stranded DNA is typically required for the successful transformation of yeast cells. The present results show that both unsubstituted and 4-methoxy-substituted isophthalic acid dianilide and 2,6- dipicolinic acid dianilide show dramatically increased transformation compared to control experiments in which no compound of Formula 1 was present.

[0063] Figure 7 is a graph confirming the surprising efficacy of the compounds of Formula 1 as transformation agents for yeast. The compound *N,N'*-*bis*-(4-methoxyphenyl)isophthalamide was absent (control) or used as the transformation agent in the presence or absence of single stranded DNA. In the presence of both *N,N'*-*bis*-(4-methoxyphenyl)isophthalamide and ssDNA, transformation was enhanced by 11-fold over controls. In the presence of *N,N'*-*bis*-(4-methoxyphenyl)isophthalamide but with ssDNA absent, transformation was enhanced by ~9-fold over the experiment in which ssDNA was present and ~100-fold over controls when ssDNA was present.

[0064] Figure 9 is a graph representing the results of viability test (MTT Assay) of HEK-293 cells following transfection with tris-arene or controls. In this experiment, transfection of a ~5.08 kb DNA plasmid in to HEK-293 cells was attempted in the

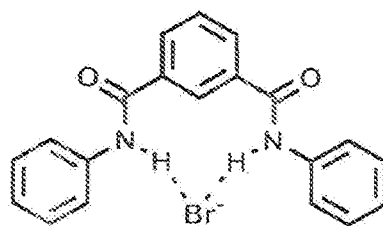
presence or absence of the compound *N,N'*-bis-(4 methoxyphenyl)isophthalamide. HEK-293 cells that were not treated for transfection were used as positive control to determine the absorbance of the number of cells in each well (5×10^4 cells/well). The x-axis represents the experiment performed at different ratios of tris-arene molecules to phosphates in DNA backbone and the controls. The y-axis represents the average absorbance of the viable cells measured at $\lambda = 540$ nm. Table 1 and 2 represent the set-up of 96-well plate for tris-arene mediated transfection of HEK-293 cells and the results of the MTT assay following the transfection procedure, respectively. The present results in figure 9, show that 4-methoxy-substituted isophthalic acid dianilide show dramatically increased survival compared to the negative control experiments in which no compound of Formula 1 was present. Higher survival of successfully transfected HEK-293 cells represents the ability of compound to transport DNA in to the cells.

[0065] Figure 10 is a graph representing the results of transfection efficiency in form of percentage of cells transfected, for HEK-293 cells following transfection with tris-arenes or controls. The average absorbance (figure 9) of three replicates was used to determine the transfection efficiency. Comparing the amount of cells containing the 5.08 kb plasmid DNA after conducting transfection, to the amount of cells present in each well provided the percentage of cells that were successfully transfected *i.e.* transfection efficiency. The x-axis represents the experiment performed at different ratios of tris-arene molecules to phosphates in the DNA backbone and the controls. The y-axis represents the transfection efficiency in the form of percentage of cells transfected. The data of transfection efficiency and fold enhancement is represented in Table 3. These results confirm the ability of tris-arenes to successfully and efficiently transfect HEK 293 mammalian cells.

[0066] The invention suggests several possible mechanisms that the *bis*(amide) compounds of Formula 1 may enhance transformation of large molecules (such as nucleic acids, peptides and proteins, and charged compounds/pharmaceuticals) through biological barriers into cellular systems. However, the description of any possible mechanism is not meant to limit in any way the exemplified function of the present invention.

[0067] Without being limited by theory, one of the mechanisms involves the formation of a complex, such as the one represented as Formula 3 in which Br^- is the bound anion. Anion binding by these compounds involves coordination of the

amide NH bonds to the anion. The amide NH group(s) typically hydrogen bond to the anion, which forms a complex of a type known in the art and shown in the structure of Formula 3.



Formula 3

[0068] Figure 8 illustrates the structure of Formula 3, *N,N'*-diphenylisophthalamide complexing a bromide anion. This structure is recorded in the Cambridge Structural Database (CSD) under the index RINGOK. The structure was reported in the *Journal of the American Chemical Society* **1997**, *119*, 2325-2326. In principle, these compounds should complex phosphate ions in the same way.

[0069] Isophthalanilides and picolinanilides are known to complex and transport such anions as Cl⁻ as described in *Chemical Communications* **2010**, 2838-2840 and phosphate anion as noted above. The complexation of Cl⁻ by related molecules is well known but we discovered that channel-like behavior (pore formation, membrane leakage) occurred for at least one of the dianilides in asolectin membranes as determined by planar bilayer conductance experiments. Membrane activity of isophthalide compounds is known in the art as shown by *Langmuir* **2001**, *17*, 6669-6674. We interpreted the pore formation in terms of a multilayer, face-to-face stack of the *bis*(amide)s upon each other. Without being limited by theory, a possible mechanism for transformation is the formation of a pore in a boundary membrane that permits transport of a DNA plasmid.

[0070] Again without being bound by theory, an alternate possible mechanism involves complexation and transport. The thickness of an aromatic ring, such as those found in *N,N'*-diphenylisophthalamide, is approximately 3.4 Å. Although this is similar to the spacing of the aromatic bases in DNA, the phosphate groups in the backbone of DNA are separated by about twice that distance. In the structure reported for B-DNA (Protein Data Bank 1BNA.pdb), the phosphate spacing varies but it is generally between 6-7 Å (as measured from the crystal structure noted above). A possible explanation for the enhanced membrane penetration is that the

bis(amide)s complex and surround the DNA plasmid, stack one upon the other, and compress the plasmid. Complexation of the phosphates and close stacking of the *bis*(amide)s would make the overall DNA-*bis*(amide) complex lipophilic and might lead to a compressed and / or coiled structure more able to diffuse through the bilayer. Presumably, the stacking of the *bis*(amide)s would be driven by exclusion of water and any favorable van der Waals interactions. Alternately, the *bis*(amide)s might simply complex the individual phosphate residues and reduce their polarity.

[0071] To the extent that compression occurred, the plasmid should become a smaller structure more amenable to diffuse through the bilayer. The compression of DNA envisioned is reminiscent of histone protein interactions as exemplified in the structure shown in *Nature* **1997**, 389, 251-260. The formation of a DNA-conducting pore or the ability to complex and reduce the polarity would be of potential utility either as a transfection agent for mammalian cells or as a transformation agent for bacterial cells. The invention further suggests that certain of the transformation and/or transfection agents enhance plasmid transport through cellular membranes and certain other compounds of the same class but differently substituted suppress gene transfer.

[0072] The following disclosed examples are merely representative of the invention which may be embodied in various forms. Thus, specific structural, functional, and procedural details disclosed in the following examples are not to be interpreted as limiting:

EXAMPLE 1

[0073] Preparation of *N',N'*-bis(4-chlorophenyl)isophthalamide. Commercially available isophthaloyl dichloride (0.35 g, 2.6 mmol, 1 equivalent) was dissolved in CH₃CN (20 mL) and was added dropwise to a stirring solution of 4-chloroaniline (1.00 g, 7.8 mmol, 3 equivalents) and triethylamine (4.4 mL, 31.3 mmol, 12 equivalents) in acetonitrile (25 mL additional for 45 mL total) held at 0 °C (ice bath). A precipitate appeared upon addition of isophthaloyl dichloride to the reaction mixture. After the addition was complete, the reaction mixture was allowed to warm to ambient temperature and stirring was continued for 24 h under an argon atmosphere. The flask and the solution within it were then cooled to ~0 °C (freezer) and held there for 4 h. The solid thus obtained was isolated by vacuum filtration, washed with cold HPLC grade CH₃CN (3 x 20 mL) and then with cold Et₂O (10 mL). The resulting solid was dried *in vacuo* for 24 h. The resulting colorless solid (0.93 g, 93%) had mp 275-278 °C). ¹H-NMR (DMSO-d₆): 7.43 (4H, d, *J* = 7.0 Hz, Anil 3/5-*H*), 7.70 (1H, t, *J* = 7.8 Hz, Isophth 5-*H*), 7.90 (4H, d, *J* = 7.9 Hz, anil 3/5-*H*), 8.14 (2H, d, *J* = 3.9 Hz, isophth 4/6-*H*), 8.63 (1H, s, isophth 2-*H*), 10.7 (2H, s, C(O)NH-anil). ¹³C-NMR (DMSO-d₆): 121.84, 126.97, 127.38, 128.53, 128.75, 130.98, 134.70, 138.13, 165.04.

EXAMPLE 2

[0074] Determination of an anion complexation constant. Anion complexation constants were determined for compounds of the present invention by a variety of methods. One such method was by use of nuclear magnetic resonance (NMR). Typically, a solution of a molecule of formula 1 was dissolved in a suitable solvent such as CDCl₃, CD₂Cl₂, CD₃SOCD₃, and the like. Solutions were prepared in the concentration range commensurate with their solubility in the chosen solvent, for example, 0.30-4.30 mM. One milliliter (mL) of this solution was transferred to standard a 5 mm diameter NMR tube and titrated with 12 - 170 mM of an appropriate salt. Using such a procedure, *N,N'*-bis-(4-nitrophenyl)isophthalamide was titrated in CH₃SOCD₃ solution with tetrabutylammonium chloride. The signals assigned to the NH protons were monitored as a function of the anion concentration to the point at which the chemical shift change reached saturation. The association constant was then calculated from the data by methods known in the art and found to be 42 ± 6.

EXAMPLE 3

[0075] Use of a bis(amide) in *E. coli* transformation. Solutions (1 mM) in DMSO of each of the compounds of Formula 1 were prepared. Control plasmid DNA (~2.6 kB, concentration = 0.1 µg/ mL), which contains an Ampicillin-resistance gene, was obtained in a buffer solution from Promega and charged into five polypropylene microcentrifuge tubes (1.5 mL, chilled on ice bath). Each tube contained 2 µL each of compound solution and DNA plasmid in buffer. Each mixture was allowed to stand at ambient temperature for 10 minutes. Competent JM-109 cells (50 µL, from the 1.5 mL polypropylene microcentrifuge tube commercial sample, Promega), thawed immediately prior to addition and gently agitated to re-suspend, were added to individually labeled tubes (chilled on ice) and 2 µL of the compound and plasmid mixture was added to the competent cells. The competent cell, compound, and plasmid mixtures were gently agitated to mix the sample. Sample was immediately returned to ice for 10 minutes. After 10 minutes, the sample was removed from the ice bath and placed in a 42 °C water bath for 45 sec. Samples were removed from 42 °C water bath and immediately placed in ice for 2 minutes. Samples were removed from ice and 950 µL of (4 °C) Super Optimal Broth with catabolite repressor (SOC) medium (Sigma-Aldrich) was added to each culture. Samples were placed in a 37 °C shaker incubator (180 revolutions per minute) for 60 minutes. Samples were removed from shaker incubator and 100 µL of sample was transferred with a micropipette to a Luria-Bertani (LB) medium Agar plate containing Ampicillin (Sigma-Aldrich, 50 mg/ mL or 143.1 mM). To carry out the selection of transformed cells, the 100 µL (1x) of the incubated SOC media containing the transformed cells were spread on to the LB Agar Ampicillin plate using a sterilized glass rod. The remaining sample in the 1.5 mL polypropylene microcentrifuge tube was centrifuged at 15,000 rpm for 1 minute. A small pellet was formed at the bottom of the tube. Supernatant (800 µL) was removed from the tube (with a micropipette) without disturbing the pellet, leaving ~100 µL of the supernatant and the pellet in the 1.5 mL microcentrifuge tube. The pellet was re-suspended in the 100 µL of remaining supernatant by gently agitating the tube. The remaining 100 µL of the sample is transferred (with micropipette) onto another appropriately labeled (10X cell concentration) LB-Agar Ampicillin plate. Cells were spread onto the LB Agar Ampicillin plate using a sterilized glass rod. LB-Agar Ampicillin plates were placed upside down in a 37 °C for 12-14 hours. Colonies were counted using a stereo

microscope. The results of the *E. coli* transformation experiments are shown in Figure 2 and 3.

EXAMPLE 4

[0076] Experimental procedure for yeast transformation with ssDNA and tris-arene: *Saccharomyces cerevisiae* URA3⁻ was used in this experiment. It is a yeast strain that lacks the URA3 gene that encodes orotidine 5-phosphate decarboxylase (ODCase) which is involved in the synthesis of uracil in yeast. *S. cerevisiae* which lacks the URA3 gene cannot grow on media lacking uracil. *S. cerevisiae* URA3⁻ was inoculated into a 15 mL test tube containing 5 mL YEPD (yeast extract peptone dextrose). The sample was placed into a 30 °C shaker incubator for 12-16 hours. The sample was removed from the incubator and a culture ~5 mL sample was transferred into a 250 mL flask containing 50 mL YEPD. The sample used should show an optical density (OD) of ~ 0.15. The culture was placed in a 30 °C shaker incubator until the OD reached 0.67. This allowed the cells to grow through two divisions, or doubling. The culture was removed from the incubator and transferred into a 50 mL centrifuge tube. The sample was centrifuged at 4000 RPM for 2 minutes. The supernatant was poured off. The cells were resuspended with 20 mL of Milli-Q (18 MΩ) H₂O. The sample was centrifuged at 4000 RPM for 2 minutes and supernatant was removed. Cells were resuspended in 1 mL of 100 mM LiOAc and transferred to a 1.5 mL tube. The 1.5 mL tube was centrifuged at 11,000 RPM for 15 seconds, supernatant (LiOAc) was removed. The sample was removed from the centrifuge tube and the cells were resuspended to a final volume of 500 µL (~400 µL of 100 mM LiOAc). Single stranded DNA (ssDNA) was placed in a hot water bath (100 °C) for 5 minutes, and then placed on ice. The cell suspension was vortexed and 50 µL aliquots were transferred into individual 1.5 mL tubes (10 tubes total). The test tubes were centrifuge at 11,000 RPM for 15 seconds. LiOAc supernatant was removed by pipette transfer. 240 µL of 50% w/v polyethylene glycol PEG 3500 was added to each of the test tubes containing cell samples. 36 µL of 1 M LiOAc was added to each test tube. 25 µL of single strand DNA (2 mg/mL) was added to each test tube. 57 µL of Milli-Q (18 MΩ) H₂O was added to each test tube. 2 µL of plasmid DNA 500 ng/ µL (or water for control) was added to each test tube. The test tube samples were vortexed vigorously until the cell pellet was completely dispersed. The samples were placed into a 30 °C incubator for 30 minutes. Samples were

removed from the incubator and placed into a 42 °C water bath for 20 minutes (heat shock). Samples were removed from 42 °C water and centrifuged at 6000 RPM for 15 seconds. Supernatant was removed by pipette. 200 µL of Milli-Q (18 MΩ) H₂O was transferred into each tube and resuspended by pipetting. The ~200 µL of cell solution was transferred onto individual plates lacking uracil (uracil minus plates). The sample was evenly distributed across the plate with a glass rod (spread plating). Plates were held for 2-3 days at 30 °C in an incubator without shaking. Plates were removed and colonies were counted.

[0077] Tris-arenes were observed to be non-toxic and non-mutagenic to bacterial cells.

EXAMPLE 5

[0078] Experimental procedure for yeast transformation with tris-arene without ssDNA: *Saccharomyces cerevisiae* (a yeast strain that lacks *URA3* a gene that encodes orotidine 5-phosphate decarboxylase (ODCase) which is involved in the synthesis of uracil in yeast. *S. cerevisiae* which lacks the *URA3* gene cannot grow on media lacking uracil. *S. cerevisiae* *URA3*⁻ was inoculated into a 15 mL test tube containing 5 mL YEPD (yeast extract peptone dextrose). The sample was placed into a 30 °C shaker incubator for 12-16 hours. The sample was removed from the incubator and a culture sample —5 mL was transferred into a 250 mL flask containing 50 mL YEPD. The sample to be used will show an optical density (OD) of ~ 0.15. The culture was placed into a shaker incubator held at 30 °C until the OD reached 0.67. This corresponds to cell growth of two divisions or doubling. The culture was removed from the incubator and transferred into a 50 mL centrifuge tube. The sample was centrifuged at 4000 RPM for 2 minutes. The supernatant was poured off. The cells were resuspended in 20 mL of Milli-Q (18 MΩ) H₂O. The sample was centrifuged at 4000 RPM for 2 minutes and supernatant was removed. Cells were resuspended in 1 mL of 100 mM lithium acetate (LiOAc) and transferred to a 1.5 mL tube. The 1.5mL tube was centrifuged at 11,000 RPM for 15 seconds, supernatant (LiOAc) was removed. The supernatant was removed and the cells were resuspended to a final volume of 500 µL (~400 µL of 100 mM LiOAc). The cell suspension was vortexed and 50 µL aliquots were transferred into individual 1.5 mL tubes (10 tubes total). The test tubes were centrifuged at 11,000 RPM for 15 seconds. LiOAc supernatant was removed with pipette. Ten Dnase/RNase free

microcentrifuge test tubes were individually labeled and 2 μ L tris-arene and 2 μ L, plasmid DNA 500 ng/ μ L were transferred into each tube. This solution was allowed to react at ambient temperature for 10 minutes. 240 μ L of 50% w/v polyethylene glycol PEG n3500 was added to each of the test tubes containing cell samples. 36 μ L of 1 M LiOAc was added to each test tube. Milli-Q (18 M Ω) H₂O (80 μ L) was added to each test tube. Plasmid DNA 4 μ L of 500 ng/ μ L and tris-arene mixture (that was allowed to incubate for 10 minutes) was transferred into each test tube. The samples in the test tubes were vortexed vigorously until the cell pellet was completely dispersed. The samples were placed into a 30 °C incubator for 30 minutes. Samples were removed from the incubator and placed into a 42 °C water bath for 20 minutes (heat shock). Samples were removed from 42 °C water and centrifuged at 6000 RPM for 15 seconds. Supernatant was removed by pipette transfer. Milli-Q (18 M Ω) H₂O (200 μ L) was transferred into each tube and resuspended by pipetting. The ~200 μ L of cell solution was transferred onto individual plates lacking uracil (uracil minus plates). The sample was evenly distributed across the plate with a glass rod (spread plating). Plates are incubated for 2-3 days at 30 °C incubator without shaking. Plates were removed and the colonies were counted.

[0079] Tris-arenes were observed to be non-toxic and non-mutagenic to yeast cells.

EXAMPLE 6

[0080] Transfection of human embryonic kidney (HEK-293) cells with Tirs(arenes) (TAs). TAs have been shown and used to enhance transformation of DNA in to prokaryotic (*E. coli*) and primary eukaryotic (*S. cerevisiae*) organisms. Here we extend our studies to mammalian cells, which are derived from multi-cellular organisms. We have tested whether TA compounds enhance transport of nucleic acids, specifically DNA, across a different type of membrane system — the mammalian plasma membrane.

[0081] A modified version of the calcium phosphate mediated (Chen, C.; Okayama, H., High-efficiency transformation of mammalian cells by plasmid DNA, *Mol. Cell. Biol.* 1987, 7, 2745-2752) or Lipofectamine™ transfection (Dalby, B.; Cates, S.; Harris, A.; Ohki, E.C.; Tilkins, M.L.; Price, P.J.; Ciccarone, V.C., Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. *Methods*, 2004, 33, 95-103) protocol was used to

determine the ability of Tas, compound *N,N'*-bis(4 methoxyphenyl)isophthalamide in particular, to transfer DNA into human embryonic kidney (HEK-293) cells.

[0082] A wide range of mammalian cells, including HEK 293 cells, are sensitive to G418 (aminoglycoside) between the concentration of 400 µg/ mL – 1 mg/mL. pcDNA3.1(-) (5088 base pairs) plasmid used in the transfection protocol confers upon the cells neomycin (aminoglycoside) resistance (Southern, P.J.; Berg, P., Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter, J. Mol. Appl. Gene. 1982, 1, 327-341). HEK-293 cells that are successfully transfected with pcDNA3.1(-) will be G418 resistant and hence can be selected in the presence of G418.

[0083] The MTT assay can be used to determine the surviving cells after treating the transfected cells with G418 antibiotic (Malich, G.; Markovic, B.; Winder, C., The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines. Toxicology, 1997, 124, 179-192). MTT is a yellow colored tetrazolium ion that is reduced to purple formazan by living cells. Purple formazan can be spectrophotometrically measured for the absorbance at a wavelength of 540 nm. A higher number of cells will result in a higher absorbance for formazan. Cells that are not transfected with DNA or treated with TAs, can be used as a positive control for cell survival. Cells treated with DNA only and selected by G418, can be used as a negative control. Toxicity of TAs and G418 to HEK 293 cells can also be determined. Different ratios of TA molecules to phosphates in the DNA backbone can be used to conduct transfection.

Procedure:

[0084] Culturing and plating HEK293 cells: Growth medium DMEM with high glucose (ATCC), 10% FBS (Sigma-Aldrich) and 10µg/ mL of blasticidin (Thermo-Fischer) was prepared. HEK 293 cells were sub-cultured or thawed out from cryo-preserved samples in 10 mL growth media, centrifuged at 500 rpm for 10 minutes to remove preservative. The cells were then resuspended in fresh growth medium and cultured using a T-75 flask (Thermo-Fischer) at 37 °C and 5% CO₂. Cells were monitored for confluence and growth medium was replaced every 48 h, until cells were placed onto a 96-well plate for transfection.

[0085] The HEK-293 cells were cultured and grown to 80-90% confluence. The spent medium was discarded using a pipette. Cells were rinsed with 2 mL of

phosphate buffered saline (ATCC). Trypsin-EDTA solution (2-3 mL of 0.25% (w/v)) was added to the flask and incubated at 37 °C for 5 minutes in the presence of 5% CO₂. The volume adjusted to 10 mL with growth media. The flask wall was repeatedly washed with the media to remove the maximum adherent cells into the solution. The trypsinized cells were spun at 500 rpm for 10 minutes, supernatant was discarded and 10 mL of media (DMEM + FBS, no antibiotics) was added. Cells were counted using a Nexcelom Cellometer AutoT4 and diluted to achieve a final cell concentration of 5×10^5 cells/mL. The cell concentration of 5×10^5 cells/mL was used to prepare a 96 well plate at 100 μ L/well to get final concentration of 5×10^4 cells/well. The plated cells were incubated for 24 hours at 37°C in presence of 5% CO₂ to obtain 90-95% confluence.

[0086] Transfection of HEK 293 cells: The media in 96-well plate was replaced with Transfection Media (DMEM + FBS, no antibiotic). Plasmid DNA (pcDNA3.1) was prepared in sterile 1.5 mL micro-centrifuge tube to obtain concentration of 500ng/ μ L. A tria-arene solution was prepared in sterile 1.5 mL micro-centrifuge tube according to the desired ratios of tris-arene:phosphate in DNA backbone (50:1 to 1:10). In a separate sterile 1.5 mL micro-centrifuge tube, mix 6 μ L of appropriate concentrations of DNA (100 ng/ μ L of pcDNA3.1(-)) and 6 μ L of appropriate concentrations of TA to get a final volume of 12 μ L. The mixture was reacted for 10 minutes at room temperature. 10 μ L of the DNA +Tris arene mixture was added to the HEK-293 cells in the 96 well plate. No DNA was added to the cells only control, no DNA was added for the TA control and no TA was added for DNA only control. The treated 96 well plate was then incubated for 24 h at 37 °C in presence of 5% CO₂. The spent media was replaced with 100 μ L of the transfection assay media (DMEM + FBS + G418). The G418 was used at 750 μ g/ mL concentration and incubated for 24 hours at 37 °C in the presence of 5% CO₂. The MTT assay was conducted to determine the cells that were transfected with pcDNA3.1 and resistant to G418.

[0087] MTT assay: The MTT solution was made by adding 3 mL PBS to dry MTT solution powder (Sigma-Aldrich) to get a final concentration of 5 mg/mL. The spent media was discarded and the cells were washed with an equal amount of PBS. Equal amount of fresh PBS was added to each well, *i.e.* 100 μ L. MTT solution (10 μ L, 10% of original culture volume) was added to the cells to get the final concentration of MTT/well to 500 μ g/mL. The 96-well plate was returned to the incubator for 3-4 h. After the incubation period, the resulting MTT formazan crystals were dissolved

using 100 μ L of MTT solvent (Sigma-Aldrich). The absorbance was measured at a wavelength of 540 nm using SpectraMax340 micro plate reader. The percentage of cells transfected (or transfection efficiency) was calculated using the following equation.

$$\text{Percent of viable cells transfected} = \frac{\text{Absorbance of transfected cells} \times 100}{\text{Absorbance of cell only control}}$$

[0088] Results: The ability of TAs to transfer 500 ng of pcDNA3.1(-) into HEK 293 was tested. DNA was reacted or mixed with TAs for 10 min before adding to the cells. TAs were used at different equivalents of phosphate in the DNA backbone. The TA to phosphate DNA ratios used were 1:10, 1:1, 10:3 (1mM), 20:3 (2mM), 10:1 and 25:1. Cells alone were used as a control to determine the amount of cells in the well. The DNA only control did not contain any TA, and was used as a negative control. TA alone was also used to determine the toxicity of the compound on the HEK-293 cells. The toxicity of TA with respect to these cells was tested at a maximum concentration of 2 mM, whereas no cells were exposed to a concentration higher than 43 μ M TA. The layout of the 96-well plate is illustrated schematically in Table 1 below.

Table 1: Set up of 96-well plate for tris-arene mediated transfection of HEK 293 cells

	1	2	Tris-arene 3	Replicates 1	Replicates 2	Replicates 3
A	Cells	Cells	0.05 mM	1:10 TA:P	1:10 TA:P	1:10 TA:P
B	Cells	Cells	0.1 mM	1:1 TA:P	1:1 TA:P	1:1 TA:P
C	Cells	Cells	0.25 mM	10:1 TA:P	10:1 TA:P	10:1 TA:P
D	Cells	Cells	0.5 mM	25:1 TA:P	25:1 TA:P	25:1 TA:P
E	Cells	Cells	0.75 mM	10:3(1 mM)	10:3(1 mM)	10:3(1 mM)
F	Cells	Cells	1 mM	20:3(2 mM)	20:3(2 mM)	20:3(2 mM)
G	Cells	Cells	1 mM	DNA only	DNA only	DNA only
H	Cells	Cells	2 mM	DNA only	DNA only	DNA only

TA:P = TA : Phosphate in DNA backbone

[0089] Table 2 below represents the absorbance of dissolved formazan crystals produced by living cells. A higher absorbance represents a greater number of viable cells. All the wells in the plate initially had an equal number of cells (5×10^4 cells/well). Columns 1 and 2 represent the cells that were not transfected but used as positive control. All the wells (except A1) in columns 1 and 2 had absorbance values between 0.5 to 0.8. The average absorbance of the viable cells was found to be 0.59. This indicated that the cells were optimally grown. Column 3 determines the toxicity of TAs to the HEK-293 cells. Well labeled E3, F3, G3, and H3 exhibits dramatic decrease in the absorbance, indicating that concentrations of TAs at 750 μ M or higher are toxic to these cells. However, the concentration of TAs used for transfection never exceeded 43 μ M.

[0090] Columns 4, 5, and 6 represent the results of transfection and the DNA only control. Moving down the column, the ratio of TA to phosphate changes as follows 1:10, 1:1, 10:1, 25:1, 1 mM (10:3) and 2 mM (20:3). Last two rows contain DNA and no TA. The MTT assay conducted on transfected cells after selection with G418 antibiotic resulted in absorbance values between 0.4 and 0.6. The average absorbance for three replicates of TA:phosphate ratio of 1:10 was 0.49, 1:1 was 0.55, 10:1 was 0.38, 25:1 was 0.36, 10:3 (1 mM) it was 0.24 and at 20:3 (2 mM) it was 0.29. The negative control showed low growth as determined from the absorbance values of 0.03-0.3. The average absorbance for 6 replicates of the negative control was 0.17 nm. The results are also represented in graph 1. It is clear that the transfected cells are resistant to G418 antibiotic, indicating that TAs can transfer DNA into HEK293 mammalian cells.

Table 2: Results (absorbance at $\lambda = 540$ nm) of MTT assay for TA mediated transfection of HEK293 cells

				4	5	6
	1	2	3	Replicate 1	Replicate 2	Replicate 3
A	0.004	0.565	0.52	0.473	0.458	0.528

B	0.528	0.773	0.679	0.611	0.477	0.567
C	0.56	0.641	0.608	0.329	0.471	0.332
D	0.543	0.662	0.452	0.35	0.373	0.368
E	0.631	0.503	0.022	0.522	0.119	0.091
F	0.89	0.702	0.043	0.464	0.016	0.38
G	0.627	0.615	0.007	0.011	0.29	0.013
H	0.621	0.643	0.006	0.039	0.031	0.253

[0091] The average absorbance of three replicates was used to determine the transfection efficiency. Comparing the amount of cells containing pcDNA3.1(-) after conducting transfection to the amount of cells in each well (columns 1 and 2), provided the percentage of cells that were successfully transfected *i.e.*, the transfection efficiency. Since the number of cells/well was always constant, the average absorbance was used to calculate the transfection efficiency. The data for transfection efficiency are represented in the table 3 below. The highest efficiency was observed at 92.8%, when the TA:phosphate ratio was 1:1. Compared to the negative control, a 5.2-fold increase in transfection efficiency was observed with 1:1 ratio of TA: phosphate DNA. With increase in TA concentration of 10:1 and 25:1, the efficiency decreased to 63.4% and 61.2% respectively. This further confirms the ability of TAs to successfully and efficiently transfect HEK293 mammalian cells. The same data are represented in graphical format in Figure 10.

Table 3: Transfection efficiency of TA mediated transfection and negative control.

TA:P Ratio	% Efficiency	Fold Enhancement
1:10	81.83985416	4.5
1:1	92.83410461	5.2
10:1	63.49740569	3.5
25:1	61.197588	3.4
10:3	41.06015987	2.3
20:3	48.24007853	2.7
Negative Control	17.86565699	Negative control

[0092] Conclusion: Isophthalic acid para-methoxy or tris-arene could efficiently transfer pcDNA3.1 DNA plasmid into human embryonic kidney 293 cells. Compared to the control, the highest efficiency was achieved when TA:phosphate DNA ratio was 1:1, *i.e.* 92.8%. This was 5.2 fold higher than the negative control (Table 3). TAs were also found to be non-toxic at the concentration used in the experiment and at concentration at least 10-fold higher. Varying the ratio of TA: phosphate DNA also provided higher transfection efficiency of at least 2.3-fold.

[0093] Tris-arenes did not exhibit any cytotoxicity to HEK-293 cells, at the concentration at least 17-fold higher than used in the transfection protocol.

EXAMPLE 7

[0094] *Arabidopsis Thaliana* protoplast plasmid transformation. *Arabidopsis thaliana* seeds were placed into Metro-Mix 360 soil and allowed to grow for 3-4 weeks in an environmentally-controlled chamber with a 12 h light at ~23 °C and a 12 h dark at ~20 °C cycle under low light (50–75 W) and (40–65%) relative humidity. Samples of true leaf numbers of 5-7 (prior to flowering) were removed at the base of the leaf with a surgical scalpel. Ten leaves were removed from the sample plant and cut into 0.5-1 mm strips and immediately transferred to a sterile petri dish containing 5 mL of enzyme solution (Cellulase from *Aspergillus niger* 10% wt/vol solution Sigma, cat. no. C-1184). This digest yields ~ 0.5×10^6 protoplast cells. The petri dish containing the leaves and digest solution was placed into a desiccator with a vacuum top. The desiccator was covered with aluminum foil to induce a dark environment and placed on a slow shaker at ~20 rpm. A vacuum hose was attached to the desiccator and the sample was vacuum infiltrated for 30 min. The desiccator was removed from the shaker and the vacuum top was closed. The desiccator containing the sample in the dark and under vacuum was allowed to incubate for an additional 3 h. The enzyme/protoplast solution containing the digested plant leaves were removed from the desiccator and 5 mL of W5 solution was added. The enzyme/protoplast solution containing the digested plant leaves and W5 solution was filtered through a 75 µm nylon mesh. The filtered solution was centrifuged at 9000 rpm for 1 min to pellet the protoplast cells. The supernatant was removed and the sample was suspended in 3 mL of W5 solution.

[0095] In three separate 1.5 mL sterile DNase/RNase microcentrifuge tubes, 10 µg DNA of a 10.3 kb plasmid containing the selective markers: enhanced yellow fluorescent protein, hygromycin B, and kanamycin resistance. The 3 microcentrifuge tubes were labeled:

1. Protoplast (no DNA or compound) Contained 12 µL Milli-Q water
2. Protoplast + DNA (10 µg DNA) Contained 10 µL DNA + 2 µL DMSO
3. Protoplast + DNA + Tris arene (10 µg DNA + a 1:1 ratio of phosphate to compound Iso OCH₃) Contained 10 µL DNA + 2 µL tris-arene solution

[0096] The 3 samples were allowed to react for 10 min. Protoplasts cells (100 µL) were added to each labeled tube and mixed gently. The samples were allowed to incubate at room temperature for 2 min. After the incubation, 110 µL of PEG–calcium transfection solution was added to all three samples and then mixed completely by gently tapping the tube. The samples were allowed to incubate at room temperature for 15 min. The samples were diluted with 400 µL W5 solution and mixed well by gently inverting the tubes to stop the transfection process. The samples were centrifuged at 9000 rpm for 2 min and the supernatant was removed. The samples containing the protoplast cells were re-suspend in 1 mL WI solution. Samples were viewed at 400x magnification with an enhanced yellow fluorescent protein setting using a Zeiss confocal microscope.

[0097] **Materials:**

[0098] Metro-Mix Series 300-360

[0099] Ingredients:

[00100] Canadian sphagnum peat moss

[00101] Vermiculite

[00102] Fine bark

[00103] Bark ash

[00104] Starter nutrient charge (with gypsum and slow-release nitrogen)

[00105] Dolomitic limestone and long-lasting wetting agent

[00106] W5 solution

[00107] Prepare 2 mM MES (pH 5.7) containing 154 mM NaCl, 125 mM CaCl₂ and 5 mM KCl. The prepared W5 solution can be stored at room temperature.

[00108] PEG–calcium transfection solution

[00109] PEG4000 Fluka, cat. no. 81240 containing 0.2 M mannitol and 100 mM CaCl_2 .

[00110] WI solution

[00111] 4 mM MES (pH 5.7) containing 0.5 M mannitol and 20 mM KCl. The prepared WI solution can be stored at room temperature).

[00112] Tables 4 and 5 represent the results for the transfection of *Arabidopsis thaliana* protoplast cells with tris-arenes or controls. In this experiment, transfection of a ~10.3 kb DNA plasmid encoding enhanced yellow fluorescent protein (EYFP) in to *A. thaliana* protoplast cells was attempted in presence or absence of the compound *N,N'*-bis-(4-methoxyphenyl)isophthalamide. *A. thaliana* protoplast cells that were not treated for transfection or were treated for transfection in the absence of the compound were used as negative control as they failed to express EYFP. Table 4 represents the number of cells expressing EYFP, observed under a Zeiss confocal microscope on a single visual spot. Table 5 represents the number of cells expressing EYFP, observed under a Zeiss confocal microscope on a visual line spot. The present results in Tables 4 and 5, show that 4-methoxy-substituted isophthalic acid dianilide dramatically increases in expression of EYFP and hence the transfection efficiency when compared to the negative control experiments in which no compound of Formula 1 was present.

Table 4

3x Visual spot count	Protoplast	Protoplast + DNA	Protoplast DNA + Tris arenes Iso (OCH_3)
Total Cells Counted	8 + 13 + 9 Total = 30	7 + 11 + 9 Total = 27	10 + 6 + 8 Total = 24
Cells expressing EYFP	0 + 0 + 0 Total = 0	1 + 1 + 0 Total = 2	5 + 2 + 3 Total = 10

Table 5

Visual line spot	Protoplast	Protoplast + DNA	Protoplast DNA + Tris arenes Iso (OCH ₃)
Total Cells Counted	47 + 53 + 41 Total = 141	51 + 45 + 56 Total = 152	46 + 49 + 59 Total = 154
Cells expressing EYFP	0 + 0 + 0 Total = 0	4 + 5 + 6 Total = 15	13 + 20 + 23 Total = 56

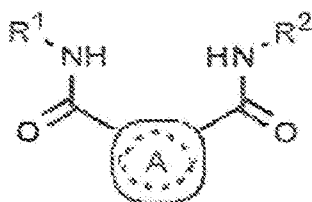
[00113] This is an approximately 70-80% increase in transformation efficiency vs. the standard protoplast plasmid transformation protocol.

[00114] While the invention has been described in connection with specific embodiments thereof, it will be understood that the inventive device is capable of further modifications. This patent application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features herein before set forth.

CLAIMS

What is claimed is:

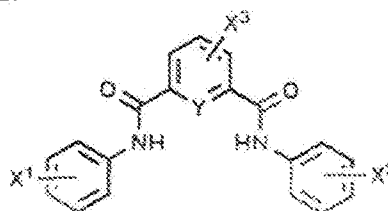
1. A method for transporting a molecular species through a cell wall or cell membrane into a cellular system, the method comprising the steps of:
 - (a) combining the cellular system, a media conducive to maintaining the integrity of the cellular system, the molecular species, and an anion complexing agent, wherein the anion complexing agent is a *bis*(amide) compound that comprises a chemical structure of Formula 1:



Formula 1; and

- (b) incubating the molecular species and anion complexing agent in the media with the cellular system to allow for transport of the molecular species through the cell wall or cell membrane of the cellular system.
2. The method of Claim 1, wherein the molecular species comprises a nucleic acid molecule.
3. The method of Claim 2, wherein the nucleic acid molecule comprises DNA.
4. The method of Claim 1, wherein the molecular species comprises a DNA plasmid of up to about 50 kilobases in size.
5. The method of Claim 1, wherein the molecular species comprises a DNA plasmid of up to about 25 kilobases in size.
6. The method of Claim 1, wherein the molecular species comprises a DNA plasmid of from about 5 kilobases to about 25 kilobases in size.

7. The method of Claim 1, wherein the molecular species comprises a polypeptide.
8. The method of Claim 1, wherein the molecular species comprises a charged compound.
9. The method of Claim 1, wherein the cellular system is selected from the group consisting of bacterial cells, yeast cells, animal cells, and plant cells.
10. The method of Claim 1, wherein the cellular system comprises bacterial cells or yeast cells.
11. The method of Claim 1, wherein the cellular system comprises animal cells.
12. The method of Claim 1, wherein the animal cells are mammalian cells.
13. The method of Claim 1, wherein the cellular system comprises plant cells.
14. The method of Claim 1, wherein the *bis*(amide) compound comprises a chemical structure of Formula 2:



Formula 2.

15. The method of Claim 1, wherein the *bis*(amide) compound is a derivative of substituted or unsubstituted dianilides of either isophthalic acid or 2,6-dipicolinic acid.
16. The method of Claim 1, wherein A is benzene, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group.

17. The method of Claim 1, wherein A is pyridine, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group.
18. The method of Claim 1, wherein:
- (a) A is benzene, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group and wherein the cellular system comprises bacterial cells; or
- (b) A is pyridine, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group and wherein the cellular system comprises bacterial cells.
19. The method of Claim 1, wherein:
- (a) A is benzene, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group and wherein the cellular system comprises yeast cells; or
- (b) A is pyridine, R¹ and R² are identical aniline derivatives which are: (i) unsubstituted in the 4-position or (ii) substituted in the 4-position by a methoxy, chloro, or cyano group and wherein the cellular system comprises yeast cells.
20. The method of Claim 1, wherein the cellular system comprises yeast cells and the method does not comprise the addition of ssDNA.
21. The method of Claim 20, wherein the anion complexing agent comprises the compound *N,N'*-bis-(4-methoxyphenyl)isophthalamide.
22. The method of Claim 1, wherein transport of the molecular species through the cell wall or cell membrane into the cellular system causes a phenotypic change in the cellular system.

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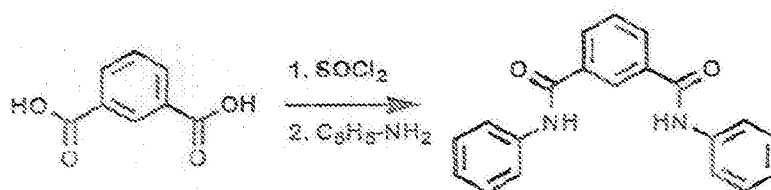


FIG. 1

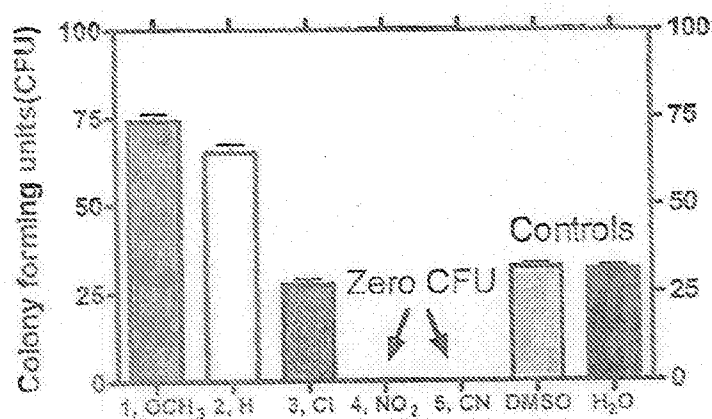


FIG. 2

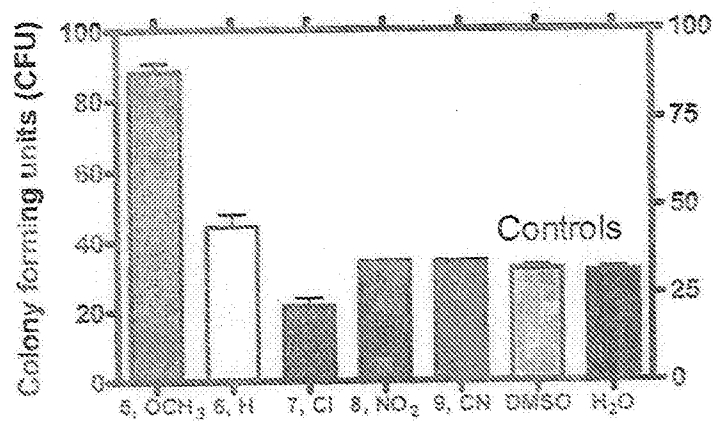


FIG. 3

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Average Colony Forming Units (CFU)
for dianilide compounds (>20kb plasmid
with JM109 competent cells)

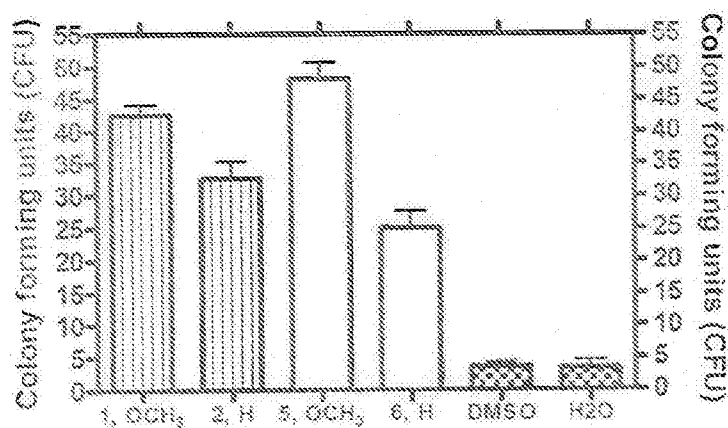


FIG. 4

Yeast transformation without ssDNA

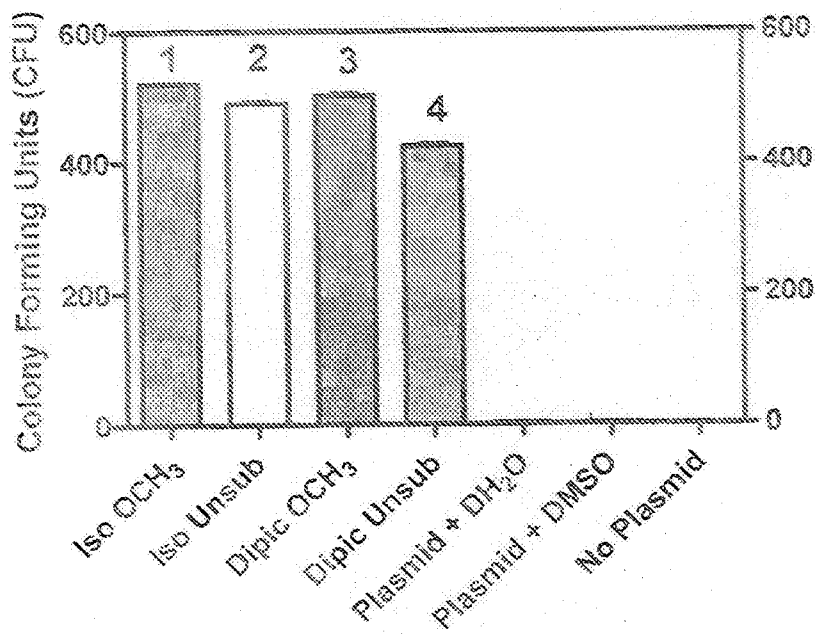


FIG. 5

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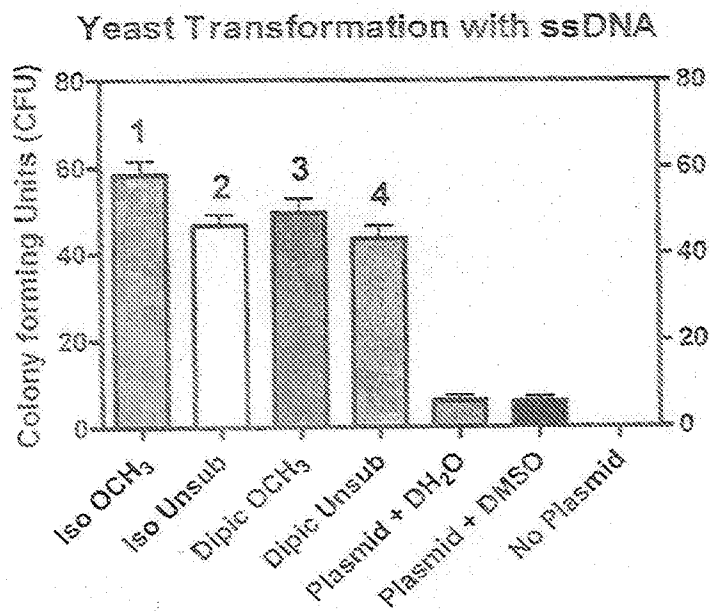


FIG. 6

Yeast Transformation with & without ssDNA

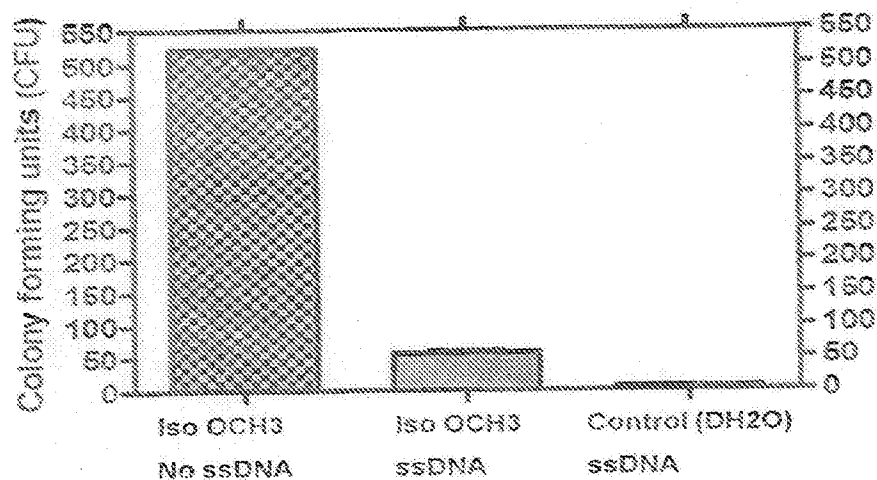


FIG. 7

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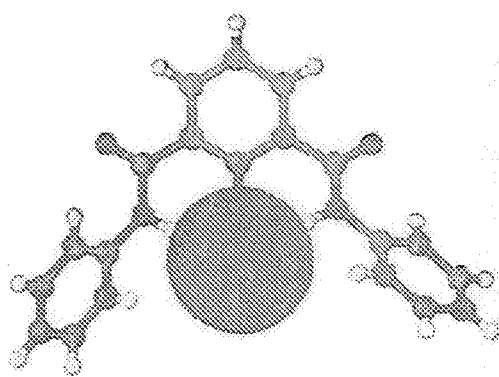


FIG. 8

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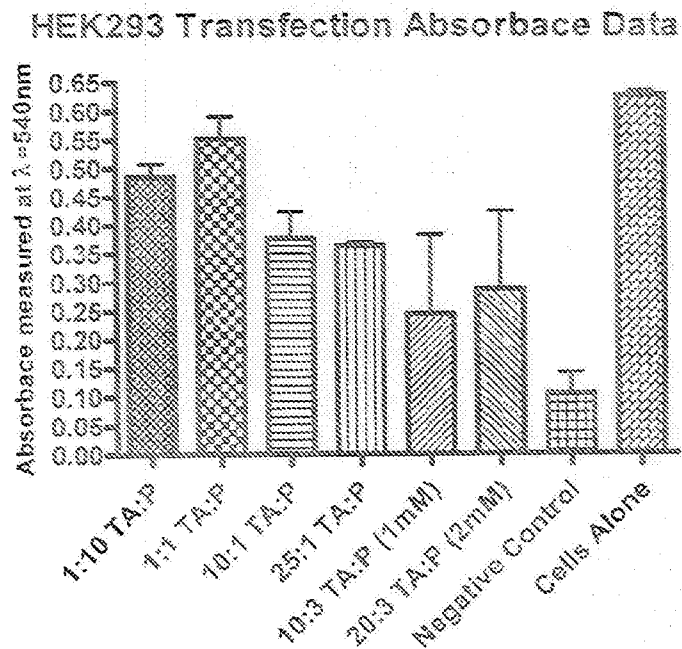


FIG. 9

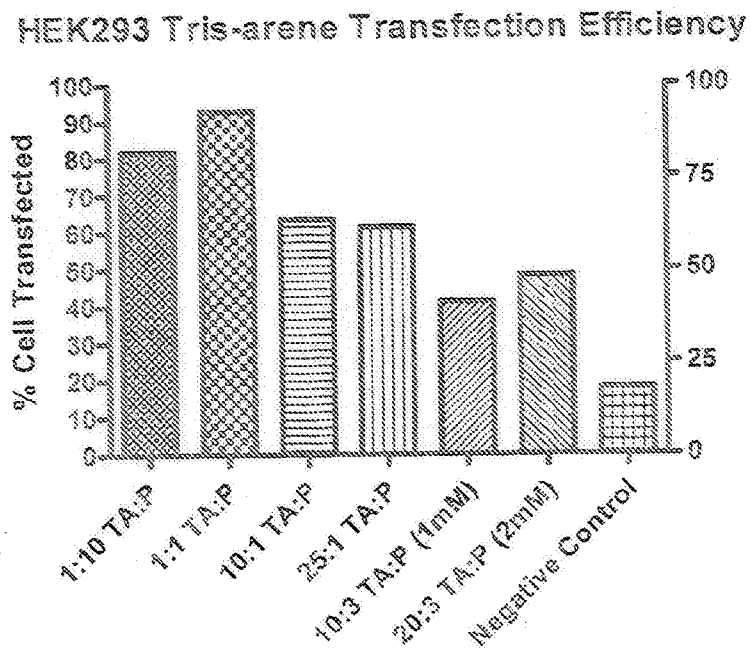


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2013/049764

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 15/87 (2013.01)

USPC - 435/455

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 31/015, 31/04, 31/16, 31/194; A61P 43/00; C12N 15/00, 15/87 (2013.01)

USPC - 435/455, 468, 476, 483; 514/1.1, 1.2, 43, 44R, 68, 107, 113, 563, 616

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC - A01N 57/14, 65/00; A61K 31/66, 31/70, 31/195, 38/00, 48/00; C07C 2101/14; C07K 14/415; C12N 15/74, 15/81 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google Patents, Google Scholar

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2006/0172418 A1 (SLATER et al) 03 August 2006 (03.08.2006) entire document	1-22
Y	YAMNITZ et al. "Dianilides of dipicolinic acid function as synthetic chloride channels." Chem. Commun. 46: Pages 2838-2840. 2010. entire document	1-22
Y	ZHANG et al. "Benzamide derivatives as dual-action hypoglycemic agents that inhibit glycogen phosphorylase and activate glucokinase." Bioorganic & Medicinal Chemistry 17 (2009) Pages 7301-7312. entire document	21
A	FYLES et al. "Membrane Activity of Isophthalic Acid Derivatives: Ion Channel Formation by a Low Molecular Weight Compound." Langmuir 17: Pages 6669-6674. 2001. entire document	1-22
A	ATKINS et al. "Anion Complexation and Transport by Isophthalamide and Dipicolinamide Derivatives: DNA Plasmid Transformation in E. coli." J. Am. Chem. Soc. 134:Pages 13546-13549. 03 August 2012. entire document	1-22

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

26 October 2013

Date of mailing of the international search report

22 NOV 2013

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