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Fluorescent Cell Chip a new in vitro approach for immunotoxicity screening

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Abstract

The Fluorescent Cell Chip (FCC) has been developed specifically for immunotoxicity screening of chemical compounds. This in vitro test is based on a panel of genetically modified reporter cell lines that regulate the expression of fluorescent protein in the same way as they regulate expression of cytokines. Thus, changes in fluorescence intensity represent changes in cytokine expression. Consequently, this technique conforms to efficiency expected from high throughput screening assay. In a pre-validation effort we analyzed 46 compounds. The experimental protocol employed five reporter cell lines derived from murine EL-4 T cells. Reporter cells were exposed to tested chemicals on a 96 well plate and analyzed for EGFP-mediated fluorescence using automated flow cytometric assay. Tested compounds reproducibly generated compound-specific patterns of changes in fluorescence that allows for the hierarchical clustering of their expected activities based on pattern similarity analysis. Resultant classification revealed correlation with available in vivo immunotoxicity data. In conclusion, FCC is a new promising approach for in vitro screening of chemicals for their immunotoxicity.

Keywords: Immunotoxicity screening; Fluorescent Cell Chip (FCC); Cytokine genes; EGFP

1. Introduction

The predictive immunotoxicity testing is a complicated process that requires the battery of tests. Most of the existing tests for immunotoxicity employ experimental animals but there are also in vitro tests that could be implemented for that purpose (Gennari et al., 2005). Most of these assays determine changes in the phenotype of cultured immune cells upon in vitro exposure to tested chemicals. The endpoints employed include cell surface antigens expression, proliferation rate, and cytokine expression (Gennari et al., 2005). Changes in cytokine expression are frequently observed upon exposure of human and animals to toxic compounds and ability of xenobiotics to induce (Dastych et al., 1999; Prigent et al., 1995) or suppress (O'Keefe et al., 1992) cytokine expression in immune cells were proposed as possible mechanism explaining their adverse effects on immune system. Therefore, measurement of cytokine expression upon in vitro exposure of immune cells to chemicals is a viable option for an endpoint useful for determination of possible immunotoxic properties of chemicals tested in vitro. Tests that relay on cytokine expression include among others whole blood assay (Langezaal et al., 2001), allergenic activity assay in established

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keratinocyte cell lines (Van Och et al., 2005), and Fluorescent Cell Chip assay (FCC) (Ulleras et al., 2005). FCC has been developed specifically for fast screening of large number of compounds and is based on a genetically modified murine T cell lines that regulate the expression of fluorescent protein in similar way as they regulate expression of cytokines. This assay was previously tested against several substances of known immunotoxic and immunomodulatory properties and showed ability to generate patterns of response of reporter gene expression that correlate with activities of these compounds observed in vivo and in other assays in vitro (Ringerike et al., 2005). We followed these original observations, modified the experimental protocol of FCC test to conform

Table 1
List of substances tested in this study

No.	Tested substance	Source of substance	IC_{10} (mM) PI test
1	2′,3′-Dideoxyadenosine	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	1
2	2′,3′-Dideoxy-2′,3′-didehydrothymidine	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	2.2
3	2′,3′-Dideoxyinosine	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	1
4	2,4-Diaminotoluene	Sigma Chemical Co., St. Louis, MO, USA	0.98
5	2-Aminoanthracene	Sigma Chemical Co., St. Louis, MO, USA	0.026
6	2-Mercaptobenzothiazole	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	1.1
7	3′-Azido-3′-deoxythymidine	Sigma Chemical Co., St. Louis, MO, USA	3.2
8	Acetaminophen	Sigma-Aldrich Chemie GmbH, Steinheim, Germany	3
9	Actinomycin D	Sigma Chemical Co., St. Louis, MO, USA	0.000008
10	Aluminum chloride	POCH, Gliwice, Poland	1
11	Amphoterycin B	Sigma Chemical Co., St. Louis, MO, USA	0.0054
12	Benzethonium chloride	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	0.027
13	Chlorpromazine	Sigma Chemical Co., St. Louis, MO, USA	0.007
14	Cisplatin	Sigma Chemical Co., St. Louis, MO, USA	0.005
15	Cobalt chloride	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	0.7
16	Cyclophosphamide	Sigma Chemical Co., St. Louis, MO, USA	1.25
17	Cyclosporine A	Calbiochem, La Jolla, CA	0.0017
18	Dapsone	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	48
19	Dexamethasone	Sigma Chemical Co., St. Louis, MO, USA	0.32
20	Dibenzo[*a,i*]pyrene	Sigma Chemical Co., St. Louis, MO, USA	0.016
21	Diethanolamine	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	1.62
22	Dimethyl sulfoxide (DMSO)	Sigma Chemical Co., St. Louis, MO, USA	5.1
23	Ethanol	Euorochem BGD Sp z o.o. Tarnów, Poland	200
24	FK 506	Fujisawa Farmaceuticals, Japan	0.0000073
25	FR 167653	Fujisawa Farmaceuticals, Japan	nd (0.1)
26	Histamine	Sigma Chemical Co., St. Louis, MO, USA	nd (1)
27	Hydrocortisone	Sigma Chemical Co., St. Louis, MO, USA	nd (1)
28	Hydrogen peroxide	Sigma Chemical Co., St. Louis, MO, USA	0.1
29	Isoniazid	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	2.2
30	Isophorone diisocyanate	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	0.00047
31	Lead acetate	POCH, Gliwice, Poland	0.1
32	Lithium carbonate	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	8.1
33	Magnesium sulfate	Sigma Chemical Co., St. Louis, MO, USA	nd (1)
34	Mercuric chloride	Ubichem limited, Eastleigh, Hampshire, GB	0.05
35	Methanol	Chempur, Piekary Śląskie, Poland	2000
36	Mitomycine C	Sigma Chemical Co., St. Louis, MO, USA	0.007
37	Nickel sulfate	POCH, Gliwice, Poland	0.1
38	Nitrofurazone	Sigma Chemical Co., St. Louis, MO, USA	0.79
39	Patulin	Sigma Chemical Co., St. Louis, MO, USA	0.0002
40	Pentamidine isethionate	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	0.25
41	*p*-Nitroaniline	Sigma Chemical Co., St. Louis, MO, USA	0.0065
42	Pyrimethamine	Sigma Chemical Co., St. Louis, MO, USA	0.001
43	Ribavirin	Sigma Chemical Co., St. Louis, MO, USA	nd (0.16)
44	Sodium bromate	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	nd (0.93)
45	Sodium lauryl sulfate	Sigma Chemical Co., St. Louis, MO, USA	0.3
46	Triethanolamine	Sigma–Aldrich Chemie GmbH, Steinheim, Germany	0.39

nd: IC_{10} was not established experimentally, the value in parenthesis is the highest concentration selected for FCC test.

with multiwell plate format and employed this assay for testing of 46 substances.

2. Materials and methods

2.1. Chemicals and reagents

Reagents for cell culture were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except for FCS that was obtained from Biowest (Nuaillé, France). The source of chemicals employed in the study as tested compounds is provided in Table 1. Stocks of chemicals were prepared by dissolving or diluting them either in H_2O, DMSO or culture medium. Desired working solutions were obtained by diluting stocks in culture medium.

2.2. Culture of EL-4 derived reporter cell lines

Five reporter cell lines stably transfected with transgene consisting of promoter regions from mouse β-actin

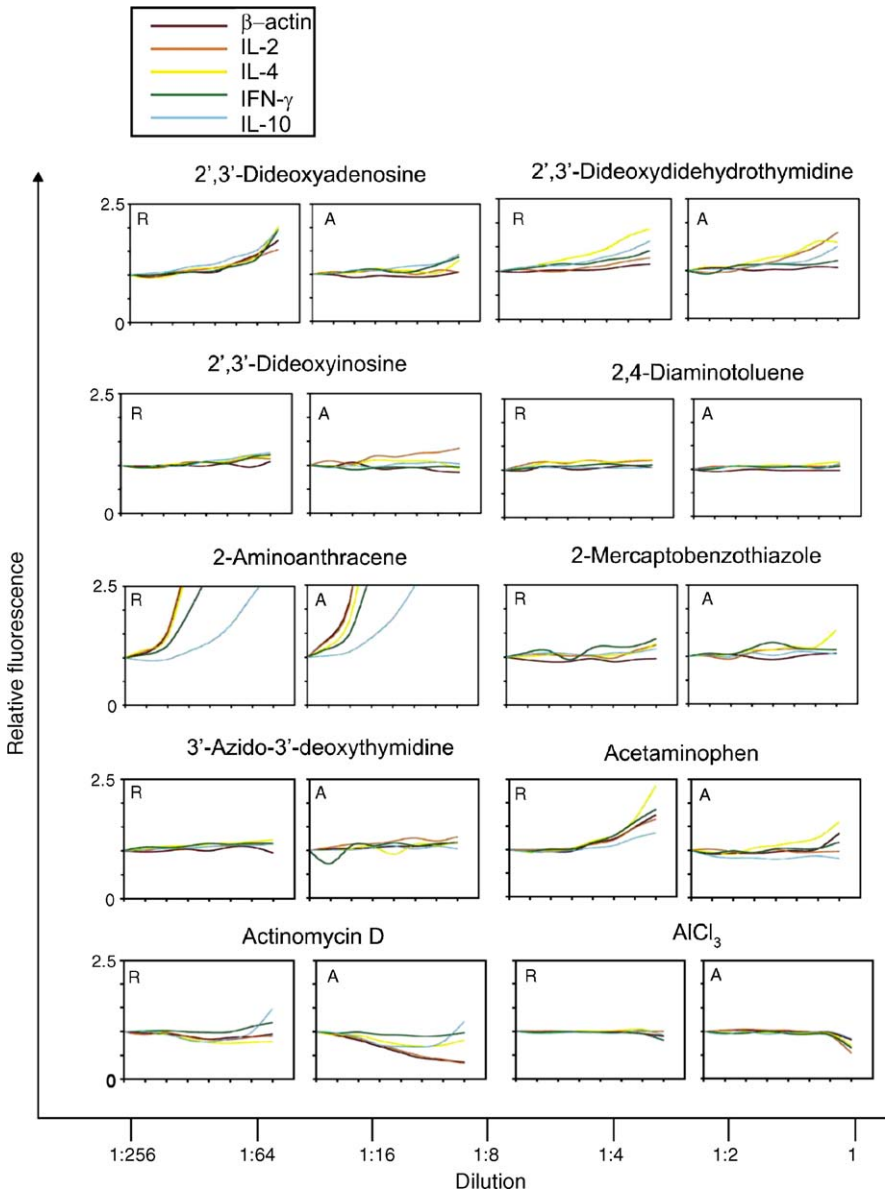
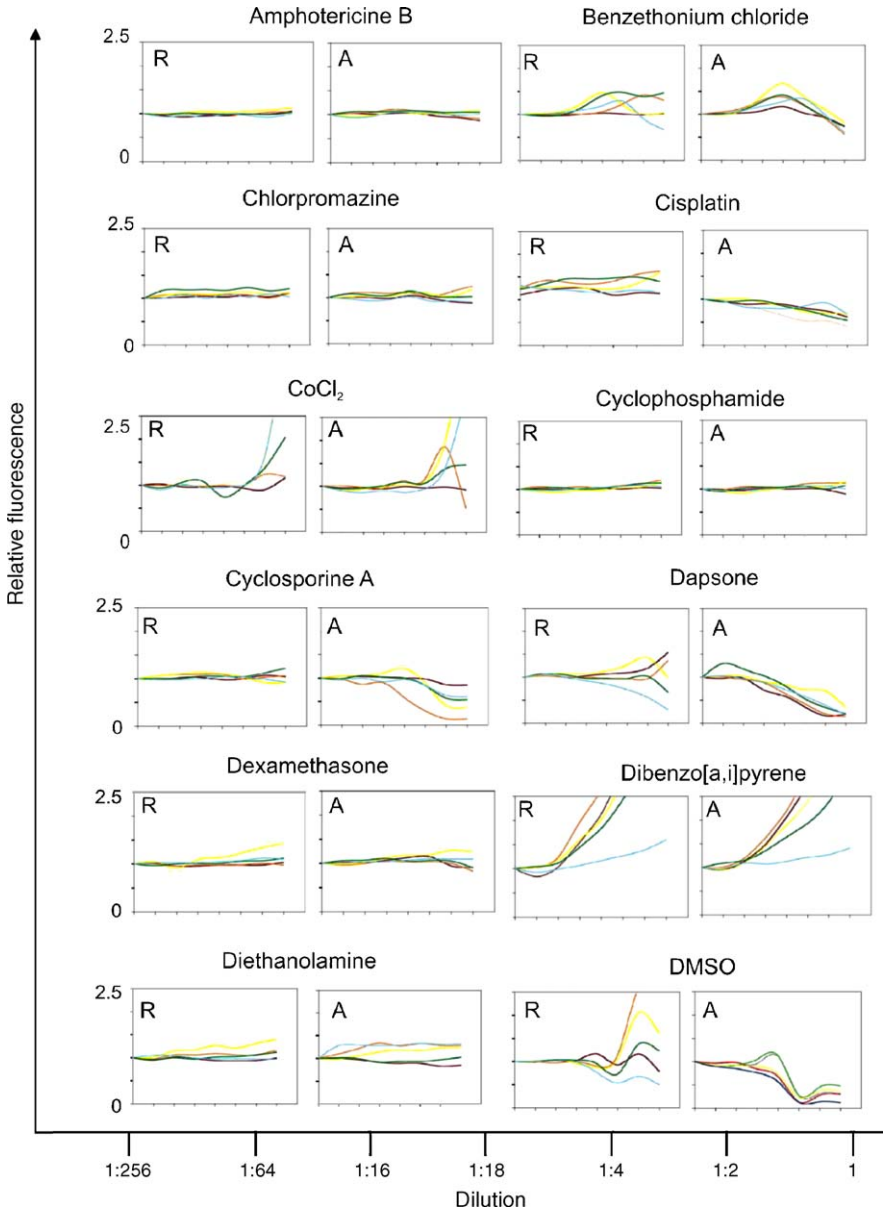


Fig. 1. The fluorescence patterns obtained with FCC test. Five reporter cell lines for β-actin (E/008/009), IL-2 (E/253/006), IL-4 IFN-γ ≲ E/552/003 and IL-10 (E/752/005) were exposed to series of dilution of tested compounds. Reporter cells were either resting (R) or activated PMA/Ionomycin (A). Fluorescence (MIF) determined for each experimental condition was normalized against control (medium only) for resting and activated control (PMA/Ionomycin) for activated. Each data point represents mean from three independent experiments.



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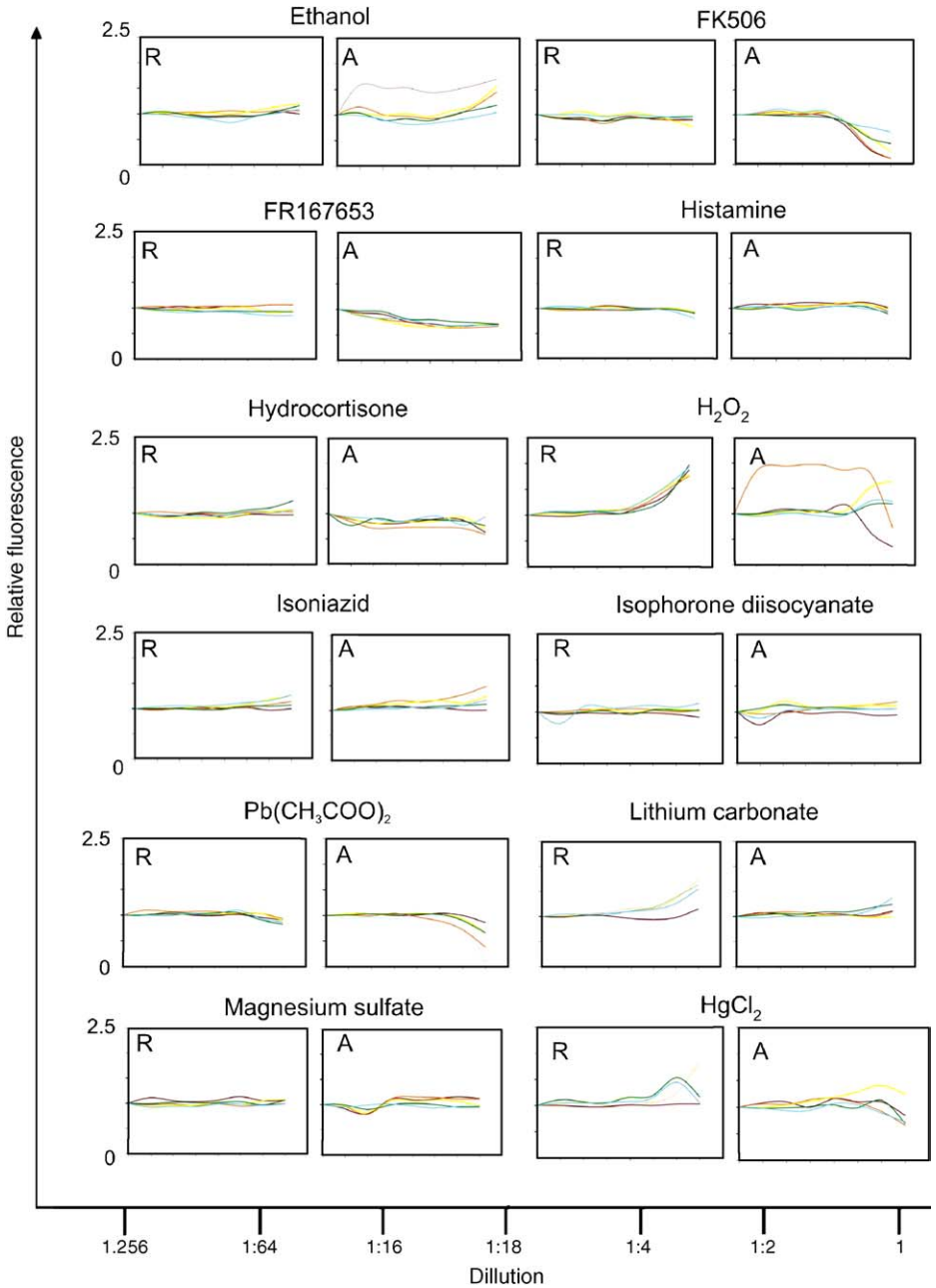
(E/008/009), IL-2 (E/253/006), IL-4 (E/452/002), IFN-γ (E/552/003) and IL-10 (E/752/005) and ORF for EGFP were described earlier (Ulleras et al., 2005). Reporter cells were cultured at density 5×10^5 to 1×10^6 cells/ml in medium RPMI 1640 supplemented with 2 mM L-glutamine, 20% FCS and 0.5 mg/ml of G418 in a humidified atmosphere with 5% CO_2 at 37 °C. EL-4 T cells (ATCC number TIB-39) were cultured in the same media but supplemented with penicillin/streptomycin rather than G418. Prior to experiments cell lines were tested for *Mycoplasma* sp. contamination with MycoTect Kit (Invitrogen Corporation, CA, USA).

2.3. Exposure to chemicals

In preliminary experiments, direct cytotoxicity of tested compounds was assessed using flow cytometric propidium iodide uptake assay with β-actin reporter cells (E/008/009). The concentration of tested compound resulting in 10% cytotoxicity (IC_{10}) was next used as the highest tested in FCC assay. If cytotoxicity was not observed, the highest tested concentration was chosen arbitrary. Next, tested compounds were evaluated for their fluorescence and/or induction of cell autofluorescence in EGFP fluorescence

emission spectra. To this end, EL-4 cells were exposed to the series of dilution of tested compound consisting of the dilutions 1 (the highest concentration equal to IC_{10}, 1:2 and 1:4 for 18 h followed by flow cytometric determination of fluorescence associated with cells on FL1 channel. For determination of the effect of tested compound on EGFP-mediated fluorescence of reporter cell lines, cells were seeded at a density of 5×10^4 per well in a 96 well plate, and incubated with the desired compound concentration in a total volume of 250 μl at 37 °C in a humidified atmosphere of 5% CO_2 for 18 h. Each plate was arranged to accommodate five reporter cell lines, either activated with PMA (50 ng/ml) and Ionomycin (1 μM) or not and exposed to tested substances at following series of dilutions: 1 (IC_{10}) > 1:2 > 1:4 > 1:8 > 1:16 > 1:64 > 1:256. For each cell line, there were two controls one consisting of cells in medium only, and the other consisting of cells activated with PMA/Ionomycin. Following 18 h incubation, cells were



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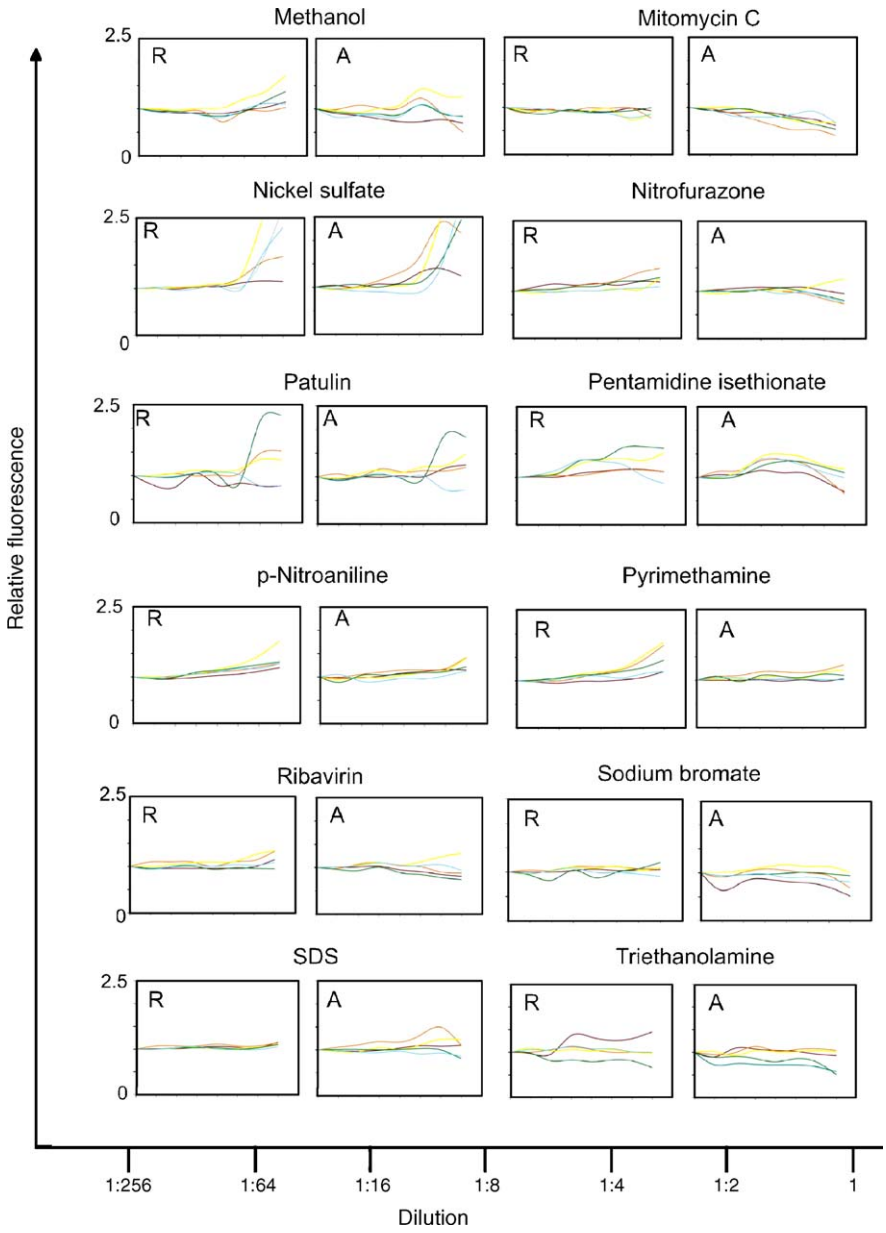


Fig. 1. (*Continued*).

analyzed by flow cytometry to determine the level of EGFP-mediated fluorescence.

2.4. Flow cytometry

Samples were analyzed in the Cytomics FC 500 MPL Beckman-Coulter flow cytometer equipped with multi plate loader and MXP Acquisition Software v.1.1 (Beckman Coulter, Inc., Fullerton, CA 92853). To determine IC_{10} for tested compounds EL-4/β-actin reporter cells exposed to increasing concentration of tested compounds were stained in situ with propidium iodide (50 μg/ml) and after 20 min incubation analyzed on FL3 channel. Untreated EL-4/β-actin reporter cell line cells were used as a negative control to create gates for live and dead cells. The gate for dead cells was then adjusted using the cell death positive control, or a sample treated with the highest concentration of drug to include the peak of PI positive cells visible on FL3 histogram. In the routine acquisition, protocols for determination of EGFP-mediated fluorescence live cells were selected based on live/dead cell gates on FS/SS plot. These gates were verified by comparison with the number of PI negative/positive cells that showed good correlation with differences not exceeding ±5% of analyzed cell population.

2.5. Data presentation and statistical analysis

Raw data from triplicate experiments were exported to MS Excel during flow cytometry analysis and values of relative means of fluorescence ± S.D. calculated. Statistical significance of observed differences was tested with ANOVA followed by Holm Sidak test using SigmaStat 2.03 software (SPSS, Inc., Chicago, IL, USA). Also the agglomerative nesting hierarchical clustering (Kaufman and Rousseeuw, 1990; the R Project for Statistical Computing, http://www.r-project.org/) was used to obtain groups of substances that affect EGFP expression in examined reporter cell line in a similar way. As a measure of similarity, the "Manhattan distance" between expressions of EGFP in reporter cell lines, which were affected by given substance, was chosen. As partitioning method "the complete partitioning" was used (two clusters were joined when the largest distance between a point in the first cluster and a point in the second cluster were smaller than given similarity level). Also other distances and other partitioning methods were investigated, but presented results were most satisfying.

3. Results and discussion

In the first step of an experimental protocol 49 compounds characterized to different degree for their immunotoxic and immunomodulatory properties were tested for their cytotoxicity against β-actin reporter cell line. For 42 chemicals, the IC_{10} values were determined (see Table 1). For four compounds, we did not observed cytotoxicity within the range of tested concentrations. Three substances were eliminated from further testing due to the increase of fluorescence observed in EL4 cells (not expressing EGFP) following incubation with these compounds (data not shown). Thus, 46 compounds (Table 1) were next tested at seven increasing concentrations employing five reporter cell lines either resting or activated (PMA/Ionomycin). Resultant data (Fig. 1) showed that many tested compounds generated pattern of fluorescence suggesting that they mediated dose dependent and reporter cell line specific changes in EGFP expression. To assess the possible relationship of observed fluorescence patterns with compound immunotoxicity observed in vivo all compounds were classified using uniform criteria (see Table 2) to assign each of the compounds into a one of categories describing compound immunotoxic and immunomodulatory activities. In a decision to label the compound as immunotoxic (IT) the clinical manifestation either adverse (increased lethality, allergy and auto-immune disease) or beneficial (prevention of graft rejection) were prerequisites. Compounds classified as immunotoxic were divided further into two subcategories. Category IT-1 consists of compound that mediate mainly immunosuppression and category IT-2 consists of compound mediating such immune disorders as contact hypersensitivity, asthma, anaphylaxis, immune complex mediated hypersensitivity and auto-immune diseases. Compounds for which only changes in selected parameters of functions of immune system were reported were labelled as immunomodulators (M). Substances that were previously used as a negative control in similar toxicity tests in vitro and substances tested in vivo with negative results were labelled as inert (N). Cyclophosphamide that is a potent immunosuppresant in vivo was labelled N to reflect its lack of activity towards immune cells in vitro. Compounds for which there is a lack of published in vivo data or published data are contradictory were labelled as unknown (U). Resultant classification of compounds is presented in Table 3. Out of all tested 24 compounds presented in Fig. 2 mediated changes in EGFP expression in reporter cell lines that met criteria

Table 2
Criteria of categorization of substances according to their known immunotoxic and immunomodulatory activities

Category	Subcategory	Description	Symbol
Inert		Substances not expected of mediating immunotoxicity	N
Immunotoxicants	Immunosuppressants	Substances suppressing immune response in human and animals including immunosuppressive drugs and other substances causing such clinical manifestation as increased incidences of neoplasia and increased lethality following infection	IT-1
	Others	Substances modulating immune response in human and animals resulting primarily in different hypersensitivity reactions such as contact hypersensitivity, anaphylaxis, and allergic asthma and substances causing auto-immune diseases	IT-2
Immunomodulators		Substances that affect certain parameters of immune system in human and animals but not known to result in clinical manifestation	M
Unknown		Substances which effect on immune system could not be determined due to the lack of data or contradictory data	U

Table 3
Categorization of substances employed in this study according to their known immunotoxic and immunomodulatory activities

Tested substance	Clinical manifestation of immunotoxicity in humans and animals	Changes in parameters of immune function in humans and animals	Other data indicating immunotoxicity/immunomodulation	Category
2′,3′-Dideoxyadenosine		AFC↓, NK↑ (Cao et al., 1990; Luster et al., 1991; U.S. NTP database)		M
2′,3′-Dideoxy-2′,3′-didehydrothymidine	Possible allergic reactions in human (Shah, 2005; Squires et al., 2000)	AFC↔, NK↔ (U.S. NTP database)		U
2′,3′-Dideoxyinosine		AFC↓, MLR↓, CSM↓, NK↔ (Phillips et al., 1997; U.S. NTP database)		M
2,4-Diaminotoluene		AFC↓, NK↓, DTH↓ (Burns et al., 1994; U.S. NTP database)		M
2-Aminoanthracene				U
2-Mercaptobenzothiazole	Contact hypersensitivity (Kaniwa et al., 1994)	Induction of DTH (U.S. NTP database) LLNA positive (De Jong et al., 2002)		IT-2
3′-Azido-3′-deoxythymidine		AFC↔, NK↓, CSM↑↓ (McKallip et al., 1995; U.S. NTP database)		M
Acetaminophen			Suggested involment of immune system in observed hepatotoxicity (Liu et al., 2004; Boulares et al., 2002)	U
Actinomycin D	Immunosuppressant preventing allograft rejection (DiSalvo et al., 1995)			IT-1
Aluminum chloride	Asthma (Desjardins et al., 1994; Vandenplas et al., 1998)		Alum stimulates humoral immune response (Jordan et al., 2004)	U
Amphotericin B			Induces cytokine production in immune cells (Kumar and Chakrabarti, 2000)	U
Benzethonium chloride	Contact hypersensitivity (Kanerva et al., 2001); (Fraki et al., 1985),	Negative DTH test in mouse model (U.S. NTP database)		IT-2
Chlorpromazine		NK↑ (Ghosh and Chattopadhyay, 1993)	Photosensitizing compound (Suzuki et al., 2000)	M
Cisplatin	Allergic reactions including anaphylaxis (Basu et al., 2002; Onoyama et al., 1997; Ozguroglu et al., 1999; Raulf-Heimsoth et al., 2000; Shepherd, 2003)	Serum IgM↔, DTH↓ (Kouchi et al., 1996), positive in popliteal lymph node assay in mice (Schuppe et al., 1997)		IT-2
Cobalt chloride	Contact hypersensitivity (Barceloux, 1999; Thomssen et al., 2001), asthma (Cugell, 1992).			IT-2
Cyclophosphamide	Immunosuppressive drug used in therapy of auto-immune diseases (Miserocchi et al., 2002), increased lethality in murine viral infection model (Selgrade et al., 1992)		Control substance for immunotoxicity test in vitro (Langezaal et al., 2002)	(IT-1) N*

Cyclosporin A	Immunosuppressant preventing allograft rejection (Bierer et al., 1993; White and Calne, 1982)	AFC↓ (U.S. NTP database)		IT-1
Dapsone	Immunosuppressive drug used in therapy of auto-immune diseases (Burrows et al., 1993; Meeker et al., 2003; Miserocchi et al., 2002; Russell and Weenig, 2004)	AFC↔, N↔ (U.S. NTP database)		IT-1
Dexamethasone	Immunosuppressant preventing allograft rejection (Kher et al., 1992; Mittal et al., 1997)			IT-1
Dibenzo[*a*,*i*]pyrene				U
Diethanolamine	Asthma (Piipari et al., 1998), contact hypersensitivity (Geier et al., 2004)	AFC↓, NK↓, CSM↓ (U.S. NTP database)		IT-2
Dimethyl sulfoxide	(Swanson, 1985)			U
Ethanol		CSM↓↑ (Cook et al., 2004); NK↓ (Zabrodskii et al., 2003)	Anti-adduct antibodies in human (Latvala et al., 2005)	M
FK 506	Immunosuppressant preventing allograft rejection (Bierer et al., 1993)			IT-1
FR167653			Inflammatory cytokines inhibitor (Nishikori et al., 2002; Sano et al., 2001)	U
Histamine		Immunomodulation (Jutel et al., 2005)		M
Hydrocortisone	Immunosuppressant preventing allograft rejection (Chutna and Hasek, 1975; Saudek et al., 1995)			IT-1
Hydrogen peroxide			In vitro model of oxidative stress (el-Hag et al., 1986)	M
Isoniazid		CSM↑↓, CT↑, Ig↓ (U.S. NTP database)		M
Isophorone diisocyanate	Contact dermatitis (Militello et al., 2004)	Induces DTH (U.S. NTP database)	Skin sensitizer in guinea pig model (Zissu et al., 1998)	IT-2
Lead acetate			Involvement in immediate and contact hypersensitivity (Laschi-Loquerie et al., 1984)	M
Lithium carbonate	Leukocytosis (Ballin et al., 1998; Capodicasa et al., 2000)	AFC↔, NK↔, CSM↑↓ (U.S. NTP database)		M
Magnesium sulfate				N
Mercuric chloride	Allergic reaction in human (Ashinoff et al., 1995), contact hypersensitivity in human (Lebrec et al., 1999), systemic auto-immune disease in rats (Pelletier et al., 1987); (Kiely et al., 1995)			IT-2
Methanol		NK↓ (Zabrodskii et al., 2003)		M

Table 3 (*Continued*)

Tested substance	Clinical manifestation of immunotoxicity in humans and animals	Changes in parameters of immune function in humans and animals	Other data indicating immunotoxicity/immunomodulation	Category
Mitomycin C	Contact hypersensitivity (Kunkeler et al., 2000; Vidal et al., 1992), Antigen-Antibody Immune complex mediated hypersensitivity (Mistry et al., 2004; Verweij et al., 1987)			IT-2
Nickel sulfate	Contact hypersensitivity (Budinger et al., 2001; Rustemeyer et al., 2004)			IT-2
Nitrofurazone (5-nitro-2-furaldehyde semicarbazone)	Possible contact sensitizer (Guijarro et al., 1999)	AFC↔, NK↔ (U.S. NTP database)		U
Patulin		AFC↔, NK↔, Ig↔, DTH↓ (Paucod et al., 1990; U.S. NTP database)	Possible immunosupression (Sharma, 1993)	U
Pentamidine isethionate		AFC↔, NK↔, CSM↔ (U.S. NTP database)	Suspected of causing immune mediated pancreatitis (Mallory and Kern, 1988). Selective inhibition of B cell proliferation (Ferrante et al., 1985)	U
p-Nitroaniline				U
Pyrimethamine		CT↓, CSM↓, NK↑, AFC↑, NK↔, Ig↓ (U.S. NTP database)		M
Ribavirin		AFC↓, Ig↓ (U.S. NTP database) DTH↑ (Tam et al., 1999)		M
Sodium bromate		Ig↑ (U.S. NTP database)		M
Sodium lauryl sulfate			Nonsensitizing irritant (Ikarashi et al., 1993)	N
Triethanolamine	Weak skin sensitizer (Geier et al., 2004)	AFC↔, NK↔, Ig↔ (U.S. NTP database)		U

↑, Increase; ↓, decrease; ↔, no change; *, category assign based on in vitro activity. AFC, antibody forming cells; CSM, cell surface markers; CT, cytotoxic T cell activity; DTH, delayed type hypersensitivity; Ig, immunoglobulin serum level; LLNA, local lymph node assay; MLR, mixed lymphocyte reaction; NK, natural killer cells activity.

of statistical significance (ANOVA followed by post hoc test). Interestingly, five out of seven compounds labelled as IT-1 and six out of nine compounds labelled as IT-2 mediated such statistically significant changes (Fig. 2). This observation prompted us to apply comparison of obtained fluorescence patterns for the purpose of classification of compounds that might be next used for prediction of similarity of unknown compound to

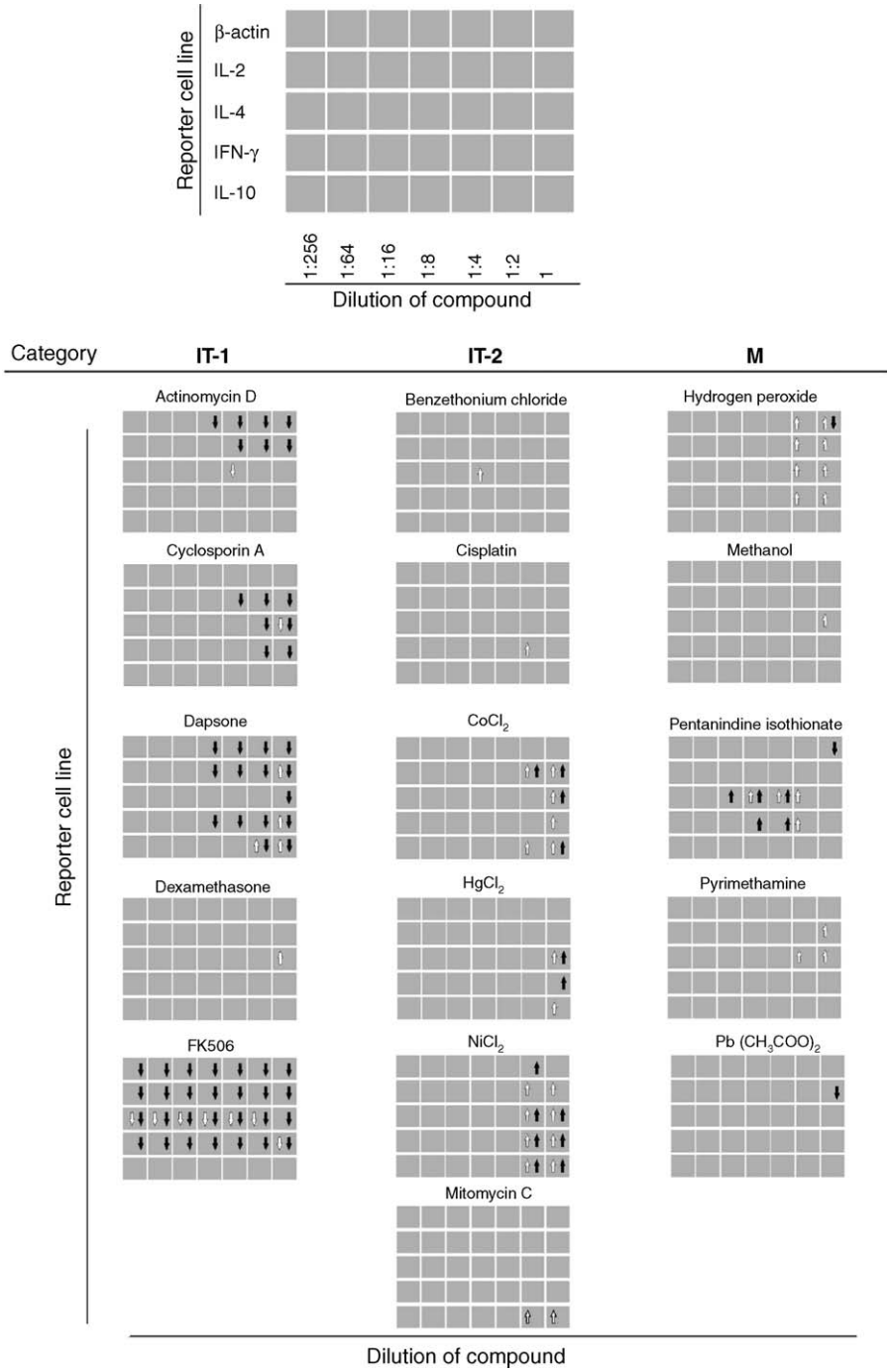
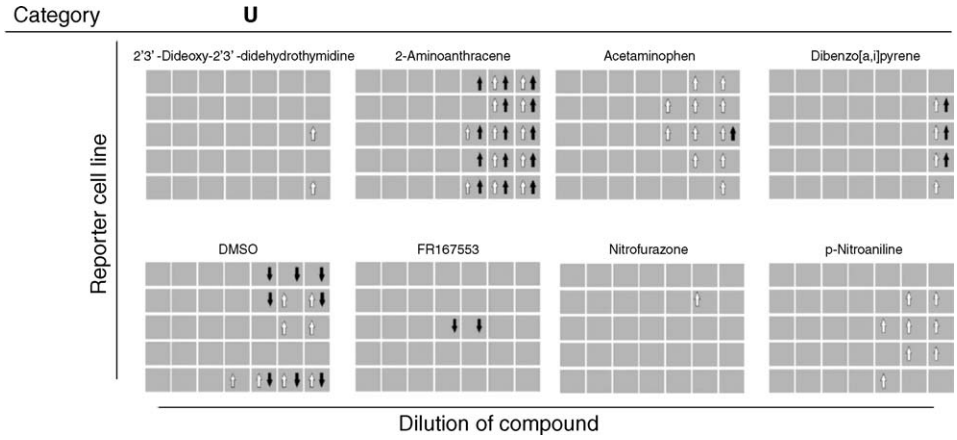


Fig. 2. The results of statistical analysis of FCC test. Each square represents combination of reporter cell line and tested compound dilution. Open arrow represents statistically significant changes ($p < 0.05$; ANOVA followed by Holm Sidak test) under "resting" condition. Solid arrow represents statistically significant changes under "activated" condition. Arrows are directed according to the direction of the observed changes in fluorescence.



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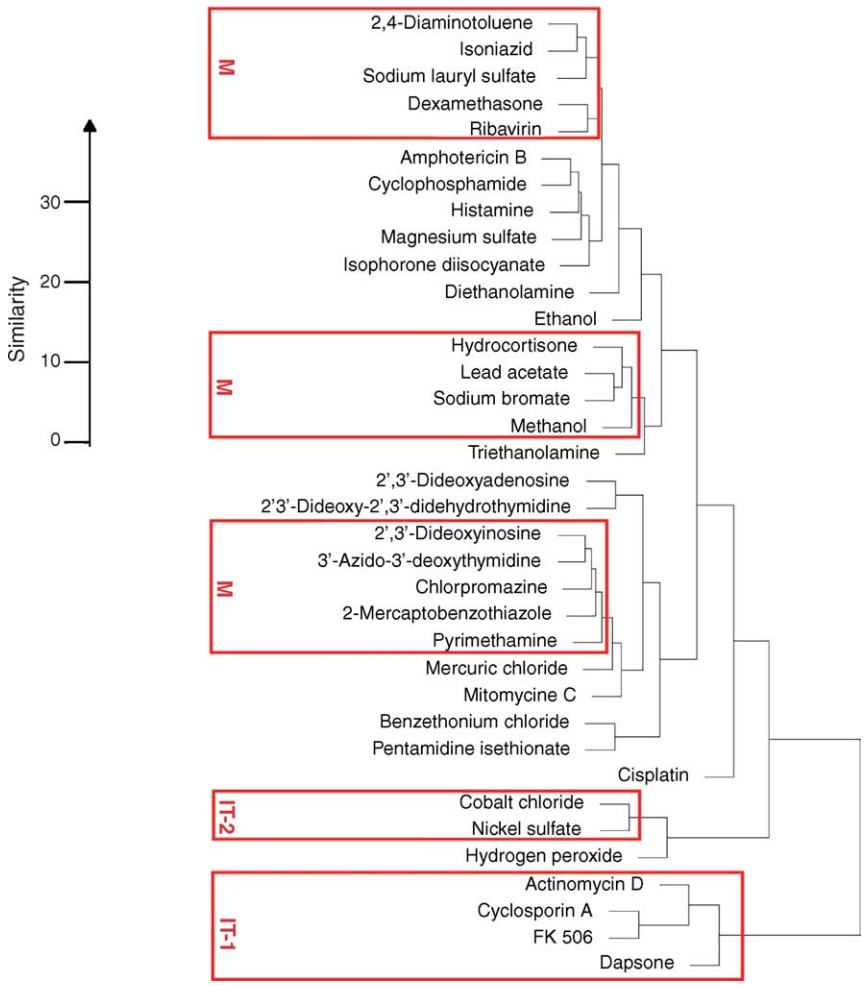

Fig. 3. Hierarchical clustering of substances based on FCC-generated patterns. Fluorescence patterns generated by compounds with assigned immunotoxic activity (IT-1, IT-2, M, and N) were compared as described in Section 2. *Y*-axis denotes similarity level, i.e., the maximal distance between every pair of substances located in the subtree located below given level.

characterized immunotoxicants. To this end, we have used the agglomerative nesting hierarchical clustering method to divide tested substances into groups that affect EGFP expression in examined reporter cell lines in a similar way. As could be seen in Fig. 3 such methods organized the set of tested compounds into several clusters. We observed, in some subtrees (marked with red rectangles) predominated substances of the same category of immunotoxic activity (Table 3). A cluster created for substances labelled IT-1 consists of four immunosuppressive drugs, Cyclosporin A, FK 506, Actinomycin D and Dapsone that strongly inhibited increase in EGFP expression in IL-2 reporter cell line upon activation with PMA/Ionomycin but did not affected EGFP expression in resting cells. Interestingly, all substances in this group have a similar mechanism of action in vivo that involves inhibition of cytokine expression in T cells. The corticosteroids (hydrocortisone and dexamethasone) did not show similarity to other compounds labelled IT1 as they did not inhibit PMA/Ionomycin-mediated EGFP expression. This is in agreement with previous observation that dexamethasone did not inhibit cytokine production and cell proliferation in PMA/Ionomycin activated T lymphocytes (Furue and Ishibashi, 1991; Furue et al., 1990). Thus, the lack of response of reporter cell lines to these compounds might result from the type of stimulants (PMA and Ionomycin) used in the assay. Another distinct group created for substances labelled IT-2 consists of $NiCl_2$ and $CoCl_2$ that have similar pathological activities observed in vivo. Both these metals have also similar molecular mechanism of action that involves generation of reactive oxygen species in exposed cells (Cavallo et al., 2003; Chachami et al., 2004; Chen et al., 2003; Leonard et al., 1998). Interestingly, hydrogen peroxide the major reactive oxygen species in living cells demonstrated high similarity to $NiCl_2$ and $CoCl_2$ in FCC test (Fig. 3). Substances labelled M and N were spread in the similarity tree to the extend that not allowed for creation of a single cluster (Fig. 3).

Thus, the preliminary analysis of similarity of fluorescence patterns generated by tested compounds showed that such approach is able to group compounds of similar activity in vivo and similar mechanism of action on immune cells. In further development of FCC technology the pattern similarity analysis could be improved by refinement of substance categorization (inclusion of additional source of data such as in vitro assays) and expansion of tested compounds list. One possible approach for using such improved analysis for predictive immunotoxicity testing is selection from the substances that belong to specified subtrees, the medoids (the most representative substances) and usage of medoids to predict the class for unknown substance.

Acknowledgments

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