Transport Screening of Drug Cocktails Through an *in Vitro* Blood-Brain Barrier: Is It a Good Strategy for Increasing the Throughput of the Discovery Pipeline?

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Purpose. The objective of the current study was to investigate whether blood-brain barrier (BBB) permeability studies *in vitro* could be accelerated by running several compounds together in the same experiment.

Methods. To address this question, we compared the transport of six compounds run separately with the results of the same compounds run together (cocktails).

Results. The study clearly demonstrated that the outcome of the experiments were totally different depending on the strategy used. Furthermore, the study highlights the importance of having the resistance to drug transport offered by filters without cells under control, as the filter membrane itself can be the rate-limiting step for some compounds; in addition, there is always a potential risk of interactions between molecules in cocktails as well as drug-drug interaction at the level of BBB transporters. In this study, the presence of several P-glycoprotein substrates in the drug cocktail was found to cause breakdown of the BBB.

Conclusions. The results demonstrate that unless a strategy that involves running several compounds in the same experiment is properly validated, the results are of little predictive value.

KEY WORDS: blood-brain barrier; drug screening; endothelial cells; *in vitro*; P-glycoprotein.

INTRODUCTION

The recent development in robotic and combinatorial chemistry synthesis in pharmaceutical research and development generates a huge number of potentially pharmacologically interesting compounds. This has led to increasing demands on the evaluation processes that are performed when selecting compounds for lead optimization. Hence, there is a need for screening methods with good capacity as well as methods that generate good-quality data for reliable prediction of the *in vivo* situation in terms of, for example, absorption, blood-brain barrier (BBB) permeability, safety, and metabolism.

The BBB is a regulatory interface that poses a formidable obstacle to the effective treatment of many disorders affecting the central nervous system (CNS). Due to the presence of this restrictive barrier between the blood and the brain, many potential drug candidates cannot be used for the treatment of CNS diseases because they do not reach therapeutic concentrations in the brain at acceptable doses. Obtaining early information about the transport characteristics of potential drug candidates across the BBB in CNS discovery programs is therefore vital but could also prove valuable for discovery programs aimed at peripheral targets and where it is desirable to keep the compounds out of the CNS to minimize the risk for side effects.

By using an *in vitro* model of the BBB, it is possible to assay most drugs in buffer solution with High Performance Liquid Chromatography (HPLC) or Liquid Chromatography Mass Spectrometry (LCMS), which means that potential drug candidates can easily be screened at an early stage of the drug discovery process for optimal transport properties.

Evaluation of drug transport to the brain *in vitro* has usually been carried out by studying the transport of individual molecules across endothelial cell monolayers. In accordance with previously demonstrated *in vitro* results in an intestinal *in vitro* model (1–5), we aimed to investigate whether permeability studies could be accelerated in an *in vitro* BBB model by running several compounds together in the same experiments.

In an attempt to address this issue, we studied the transport of the compounds separately or together. The compounds were chosen in such a way that a number of properties known to affect transport could be addressed. Caffeine and antipyrine penetrate the BBB well whereas inulin is regarded as relatively impermeable; finally, vincristine, cyclosporin A, and doxorubicin are well-known substrates for P-glycoprotein. All the transport studies were performed in the presence of a paracellular marker, [^{14}C]-sucrose, in order to monitor potential toxic effects on the BBB exhibited either by single compounds or the drug cocktails.

MATERIALS AND METHODS

Chemicals and Antibodies

[U-^{14}C]sucrose (615 mCi/mmol) and [^{3}H]inulin (540 mCi/mmol) were obtained from Amersham Laboratories (Les Ulis, France). Caffeine, antipyrine, vincristine, doxorubicin, and cyclosporin A were obtained from Sigma (St. Quentin fallavier, France). The mouse anti-vimentin antibody, the rabbit polyclonal antibodies against ZO-1, occludin, and claudin-1 were from Zymed Laboratories Inc. (San Francisco, CA, USA). Primary antibodies were detected with appropriate combination of fluorescently labeled secondary antibodies from Molecular Probes, Inc. (Eugene, OR, USA): CyTM3-conjugated goat anti-mouse IgG, Bodipy-conjugated goat anti-rabbit IgG, and Alexa Fluor 568-conjugated goat anti-rabbit IgG, respectively.

Cell Culture

Primary cultures of mixed glial cells were made from newborn rat cerebral cortex. After the meninges had been

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ABBREVIATIONS: BBB, blood-brain barrier; BCEC, brain capillary endothelial cell; CNS, central nervous system; CsA, cyclosporin A; Dox, doxorubicin; Pe, permeability coefficient of endothelial cells; PSf, filter permeability surface product area; PSt, total permeability surface product area (filter and endothelial cells); Vcr, vincristine.

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removed, the brain tissue was gently forced through a nylon sieve, as described by Booher and Sensenbrenner (6). Glial cells were plated in a 6-well dishes at a concentration of 1.2×10^5 cells/ml in 2 ml of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (Integro, Leuvenheim, NL), and the medium was changed twice a week.

Three weeks after seeding, glial cells were stabilized, and coated filters were set in. Brain capillary endothelial cells (BCECs), isolated and characterized as described by Méresse *et al.* (7), were plated at a concentration of 4×10^5 cells/ml on the upper side of the filters (Millicell PC 3 μM, 30-mm diameter, Millipore Corp, Bedford, MA, USA) coated with rat tail collagen prepared by a modification of the method of Bornstein (8). The medium shared by both cell types was DMEM supplemented with 10% (v/v) heat-inactivated calf serum and 10% (v/v) horse serum (Integro, Leuvenheim, NL), 2 mM glutamine, 50 μg/ml gentamycin, and bFGF (1 ng/ml). The medium was changed every other day. Under these conditions, BCECs formed a confluent monolayer after 7 days. Experiments were performed 5 days after confluence.

Fluorescence Microscopy

Endothelial cells grown on porous filter were fixed with 4% paraformaldehyde and permeabilized with cold aceton (–20°C). The samples were washed with PBS and soaked in a blocking solution: Tris-buffered saline (20 mM Tris·HCl, 0.5 M NaCl, pH 7) containing 5% ovalbumin and 1% heat-inactivated normal goat serum. They were then incubated with the mouse anti-vimentin antibody. After rinsing, the cells were incubated 1 h with the secondary antibody, CyTM3-conjugated goat anti-mouse IgG.

For the localization of tight junction–associated proteins, BCECs were fixed with 4% paraformaldehyde for occludin, 1% paraformaldehyde for ZO-1, and with cold methanol (–20°C) for claudin-1. Then, in all staining protocols, cells were permeabilized with 0.1% Triton X-100 in PBS and soaked in a blocking solution: PBS containing 10% heat-inactivated normal goat serum. BCECs were then incubated with appropriate combination of primary and secondary fluorescently labeled antibodies in PBS added with 2% heat-inactivated normal goat serum. Following three washes in PBS, the filters and their attached monolayers were mounted on glass microscope slides with Mowiol mountant (Hoechst, Frankfurt, Germany). The specimens were visualized and photographed with Leica fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Transport Experiments

All transport studies were conducted at 37°C in buffered Ringer's solution, pH 7.4. After a previous dissolution in water (antipyrine, caffeine, doxorubicin, and inulin) or in methanol (cyclosporine A and vincristine) at the concentration of 1 mM, all compounds were diluted in buffered Ringer's solution to the tested concentration (1 μM or 500 nM). To establish that all six compounds were really dissolved when tested alone or in cocktails, five injections of the test solutions (1 μM or 500 nM) were performed. The good reproducibility of the LCMS analyses ensured good dissolution of all compounds (data not shown).

Prior to the transport experiments, cell monolayers were washed twice with Ringer's solution. At the initiation of the experiment, 2.5 ml of buffered Ringer's solution were added to wells of a 6-well plate. One insert containing a confluent BCEC monolayer was placed in the first well of the 6-well plate. Then, tested compounds, alone or together, were added at a concentration of 1 μM to the apical compartment. Different cocktails were assayed containing only 1 (Cocktails B), 2 (Cocktails C) or 3 (Cocktail A) P-glycoprotein substrates. The integrity of the BCEC monolayers was checked by adding [^{14}C]sucrose as a radioactive tracer (80 nM) in each upper compartment.

The plates were then placed on a rocking platform. At selected times, 20, 60, and 120 min after the addition of the test solution, the inserts were subsequently moved to other wells to minimize back diffusion of compound to the upper compartment.

At the end of the experiments, aliquots were taken from each lower compartment. The amount of radiotracers was measured in a liquid scintillation counter (Tri-carb 2100TR, Packard Instrument, Meridian, CT, USA), and the amount of tested compounds was evaluated by LCMS analysis (Thermofinnigan, LCQ duo, P4000, DAD 6000).

Data Analysis and Calculation

The cleared volume was calculated, as described by Siflinger-Birnboim *et al.* (9), by dividing the amount of compound in the receiver compartment by the drug concentration in the donor compartment at each time point. The average cumulative volume cleared was plotted vs. time and the slope estimated by linear regression analysis to give the mean and the standard deviation of the estimate. The slope of the clearance curve with inserts alone and inserts with BCEC monolayers is equal PSf and PSt, respectively, where PS is the permeability surface area product. The units of PS and S are microliters/minute and square centimeters, respectively. The PS-value for endothelial monolayer (PSe) was obtained as follows:

$$1/PSe = 1/PSt - 1/PSf \quad (1)$$

To generate the endothelial permeability coefficient, Pe (cm/min), the PSe-value was divided by the surface area of the insert.

All the values presented are the mean of triplicate inserts and are representative of three series of independent experiments.

RESULTS

BCEC Monolayer Characterization

In Fig 1A, phase contrast micrograph of BCECs co-cultured 12 days with glial cells on an insert coated with rat tail collagen is depicted. The cells form a homogenous monolayer of non-overlapping and contact inhibited cells. The distribution of immunofluorescent staining of specific tight junction–associated proteins, such as, ZO-1 (Fig. 1B), occludin (Fig. 1C), and claudin-1 (Fig. 1D), around the cell borders clearly reveal that the barrier tight junctions are well developed. This in addition to our numerous previous works concerning the characteristics of this *in vitro* system such as high transendothelial electrical resistance (TEER) (500–800 Ohms.cm^2), low permeability of sucrose and inulin (10), presence of specific transporter proteins such as P-glycoprotein (11), and a good *in vitro-in vivo* correlation (12,13) supports the use of this co-culture system as relevant model for making predictions of drug transport to the brain.

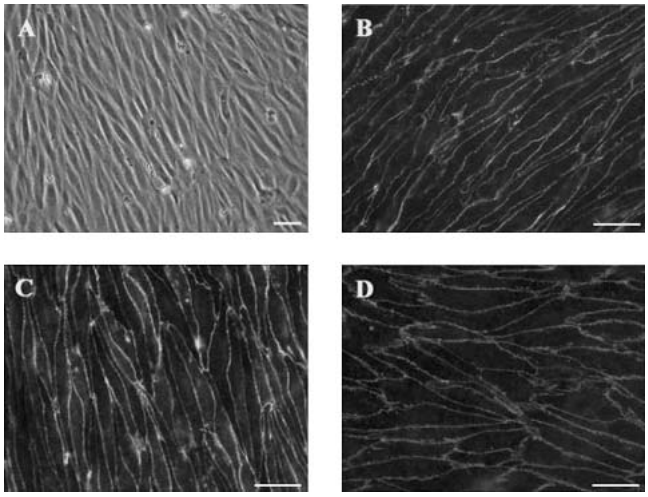

Fig. 1. (A) Phase-contrast micrographs of confluent BCECs. Immunostaining of specific tight junction associated proteins: (B) ZO-1, (C) occludin, and (D) claudin-1. Bars = 50 μm.

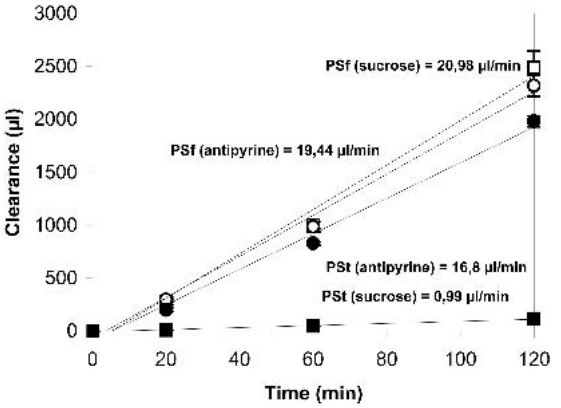

Fig. 2. Apical to basolateral transport of sucrose (□, ■) and antipyrine (○, ●) across BCEC monolayers. Confluent monolayer of BCECs and filters coated with collagen were incubated with 1 μM of sucrose or antipyrine for 120 min at 37°C. To obtain a concentration-independent transport parameter, the clearance principle was used. Clearance for insert coated with collagen and EC monolayers (black) and clearance for inserts coated with collagen (white) were plotted vs. time. The slopes of the clearance curves gave PSt and PSf and allow us to calculate the Pe values as described in "Materials and Methods." Each point is mean of three different filters, and the curves are representative of three independent experiments.

Transendothelial Transport Studies

As described previously, the permeability coefficients across the BCEC monolayers were calculated from compounds either run separately or together as cocktails. The transport assay was performed using triplicate inserts with BCEC monolayers to generate a PSt value or with triplicate inserts only coated with collagen to obtain the PSf value.

Endothelial permeability coefficients (Pe) were first determined for each compound run separately at a concentration of 1 μM. By right of example, detailed results are shown for sucrose and antipyrine and as shown in Fig. 2, the slopes of the clearance curves were linear up to 120 min for both compounds. The linearity was the same for all tested compounds. Resulting Pe values are listed in Table I. The PSf values obtained ensure that the passage of the compounds was not rate-limited by the resistance offered by the filter membrane except for vincristine and cyclosporin A, where PSf values were very low (5.08 μl/min and 1.44 μl/min, respectively). The Pe values ranged between $67.6 \pm 27.4 \times 10^{-3}$ cm/min for a molecule such as caffeine, which is known to penetrate the BBB well, to $0.05 \pm 0.004 \times 10^{-3}$ cm/min for a relatively impermeable molecule such as inulin. For each molecule, the integrity of the tight junctions of the endothelial monolayer was monitored by incubating the tested compounds with [^{14}C]sucrose, which diffuses very slowly across the BBB both *in vitro* and *in vivo* (12,14). No compound alone had any effect on the tight junctions, which remained intact with $Pe_{sucrose}$ ranging from 0.25 ± 0.01 to $0.5 \pm 0.03 \times 10^{-3}$ cm/min, depending on the experimental conditions (data not shown).

In a subsequent experiment, the Pe values were determined for six different compounds run together (caffeine, antipyrine, doxorubicin, inulin, vincristine, and cyclosporin A) (cocktail A, including three P-glycoprotein substrates). Each compound in the cocktail had a concentration of 1 μM. As in the previous experiments, [^{14}C]sucrose was added to monitor monolayer integrity during the experiments.

When the cells were incubated with cocktail A, there was a 24-fold increase of [^{14}C]sucrose permeability indicating a breakdown of the *in vitro* BBB [$Pe_{sucrose} = 5.95 \pm 0.95 \times 10^{-3}$ cm/min vs. $0.25 \pm 0.01 \times 10^{-3}$ cm/min (control)]. This leaky barrier obviously made the calculated Pe values for the different compounds in the cocktail misleading.

The same results were obtained when the experiment was performed with the same cocktail of compounds at the concentration of 500 nM ($Pe_{sucrose} = 6.42 \pm 1.27 \times 10^{-3}$ cm/min).

In contrast, no toxic effects were detected when the cells were incubated with cocktails containing only one P-glycoprotein substrate (cocktails B), as evidenced by $Pe_{sucrose}$ varying only between 0.34 ± 0.03 to $0.58 \pm 0.06 \times 10^{-3}$ cm/min depending on the experimental conditions (Fig. 3). The Pe value for each molecule was subsequently determined and found to be in the same range as when the respective molecules were run separately. As an example, the Pe values obtained for all compounds in a cocktail containing only doxorubicin as P-glycoprotein substrate [cocktail B (Dox)] are listed in Table I.

These results were confirmed by immunofluorescent staining of vimentin (a cytoskeleton marker) and ZO-1 after the endothelial cells had been submitted to these different experimental conditions. As shown in Fig. 4, vimentin and ZO-1 stainings after incubation with cocktail B (Dox) indicates a slight stress effect on the endothelial cell monolayers

Table I. Permeability Values for Analytes Tested Alone or in Cocktail B (Dox)

	Alone Pe ($\times 10^{-3}$ cm/min)	Cocktail B (Dox) Pe ($\times 10^{-3}$ cm/min)
Caffeine	67.6 ± 27.4	80.4 ± 48.4
Antipyrine	29.4 ± 5.3	33.8 ± 12.6
Vincristine	0.83 ± 0.06	–
Doxorubicin	0.45 ± 0.03	0.64 ± 0.21
Cyclosporin A	0.17 ± 0.06	–
Inulin	0.05 ± 0.004	0.10 ± 0.002
Sucrose	0.25 ± 0.01	0.58 ± 0.06

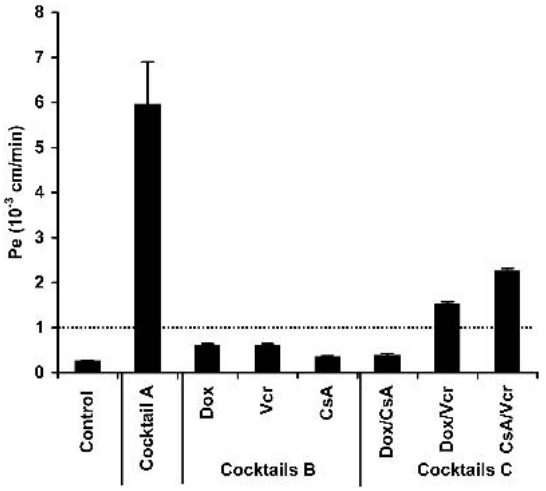

Fig. 3. Endothelial permeability coefficients (Pe) for sucrose in absence (control) or presence of three (cocktail A), one (cocktail B), or two (cocktails C) P-gp substrates. Each Pe value obtained is the mean of three different inserts and is representative of three independent experiments. $Pe_{sucrose} = 1 \times 10^{-3}$cm/min (dotted line) is considered as the threshold value for BBB integrity.

compared to the control but never displayed shriveled cytoskeleton proteins or gaps between the cells as observed after incubation with cocktail A with both stainings. Furthermore, fluorescent cell nuclei can be observed in the BCECs incubated with cocktail A due to accumulation of doxorubicin.

When the same transport experiments were performed in the presence of cocktails containing two P-glycoprotein substrates (cocktails C), there was a 6- and 9-fold increase in the sucrose permeabilities in the presence of cocktail B containing (Vcr/Dox) and (Vcr/CsA), respectively, suggesting a compromised BBB function (Pe sucrose $> 1 \times 10^{-3}$ cm/min). Interestingly, no toxicity was observed when the cells were incubated with cocktail C (Dox/CsA) as evidenced by $Pe_{sucrose}$ varying between $0.37 \pm 0.05 \times 10^{-3}$ cm/min and $0.25 \pm 0.01 \times 10^{-3}$ cm/min (control).

DISCUSSION

The development of a cell culture system that mimics an *in vivo* BBB requires endothelial cells to be cultured on microporous supports. In order to reconstruct some of the complexities that exist *in vivo*, an *in vitro* model of the BBB was developed by growing endothelial cells on one side of a filter and glial cells on the bottom of 6-well plastic dishes (12,15). In this BBB co-culture system, the culture medium is shared by both cell populations and allows selective studies on either cell type.

The transport experiments were first carried out with the test molecules separately, and the endothelial permeability coefficients (Pe) were determined for each drug. In the calculations of the Pe, special attention was given to the permeability of the compounds through filters without cells. These values were subsequently subtracted from their respective permeability values across filters with cells according to Eq. 1. In our experience, we generally regard the filter as the rate-limiting step if the PSf value is found to be below 8 μl/min.

We have demonstrated that depending on the properties of the molecules tested, the resistance offered by the filter membrane is clearly very different and needs to be taken into account in order to avoid incorrect conclusions about the permeability characteristics of compounds in the screening cascade. If not, compounds may erroneously be classified into the poor permeability range although it is the filter membrane that is the actual rate-limiting step and not the cell monolayer. Furthermore, if compound properties are very different, there is clearly a potential risk that interactions with the filter membrane will also be different and in some cases impose restrictions on using drug cocktails as a means of increasing the throughput of the discovery pipeline. In contrast, when the compounds are tested separately, it is possible to test and chose the appropriate cell culture insert for transport permeability studies: for example, the PSf for cyclosporin A can vary from 1.44 ± 0.06 μl/min on Millicell PC 3 μm to 32 ± 1.73 μl/min on Falcon 3 μm.

The calculated Pe according to Eq. 1 for the molecules

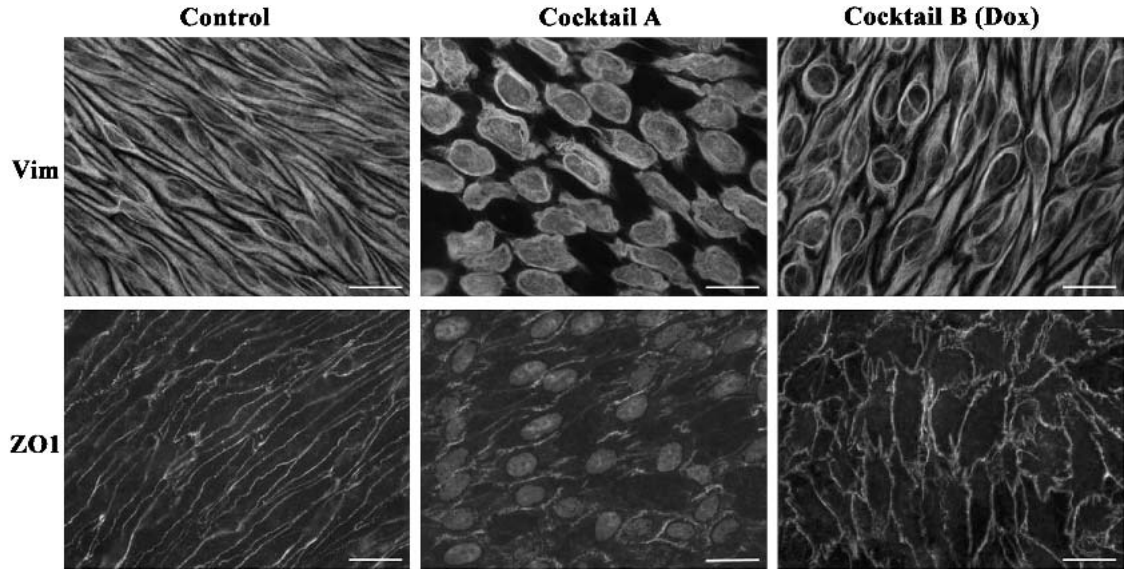

Fig. 4. Immunofluorescent staining of BCEC cytoskeletal protein, vimentin (vim) and tight junction–associated protein, ZO-1, after a 120-min incubation time with buffered Ringer's solution (Control), cocktail A or cocktail B (Dox). Bars = 50 μm.

run separately in this study were all found to be in agreement with previous data *in vitro* and *in vivo* (11,13,16).

The low sucrose and inulin endothelial permeability coefficients confirmed once more that endothelial cells are sealed by highly differentiated tight junctions as demonstrated by our cortical specific tight junction associated protein stainings (ZO-1, occludin, and claudin-1).

The choice of the test concentration was guided by the detection limits of LCMS analysis. Indeed, the concentration of 1 μM allows the detection of all tested compounds considering that some of these compounds have a low brain penetration.

The transport experiments with the drug cocktail of six compounds resulted in a huge increase in sucrose permeability indicating a breakdown of the BBB. We can note that we do not see any differences within the exposure range of 500 nM to 1 μM. Based on these data, we rather suggest that the BBB breakdown could be due to some specific P-glycoprotein substrates interactions.

Indeed, the toxic effects observed have involved the presence of at least two P-glycoprotein substrates, one of which was always vincristine. It can be speculated whether vincristine is either toxic in itself, or indirectly by acting as a P-glycoprotein inhibitor, and thereby inducing a toxic effect of doxorubicin or cyclosporin A (11,17–21).

These properties could allow doxorubicin, an anticancer drug with fluorescent properties (22) that interacts with DNA, to enter into the cell and explaining the fluorescent cell nuclei observed in the experiments of this study with cocktail A.

In conclusion, using drug cocktails as strategy in the screening process leads to potential risks of drug-drug interactions at the level of transporters or metabolic pathways in addition to potential complexities arising from interactions with different materials used in various assays. Such a strategy therefore needs careful validation that may offset potential advantages. Rapid information and feedback of generated *in vitro* data to medicinal chemists is important to allow for optimal rational drug design and support to discovery programs within the pharmaceutical industry. To meet the increasing demands generated by combinatorial chemistry, we believe that the trends toward miniaturization and automatization of the experimental design will be essential and prove more fruitful than using "drug cocktails" and that this process will be fueled not only by the belief that one can greatly decrease the demand for drug and costs of assay reagents but also allow for the screening of a greater number of compounds.

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