BMC Molecular and Cell Biology

RESEARCH

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Method for determining of cytotoxicity based on the release of fluorescent proteins

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Abstract

This paper describes a method for determining the cytotoxicity of chemical compounds based on the detection of fluorescent proteins—in this case, green fluorescent protein (GFP) and red fluorescent protein (RFP), which are released into the medium from dead cells. This method is similar in principle to the lactate dehydrogenase test (LDH test), but it does not require a reaction with a chromogenic substrate. This method also makes it possible to independently determine the viability of different lines when used in cocultures. Experiments were performed on a classical monolayer, spheroids and 3D cultures in alginate hydrogel. Capecitabine was used as a model cytotoxic agent. We included liver cells (Huh7) in a coculture model and determined changes in the cytotoxicity levels of capecitabine against NCI-H1299 cells. The experimental part also found that there were differences in sensitivity to capecitabine depending on the type of 3D cultures used.

Keywords Cell culture, Pharmacology, Toxicology, Metabolism, Spheroids

Introduction

Currently, many methods are used to determine the cytotoxicity of various chemicals and biological products. They can be classified by performance (screening versus single samples), applicability to cell culture type, and underlying principles. The most widely used screening tests are MTT and LDH, the most widely used methods applicable to individual samples are PI and trypan blue staining. All these methods have certain limitations and advantages.

Unfortunately, there is no single method that allows one to reliably determine TC50/EC50 with good inter-methods convergence. Based on our literature review, many commonly used methods exhibit some bias when compared with other methods [1]. Sometimes these differences are twofold or more [2]. In other cases, this bias may be caused, presumably, by the contribution of the drug itself to the absorption spectrum—when using spectrophotometric methods [3]. Bias may also arise due to the intrinsic nuances of the method—for example, non-compliance with time intervals, errors in seeding the required number of cells, metabolic parameter which is taken as an indicator of viability, or the effect of the dye itself [4–6].

In this paper, we propose a new method that would minimize operator influence and maximize convergence at least within a single method. The described method is mainly suitable for mass high-throughput screening. The principle of the method is based on the use of modified cell lines expressing fluorescent protein (this can be GFP, RFP, BFP, etc.). If a cell dies, the fluorescent protein is released into medium (similar to the LDH enzyme

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in the test of the same name) and can be detected by spectrofluorimeter.

General principle of the proposed method and its workflow:

1) Generation of a cell line stably expressing a fluorescent protein. For method reproducibility and stable expression, we recommend the use of lentiviral vectors.
2) Seeding of cells into wells of the plate. The first experiments described in the sections below were performed on a 96-well plate, however, for convenience, 48-well plates can also be used. Also, spheroids and other 3D cultures can be formed from modified cells.
3) Addition of test compounds to evaluate their cytotoxicity. After the required period of time—lysis of cells in the wells designated as a negative viability control with 0.5–1% Triton X100 (or other detergent of choice).
4) Centrifugation of the plates for sedimentation of debris and detached cells.
5) Sampling a growth medium for fluorimetry.

Schematically the principle of the method is shown in Fig. 1.

Advantages and disadvantages of the method—

Advantages:

1) The method does not require chemical reagents other than TritonX100 (for negative control of viability).
2) The method is not very sensitive to handling and accuracy of the operator.
3) The method can be applied to 3D-cultures and co-cultures. With the correct choice of excitation and emission wavelengths, it is possible to detect up to 2–3 signal bands from unmodified fluorescent proteins. The capabilities and palette of the method can theoretically be expanded by using narrow-band engineered fluorescent proteins or developing strategies to compensate the fluorescence spillover.
4) The method is easily scalable and can be applied to high-throughput screening in drug development.

Disadvantages:

1) The method has little justifiable applicability outside of high-throughput screening, because generating each stable cell line is a rather labor-intensive process.

   To mitigate this drawback, we propose a solution in the form of mass generation of xFP-expressing cell lines from the NCI60 list [7]. A set of such stably-modified cell lines can be used by a screening laboratory on a routine basis.
2) To successfully perform this method, there are certain requirements for the formulation of the growth medium—namely the absence of phenol red [8]. Otherwise the method has a low signal-to-noise ratio. In addition, this method has limited applicability if the substances of interest have fluorescence properties.

Selection of drugs and cell lines for experiments

We selected cell lines Huh7 and NCI-H1299 for these experiments.

Huh7 line, established in 1982 from a well differentiated hepatocyte derived cellular carcinoma cell line that was originally taken from a liver tumor in a 57-year-old Japanese male [9]. This cell line retained many phenotypic features of the original tissue, namely, the expression of a wide range of cytochromes responsible for the metabolism (activation, conversion, detoxification) of xenobiotics [10]. This property is especially important in in-vitro toxicological studies and translational research [11]. Cell line NCI-H1299 was established from a lymph node metastasis of the lung from a patient who had received prior radiation therapy [12]. This cell line is one of the most common in lung cancer research [13–15], radiotherapy [16–18] and chemotherapy [19–21].

For the experiments described in this paper, we chose capecitabine as an example, because this drug is mostly metabolized (activated) by liver cells.

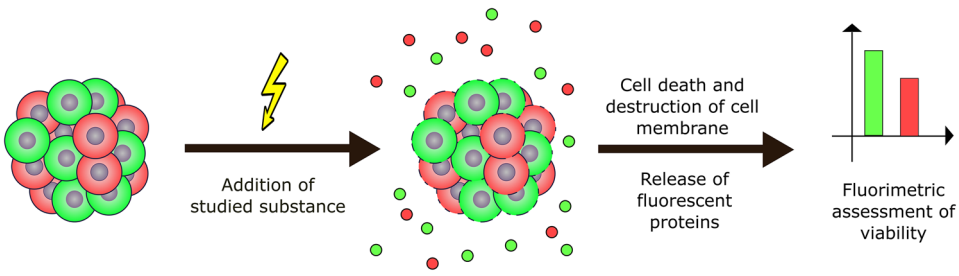

Fig. 1 General principle of the proposed method in case of co-culture of two cell lines, one of which expressing GFP and another—RFP

Experimental section

Materials and methods

As a basal growth medium we used phenol red-free F12 (PanEco, S600p). A basal growth medium was supplemented with fetal bovine serum (FBS) (HiMedia, RM10971-500ML), penicillin-streptomycin (PanEco, A073p') and L-glutamine (PanEco, F032). As dissociating agents during passaging, we used a versene solution (PanEco, P080p) and trypsin-EDTA (PanEco, P039p).

Generation of the modified cell lines

In this paper, we used cell lines Huh7, NCI-H1299 and their genetically modified derivatives, which were created by stable transduction with lentiviral vectors carrying the Tag-GFP2 (Evrogen, LP004 [22]) and Tag-RFP (Evrogen, LP001 [23, 24]) genes [25].

Thus, after the stages of cloning and selection, performed according to standard methods [26], we created the following modified cell lines—Huh7-GFP, NCI-H1299-GFP, NCI-H1299-RFP.

For the sake of reproducibility, we strongly advise against using lines obtained by simple transfection with a non-integrating vector, because the presence of selecting antibiotics (e.g. G418) may interfere with viability measurements.

Design of proof-of-concept experiment

The described experiment serves to establish the functionality of the principle of the method and answer the questions—

1) Is it possible to detect a fluorescent signal from fluorescent proteins that were released as a result of cell death or lysis?
2) Is it possible to independently measure the fluorescence of released proteins in at least two different channels?

The experiment involved 2 cell lines, derivatives of NCI-H1299 – NCI-H1299-GFP and NCI-H1299-RFP, expressing GFP and RFP respectably. Non modified NCI-H1299 was also taken as a negative control to measure intensity of autofluorescence.

The cells were seeded into the wells of a 96-well plate in the amount of 20 thousand cells per well. The cells were seeded in 6 rows of wells—2 rows for a line expressing green fluorescent protein, 2 rows for a line expressing red fluorescent protein, and 2 rows for a mixture of cells from both lines. Of all the groups of cell lines, one row was for the experiment, the other for the control.

After 24 h of incubation in a humidified CO2 incubator at 37 °C and 5% CO2, 10 µl of medium was added to the control wells, and 10 µl of 10% Triton-X100 was added to the experimental wells. The contents of the wells, to which the detergent was added, then mixed by pipetting. Then the culture plate was placed in the incubator for 20 min to lyse the cells, then the contents of the wells with detergent were once again carefully mixed by pipetting. The plate was placed in a centrifuge with adapters for the plates and centrifuged at 1000*g* for 10 min. 50 µl was taken from each well and transferred to a new plate. 50 µl of complete growth medium was added to three empty wells to use as a blank control. This new plate was then measured on the BMG Clariostar plate reader in fluorimetry mode. The results for RFP are shown in Fig. 4a, for GFP in Fig. 4b.

Readings were performed on these wavelengths:

For RFP: excitation—554 nm, emission—585 nm.
For GFP: excitation—488 nm, emission—516 nm.

Experiment to determine the toxicity of capecitabine using described method

For this experiment, cell lines NCI-H1299-RFP and Huh7-GFP were taken. Capecitabine was chosen to demonstrate the capabilities of the method.

Cells were seeded at a concentration of 20,000/well of a 96-well plate. The following combinations were used: NCI-H1299-RFP monoculture, Huh7-GFP monoculture, NCI-H1299-RFP/Huh7-GFP coculture, and NCI-H1299-RFP/Huh7 coculture. For cocultures, 10,000 cells of each line were seeded per well. The experiment was carried out in triplicate. For each mono- and coculture, their own wells were allocated for intact control (positive viability control) and negative viability control (at the end of the experiment, the cells in these wells will be lysed). After 24 h, the growth medium in the wells was replaced with a medium containing the studied compound (capecitabine), in the wells for controls a simple replacement of the medium was performed.

After 48 h of incubation in a humidified CO2 incubator at 37 °C and 5% CO2, 10 µl of medium was added to the control wells, and 10 µl 1% Triton X100 was added to the experimental wells. The contents of the wells to which the detergent was added were mixed by pipetting. Then the culture plate was placed in the incubator for 20 min to lyse the cells, then the contents of the wells with detergent were once again carefully mixed by pipetting. The plate was placed in a centrifuge with adapters for the plates and centrifuged at 1000*g* for 10 min. 50 µl was taken from each well and transferred to a new plate. 50 µl of complete growth medium was added to three empty wells to use as a blank control. This new plate was then measured on the BMG Clariostar plate reader in fluorimetry mode. The results of the experiment are shown in Fig. 5a–c.

Experiment to determine the applicability of the method to 3D cultures (spheroids and alginate hydrogel)

Spheroids

For this experiment, cell lines NCI-H1299-RFP and Huh7-GFP were taken. The spheroids were obtained by the hanging drop method. Briefly, rows of drops with a volume of 25 µl were placed on the inside of the top lid of a Petri dish, each drop containing 2000 cells. 10 ml of PBS was then added to the bottom of the Petri dish and the top lid was carefully inverted and placed on top. After 5 days, the spheroids were formed.

The medium was then changed twice, 10 µL at a time. Then after, 5th day, 10 µl of medium in droplets, which contained spheroids for the experiment, was replaced by 10 µl of complete culture medium containing 375 µM capecitabine. Thus, the final concentration of capecitabine in 25 µl was 150 µM. This concentration was chosen based on the results of a previous experiment.

After 48 h of incubation in a humidified CO2 incubator at 37 °C and 5% CO2, 10 µl of medium was taken from each group of droplets and pooled into their respective wells. The entire contents of the drops designated as "negative viability control" were taken along with the spheroids and transferred into a 250 µl PCR tube. 6 µl of Triton-X100 was added there. The tube was then vortexed and left to incubate on a rotator at room temperature for half an hour before being mixed again and centrifuged at 1000*g* for 10 min to pellet debris. 60 µl from this tube was transferred to the corresponding well of the plate. This new plate was then measured on the BMG Clariostar plate reader in fluorimetry mode. The results of the experiment are shown in Fig. 6a–c.

Alginate hydrogel

For the purposes of this work, an experiment was conducted to create three-dimensional "droplets" of cross-linked sodium alginate with cells enclosed inside. The protocol outlined in the work [27] with minor modifications was taken as a basis. The volume of spheroids was changed—100 µl, the order of addition and composition of the cross-linking solution CaCl2, the concentration of sodium alginate and the number of cells were changed. The sodium alginate concentration of 2.5% stated in Smit et al. [27] turned out to be irreproducible in practice—uniform stirring and manipulation of such a solution turned out to be impossible due to its excessive viscosity. After a series of preliminary experiments, it was decided to settle on a concentration of 1.1%, and use 1% CaCl2 in F12 as a crosslinking solution.

Before starting the experiment, a solution of 1.1% sodium alginate in Hanks' buffer was prepared. 1.1 grams of sodium alginate was added to 100 ml of Hanks' solution in a heat-resistant flask. Stirring was carried out with a magnetic armature for 4 h at a temperature of 55 °C. After stirring all visible lumps and inhomogeneities, the temperature on the magnetic stirrer was raised to 200 °C and the solution was brought to a boil twice for sterilization (since its sterilization through filters is impossible due to its viscosity). After which the flask with the solution was introduced into the laminar flow and the solution was aseptically transferred into a test tube for cooling.

A solution for "crosslinking" was also prepared—F12 with the addition of sterile calcium chloride to a concentration of 1%. To perform this experiment, cell lines NCI-H1299-RFP, NCI-H1299-GFP, Huh7-GFP and Huh7 were taken. Cell cultivation was carried out according to standard procedures. Upon reaching 80–90% confluency, Petri dishes with cultures were washed with versene, the cells were trypsinized with a 0.25% trypsin-EDTA solution, and the cell suspension was transferred to centrifuge tubes. After centrifugation at 1000 *g* for 5 min, the supernatant was removed and the cells were resuspended in 2 ml of fresh F12 medium. The concentration of the suspensions in all cell lines was adjusted to 1 million/ml, and 1 ml of the suspension of each line was transferred into a 1.5 ml Eppendorf, in the case of cocultures—0.5 ml of each.

Thus, the following were prepared for the experiment:

1) Monocultures—NCI-H1299-GFP, NCI-H1299-RFP, Huh7-GFP
2) Cocultures—NCI-H1299-GFP/Huh7, NCI-H1299-RFP/Huh7 and NCI-H1299/Huh7-GFP.

After pippeting, tubes with cell suspensions were centrifuged at 1000*g* for 10 min. In parallel, two 12-well plates were taken, and 4 ml of cross-linking solution was added to each well. After centrifugation, the supernatant was removed and 1 ml of Hanks' solution with sodium alginate was added to each tube. After slow and careful mixing, 2 drops of a cell suspension in sodium alginate were dripped into the wells of the plate from a height of 5–7 cm. The volume of each drop is 100 µl. This height is optimal for such viscous droplets that they acquire a spherical shape upon contact with the cross-linking solution. The excavation was carried out according to the following scheme indicated in the Table 1.

The crosslinking solution was then removed from all wells, the wells were washed twice with F12, and 2 ml of fresh F12 was added to each well. 3D cultures in alginate drops were cultivated for 14 days, the medium was replaced every 2 days. As visible three-dimensional structures grew and formed in the alginate droplets (shown in the figures), the formation of spheroids was monitored Fig. 2).

Table 1 Scheme of sodium alginate droplets

Cell line	Capecitabine	Negative viability control	Positive viability control
	Plate №1		
NCI-H1299-GFP	2 droplets	2 droplets	2 droplets
NCI-H1299-RFP	2 droplets	2 droplets	2 droplets
Huh7-GFP	2 droplets	2 droplets	2 droplets
	Plate №2		
NCI-H1299-RFP/Huh7-GFP	2 droplets	2 droplets	2 droplets
NCI-H1299-RFP/Huh7	2 droplets	2 droplets	2 droplets
NCI-H1299/Huh7	2 droplets	2 droplets	2 droplets

Interestingly, the Huh7 and Huh7-GFP cultures, when growing in an alginate matrix, along with spheroids (at the later stages of cultivation—days 9–12) began to form elongated, radially located formations (Fig. 3).

On the 14th day of cultivation, the medium in the wells was replaced with a medium containing 150 μM capecitabine (for the corresponding wells). In control wells, the medium was replaced with regular F12 medium. After 36 h, 20 μl of Triton X100 was added to the negative control wells. After another 12 h, 100 μl of medium was taken from each well of each culture into the wells of a 96-well plate, and fluorescence was measured in the GFP and RFP channels on a CLARIOStar BMG spectrofluorimeter. Another day later, an additional measurement was performed. Thus, we got 2 time points—48 and 72 h. The results of the experiment are shown in Fig. 7a–f.

Demonstration of the limits of applicability of the method and its use for assessing the cytotoxicity of other compounds

To identify the limits of the method's applicability, we conducted another experiments—

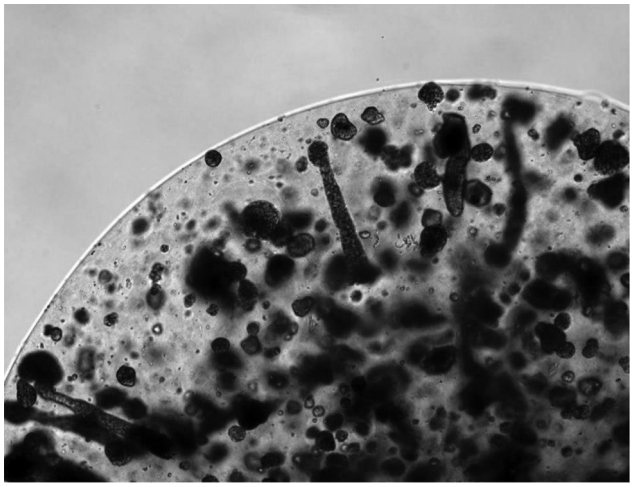

Fig. 3 Huh7 culture, 11th day of cultivation, phase contrast microscopy. 100x magnification

The first one was to establish how heavy metals (Co, Pt) affect the measured fluorescence intensity. As is known from publications [28], platinum compounds can inhibit LDH and bias the results of viability analyses which based on measuring its activity. In addition, cobalt can also inhibit this enzyme. However, in the method described in this article, the reporter proteins are fluorescent proteins, and we conducted the experiment with them.

In a second experiment, we measured how drug metabolites themselves can bias viability measurements. For this experiment, we used paracetamol as a model compound. It was chosen because its main metabolite, NAPQI (N-acetyl-p-benzoquinone imine), is capable of depleting glutathione stores and forming conjugates with proteins, thus inhibiting them and/or disrupting their spatial structure.

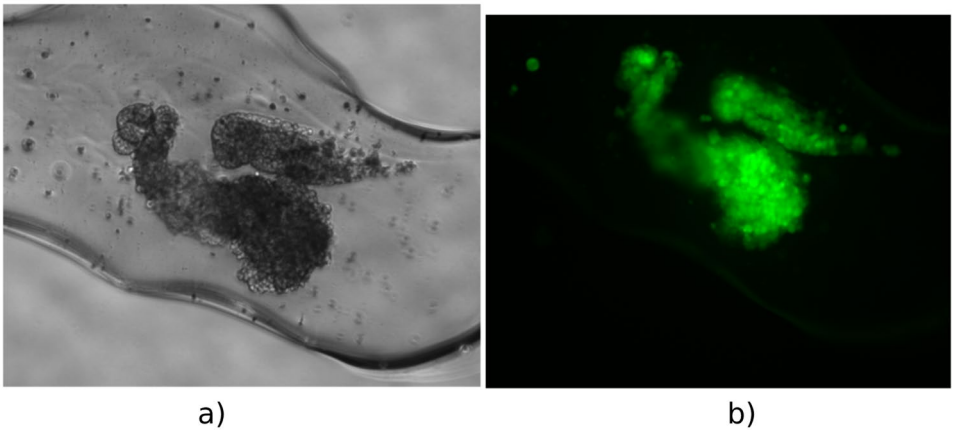

Fig. 2 **a** NCI-H1299-GFP culture growth, phase contrast microscopy. 5th day of cultivation, **b** NCI-H1299-GFP culture growth, GFP fluorescence channel. 400x magnification

Experiment to determine the influence of heavy metals on measurement results

NCI-H1299-GFP and NCI-H1299-RFP cells were prepared for the experiment and lysed using the same protocol as described in the chapter "Design of proof-of-concept experiment". Then, 1 M $CoCl_2$ solution was prepared in serum-free medium, a solution of carboplatin with a concentration of 10 mg/ml was also taken.

50 µl of lysate were added to the wells of 96-well plates, in groups—lysate of cells expressing GFP, and lysate of cells expressing RFP. Then 50 µl of solutions of the studied substances were added to these wells (final concentrations are indicated): CoCl2 from 500 to 0.5 mM, carboplatin—from 1000 to 10 µg/ml. Wells in which 50 µl of serum-free medium was added to the lysate were used as a positive control; wells without lysate, which contained 100 µl of serum-free medium, were used as a blank. The plates were mixed on an orbital shaker and then incubated at 37 °C overnight. After that, fluorescence was measured in the GFP and RFP channels. The results of the experiment are shown in Fig. 8 (for $CoCl_2$) and 9 (for carboplatin).

Experiment to assess the effect of drug metabolites on fluorescent proteins

Huh7, Huh7-GFP, NCI-H1299-GFP and NCI-H1299-RFP cells were prepared for the experiment using the same protocol as described in the chapter "Experiment to determine the toxicity of capecitabine using described method". The cells were seeded in a 96-well plate at 10,000 cells/well in the same combinations. After 24 h, paracetamol was added to the cells at final concentrations of 300 to 10 mM. After another 48 h, the culture medium was taken for fluorimetry in GFP and RFP channels. The results of the experiment are shown in Fig. 10a–c.

Results

Proof-of-concept experiment

The results show great specificity of detection in the respective excitation and emission channels (Fig. 4).

Determination of capecitabine cytotoxicity (2D cultures)

After measuring the fluorescence level, the results were calculated using the formula to determine viability:

$$via\% = \frac{(\mathrm{F\ exp} - \mathrm{F\ min} - \mathrm{F\ blank})}{(\mathrm{F\ max} - \mathrm{F\ min} - \mathrm{F\ blank})}$$

Where:

F blank—fluorescence of fresh medium
F exp—fluorescence of a medium from wells with previously added capecitabine
F min—fluorescence of a medium from wells with intact cells (positive control of viability)
F max—fluorescence of a medium from wells with lysed cells (negative control of viability)

Results from this formula can be expressed as any decimal fraction between 0 and 1 and then converted to a percentage. Where 0 is the absence of cell death, and 1 is—100%, a full cell death.

Using this formula, results were obtained for the toxicity of capecitabine in mono- and co-cultures against

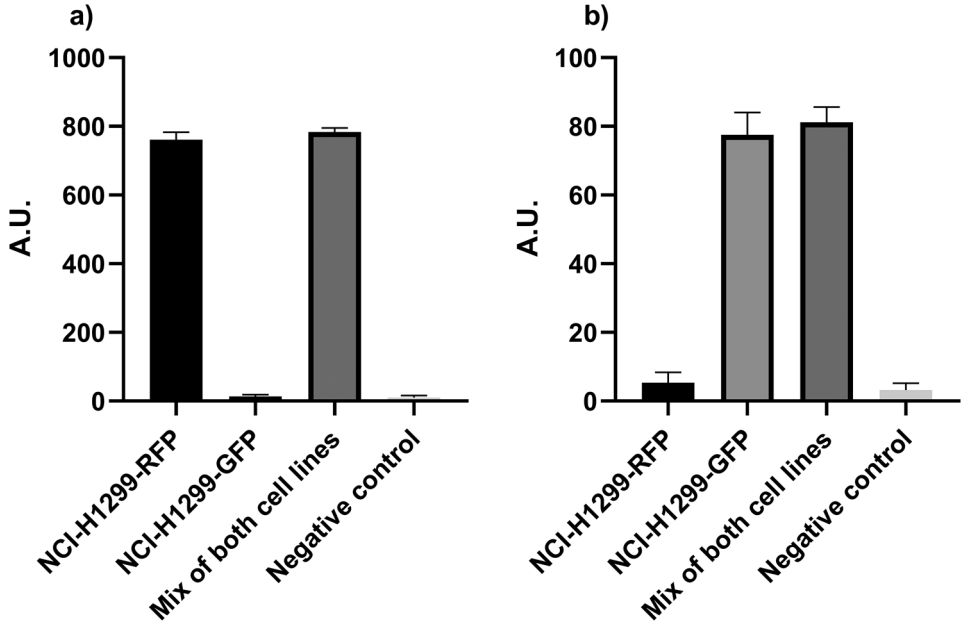

Fig. 4 **a** specificity of cell lysate fluorescence detection in RFP channel, **b** specificity of cell lysate fluorescence detection in GFP channel

NCI-H1299-RFP, NCI-H1299-GFP and Huh7-GFP cells (Fig. 5). Note that the calculated TC50 (toxic concentration), in case of NCI-H1299-RFP and NCI-H1299-GFP, differs between RFP and GFP channels, possibly due to cell decomposition products which have a great fluorescence in GFP-like range and thus interfering with a results.

Calculated IC50 for capecitabine:

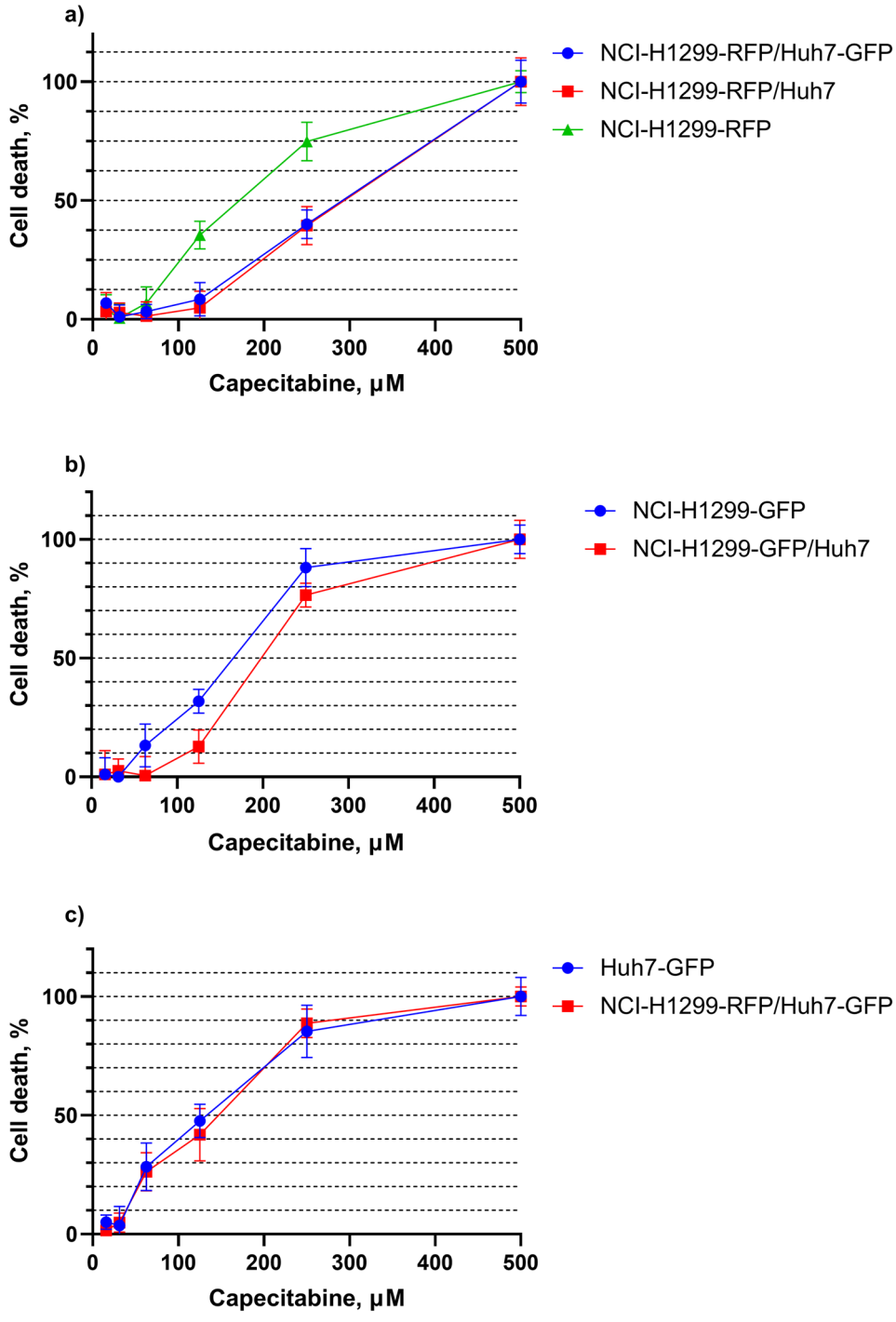

Fig. 5 **a** viability of NCI-H1299-RFP in cases of mono- and cocultures, **b** viability of NCI-H1299-GFP in cases of mono- and cocultures, **c** viability of Huh7-GFP in cases of mono- and cocultures

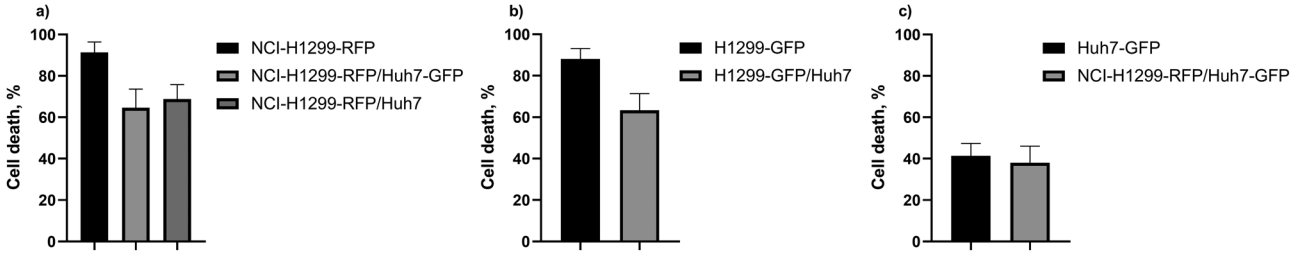

Fig. 6 **a** viability of NCI-H1299-RFP cells in mono- and cocultures, **b** viability of NCI-H1299-GFP cells in mono- and cocultures, **c** viability of Huh7-GFP cells in mono- and cocultures

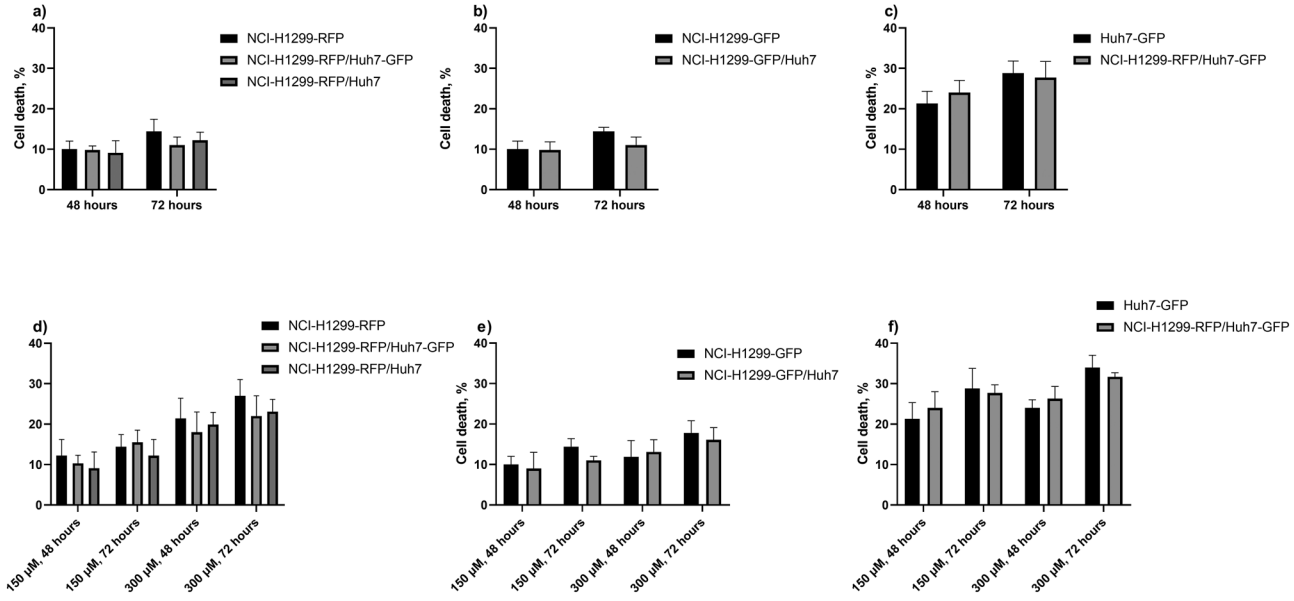

Fig. 7 **a** viability of NCI-H1299-RFP cells in mono- and cocultures, **b** viability of NCI-H1299-GFP cells in mono- and cocultures, **c** viability of Huh7-GFP cells in mono- and cocultures; **d** comparison of NCI-H1299-RFP cells viability in the presence of 150 and 300 µM capecitabine, **e** comparison of NCI-H1299-GFP cells viability in the presence of 150 and 300 µM capecitabine, **f** comparison of Huh7-GFP cells viability in the presence of 150 and 300 µM capecitabine

NCI-H1299-RFP—175,6 µM
NCI-H1299-RFP (coculture with Huh7-GFP)—309,9 µM
NCI-H1299-RFP (coculture with Huh7)—282,4 µM

NCI-H1299-GFP—158,2 µM
NCI-H1299-GFP (coculture with Huh7)—197,2 µM

Huh7-GFP—137,8 µM
Huh7-GFP (coculture with NCI-H1299-RFP)—146,3 µM

Determination of capecitabine cytotoxicity (3D cultures)

Spheroids

The results show a significant reduction in the toxicity of capecitabine in the presence of metabolizer cells (Huh7) against NCI-H1299 cells (Fig. 6a and b). Note that the viability of Huh7 cells in monoculture and coculture does not change as much, which indicates that the presence of Huh7 influences NCI-H1299, and not vice versa (Fig. 6c).

Alginate hydrogel

The results show a dramatic reduction in sensitivity to capecitabine. Interestingly, this happens in all cases (mono- and cocultures) (Fig. 7). An additional experiment was carried out using the same method, where 300 µM capecitabine was taken into the experiment—sensitivity (i.e. cell death) increased, but not much (Fig. 7d, e, f).

Experiment to determine the influence of heavy metals on measurement results

The results of the experiment to assess the level of influence of heavy metals (Co) show that even small amounts of cobalt (500 µM) reduce GFP fluorescence to 57% and RFP to 84% of the initial lysate fluorescence (Fig. 8). In the case of carboplatin, no significant effect of the

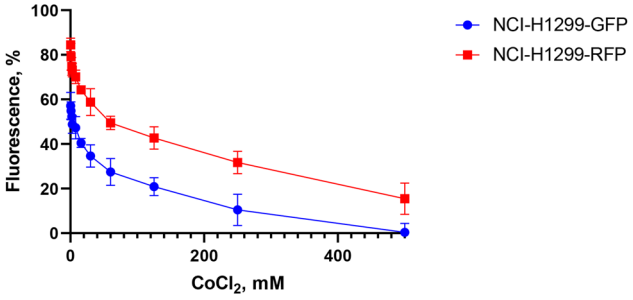

Fig. 8 Fluorescence level of cell lysate containing GFP and RFP proteins in the presence of $CoCl_2$. Fluorescence is normalized relative to each group, with fluorescence of the intact sample taken as 100%

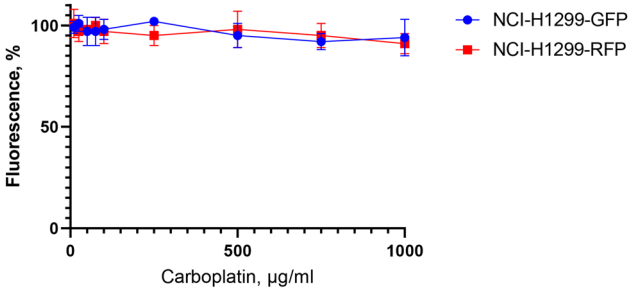

Fig. 9 Fluorescence level of cell lysate containing GFP and RFP proteins in the presence of carboplatin. Fluorescence is normalized relative to each group, with fluorescence of the intact sample taken as 100%

presence of Pt on protein fluorescence was observed (Fig. 9).

Experiment to assess the effect of drug metabolites on fluorescent proteins

The results shown at Fig. 10a–c, and it reveals two interesting effects at once—on the one hand, there is a "hump" on the cell death graph, on the other hand, with increasing concentration this "hump" is smoothed out, and the fluorescence level decreases. This effect can be explained by the accumulation of NAPQI and its interaction with glutathione, and then with cysteine in proteins. It can also be noted that when the Huh7 line is added to the co-cultures, the death of NCI-H1299 cells increases. This can be explained by the fact that Huh7 is a liver cell line, which itself has increased glutathione reserves (i.e. has a greater capacity for detoxifying NAPQI), but also more intensively metabolizes paracetamol into NAPQI, poisoning the surrounding cells (NCI-H1299) that do not have the same great capacity for detoxifying NAPQI. This effect makes it impossible (or at least extremely difficult) to use this method to accurately measure the cytotoxicity of substances that (or their metabolites) indiscriminately disrupt the structure of proteins by forming adducts.

Conclusion

This work was intended to demonstrate a proof-of-concept of the possibility of independent detection of cell death of different cell lines in cocultures. Currently, there are not many methods for detecting cell death in 3D cultures, and, as far as the authors know, there are no methods for assessing cell death in cocultures that would allow, at least semi-quantitatively, estimating the percentage of death of each of given lines included in the coculture. Moreover, the described method can be used not only for the purposes of screening promising drugs—the method can be used in modeling pathological conditions when endogenous metabolites produced by one type of cells can negatively affect another type of cells. The results showed an effect of Huh7 cells and their metabolism on NCI-H1299 cells: the presence of Huh7 reduces the toxicity of capecitabine to NCI-H1299 cells, but there is no reciprocal effect. Most likely, this may be due to the fact that capecitabine is a prodrug of 5-fluorouracil, most of which is metabolically activated by liver cells, where it exerts most of its RNA synthesis inhibition locally. In the case of paracetamol, the situation is different—the amounts of NAPQI and the rate of its formation are so high that the concentration in the presence of liver cells increases so quickly that it affects the surrounding cells. Quite interesting results obtained as a result of this work include the identified difference in sensitivity to capecitabine when culturing cells in spheroids and in alginate hydrogel. We found differences in sensitivity to cytostatics between 3D cultures in which assembly processes predominate (spheroids) and cultures in which growth and division processes predominate (alginate hydrogel). Currently, this field of comparative in vitro toxicology is virtually unexplored—anecdotal findings include the results of the Senkowski group, who discovered a difference in sensitivity to nitazoxanide when culturing cells in 2D monolayer and in 3D spheroids [29]. The disadvantages of the method identified during the work include: the method is semi-quantitative, the method is sensitive to the presence of heavy metals that easily form bonds with proteins (e.g. cobalt) and the method is of little use in conditions where metabolites of the drug, or the drug itself, easily form adducts with sulfur-containing amino acids. The latter disadvantage can be resolved by using cysteine-free fluorescent proteins that are resistant to the formation of such adducts—for example, cfSGFP2 [30].

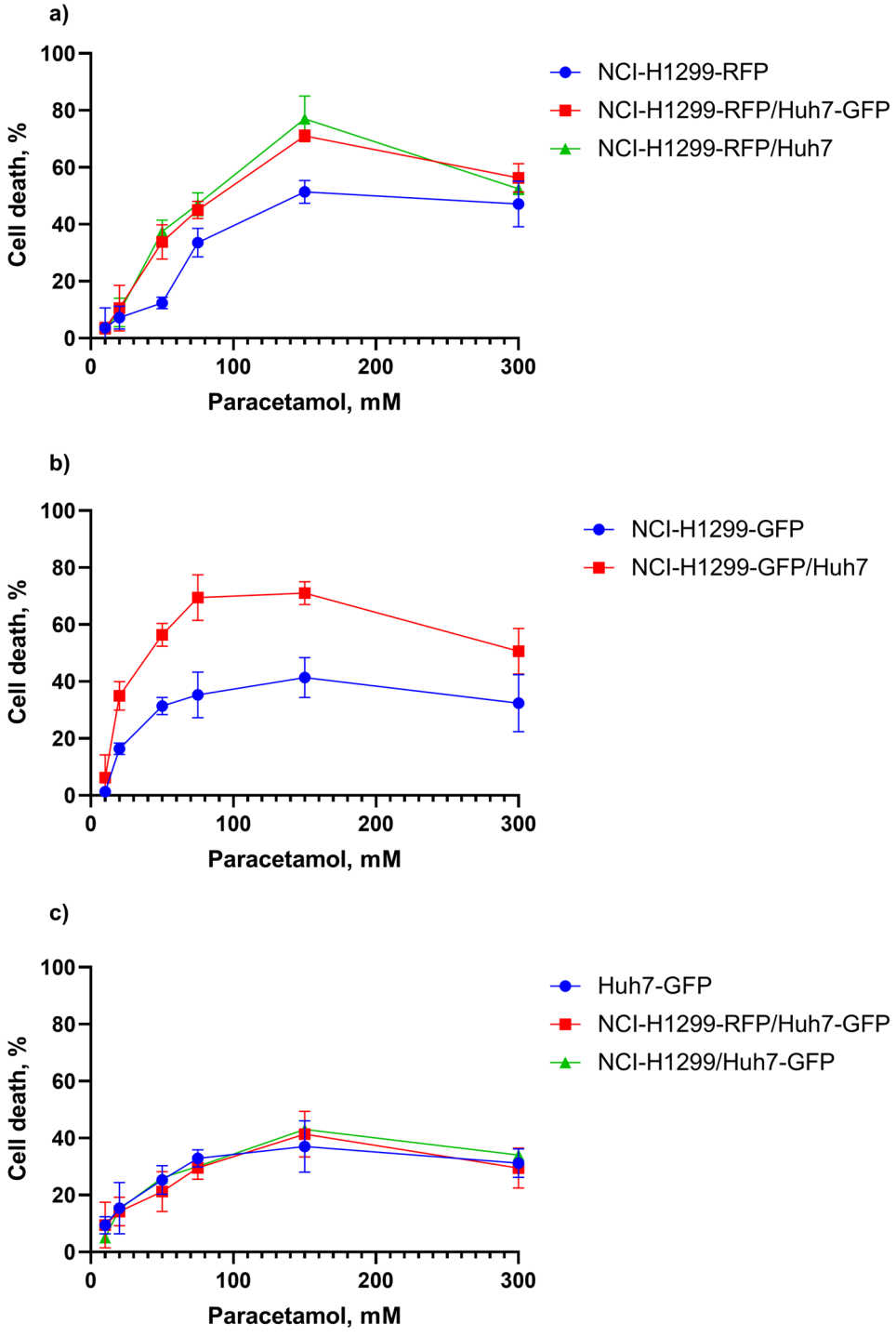

Fig. 10 **a** Comparison of NCI-H1299-RFP cells viability in the presence and absence of Huh7 liver cells, **b** Comparison of NCI-H1299-GFP cells viability in the presence and absence of Huh7 liver cells, **c** comparison of Huh7-GFP cell viability in the presence and absence of NCI-H1299 cells

Acknowledgements
Not applicable.

Author contributions
L.D., E.S. and D.Z. carried out the experiments; L.D., A.K., K.G. and E.P. wrote the main manuscript text; L.D., K.G. and E.P. performed review and corrections; E.S. and A.K. prepared the figures. All authors reviewed the manuscript.

Funding
This scientific project was financed on the basis of "The Agreement on the provision of subsidies for financial support of the execution of the state task for the provision of public services (performance of work) No. 141-03-2024-014 from January 18, 2024—Development of molecular genetic diagnostic methods for the quantification of sanogenesis in healthy people (norm) 122030900062-5".

Data availability
All data generated or analysed during this study are included in this published article

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Clinical trial number
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 15 October 2024 / Accepted: 21 January 2025
Published online: 28 January 2025

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