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Implementation of Geneticin in the *in vitro* cell culture and *in vivo* studies

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ABSTRACT

Geneticin, widely known as G418, is an aminoglycoside antibiotic produced by a bacteria *Micro-monospora rhodorangea.* Geneticin blocks the polypeptide synthesis in both prokaryotic and eukaryotic cells by inhibiting the elongation step. In consequence, rapidly dividing cells are affected more than those with a small proliferation rate. Therefore, geneticin is frequently used in molecular biology as a selective agent for mammalian cells with a different time of population doublings and during cell transfection. The dose of the G418 that is used for the selection of the transfected eukaryotic cells depends on many factors like: the type of the cells and organism from which they were isolated, as well as the growth conditions and the selected culture media. Which is why, the concentration of this antibiotic should be selected for each cell line individually.

Here, we present an overview regarding the spectrum of applications, in which geneticin can be imple-mented as a general selective agent in normal and cancerous cell culture studies.

Keywords: Geneticin G418, cells' selection, cell culture, cancer

INTRODUCTION

In 1974 a new aminoglycoside produced as the major component by a new species of a gram-positive and spore-forming bacteria *Micromonospora rhodorangea* was introduced to the public and named Geneticin. The production of geneticin was carried out by the submerged fermentation in a soybean-dextrin medium, and next subsequent adsorption from the fermentation broth by an ion-exchange procedure (Wagman et al. 1974).

Geneticin is composed of three rings functionalized with ammonium, hydroxyl and methyl groups, thus its structure is similar to gentamicin (especially gentamicin B1 and gentamicin A – other aminoglycoside antibiotics known since 1944; Aubrecht et al. 2011), therefore it may be called as gentamicin G or by a well-known abbreviation G418 (Vicens and Westhof 2003). The lyophilized base is a white powder soluble in water or methanol, whereas sulfate is soluble only in water. A typical stock solution is of a 50 mg/ml concentration (Wagman et al. 1974).

Although with some limitations geneticin retains a high biological and pharmaceutical compound of interest. Geneticin blocks the polypeptide synthesis due to the irreversible binding to the 70S and 80S cell ribosomes and inhibition of the protein elongation. In consequence, geneticin presents broad toxicity against bacteria, yeast, protozoa, helminths and mammalian cells. Resistance to G418 is conferred by the bacterial gene for aminoglycoside-3'-phosphotransferase (APH(3')) that can be expressed in eukaryotic cells (Vicens and Westhof 2003, Prokhorova et al. 2017).

SEARCH STRATEGY AND SELECTION CRITERIA

For the literature analysis performed with the use of the PubMed and Google Scholar databases, the combination of the search terms "geneticin – G418 – cell culture – transfection – cell selection" has been implemented. As the selection criteria we have chosen articles in which geneticin was used as a means for the cell selection or treatment purposes. Moreover, during hand-search some additional articles were also investigated for a better comprehension of the subject.

STATE OF ART

Geneticin presents a strong influence on all types of cells from prokaryotic to the eukaryotic, especially for those with a high proliferation rate. G418 mode of action mainly results in the inhibition of protein synthesis, the activation of phosphati-dylinositol phospholipase C (which leads to the release of GPI-anchored proteins), as well as the increase of dihydroxyacetone phosphate acyltransferase and the peroxisomal β-oxidation activity (Jin et al. 2004). Some of these functions, however, may be reduced. For example, the irreversible binding of G418 to ribosomes may be compro-mised by the bacterial aminoglycoside phosphotransferases APH(3')II and APH(3')I encoded by the genes on transposons Tn5 and Tn601 (903), respec-tively. As a con-sequence, cells transfected with genes of neomycin resistance (neo) from transposon Tn5 or Tn601 gain resistance to G418 and grow in media supplemented with this antibiotic. The selection strategy should be applied individually for various types of cells, as geneticin's effective concentration differs according to the growth medium, culture conditions as well as metabolic rate of the cells (Davies and Jimenez 1980, Scholar 2007).

Response to G418 depends on cell metabolism, i.e. some cell lines or clones are able to better tolerate a metabolic load than others. Moreover, geneticin may differently influence cell growth and their proliferation rate depending on the composition of the culture media (serum, glutamine and insulin concentration). As an example, the addition of G418 may cause the change of the flux of glucose from the lactate production towards either the TCA cycle to provide energy or other biosynthetic pathways (Yallop and Svendsen 2001). Further action of geneticin on cells can include caspase-3-dependent apoptosis (initiated by at least two pathways: cytochrome c release and endoplasmic reticulum (ER) stress) with cell shrinkage and nuclear fragmentation (Jin et al. 2004).

REVIEW AND DISCUSSION

In general, the use of geneticin may be implemented in case of one of these three applications: (i) for selecting stable clones after transfection, (ii) to eliminate the quickly dividing cells from the mixed cell culture and (iii) as a potential treatment for several diseases. Each application requires an individual analysis regarding the optimal concentration of G418, time of treatment, as well as the application of the culture medium and other conditions.

G418 FOR THE SELECTION OF STABLE TRANSFECTANTS

Transfection is a process of introducing exogenous genetic material in the form of DNA, RNA, messenger RNA (mRNA), small interfering RNA (siRNA), microRNA (miRNA) and short hairpin RNA (shRNA) into mammalian cells, that is widely used in the study of genes and protein expression. Two types of transfection can be obtained depending on the applied methodology: transient (chemical or physical) and stable (biological and physical). In principle the physical method is based on the direct injection of the genetic material, biolistic particle delivery, electroporation or laserfection; whereas the biological method is connected with the virus-mediated gene transfer (the so-called transduction). On the other hand, the chemical method requires the usage of cationic polymers, cationic lipids or calcium phosphate. The selected approach depends on the cell type and the object (gene or protein) of the experiment (Sheikh et al 2017).

The usability of geneticin as a selective agent after transfection has been known since 1980, because only positively transfected cells and resistant to geneticin are able to grow in culture media supplemented with this antibiotic (Davies and Jimenez 1980). Typically, 24 or 48 hours after cell transfection the old medium is replaced by the full growth medium with the appropriate G418 concentration and the incubation is carried out though 1-4 weeks with the G418-medium exchange every 3-4 days. Articles describing various protocols of the cell selection methods after transfection were gathered in Table 1, e.g. the physical method – electroporation (Fountain et al. 1988), biological – with virus (Belsham et al. 2004) and chemical – with Lipofectamine 2000 reagent (Yang et al. 2019). Due to the fact, that the optimal dose of G418 for the selection of transfected eukaryotic cells depends on numerous factors (e.g. cell type, culture medium, growth conditions and the cells' metabolic rate), the most suitable approach is to self-determine the sensitivity of each cell line to geneticin before performing the transfection (Tab. 2). Several concentrations of G418 ranging from

100 µg/ml to up to even 2 mg/ml should be tested. The treatment should be carried out for at least 48 hours. Next, the cytotoxic effect of G418 on cells can be checked with the MTT test (the conversion of tetrazolium salt into formazan by living cells and calculation of the half maximal inhibitory concentration – IC_{50}) (Sehati et al. 2019, Sadeghiyeh et al. 2019). If the antibiotic concentration is too low or the cell seeding density is too high, the cells can escape the selection. Thus, the selected drug concentration should be approximately 25-50% higher than the minimal concentration required to kill 100% of the cells within 7-14 days (Mally et al. 1992). Another matter to discuss is the growth medium used for the cell culture. Some medium components (e.g. serum, glutamine, insulin) may significantly influence the effect of geneticin (tab. 3), which is why the experiment aimed at the selection of a proper G418 concentration should be performed in the medium appropriate for the study (Yallop and Svendsen 2001).

Table 1. Sample conditions for cell transfection and next clone selection with geneticin (***shortcuts: Biol.TM – biological transfection method, Chem.TM – chemical transfection method, Phys.TM – physical transfection method; CF- calf serum, HS – horse serum)

Cell type used for transfection (cell line)	Growth medium	Transfection method (TM)	Clone selection with G418 (concentration and time)	Reference
Human skin fibroblasts (HSF)	EMEM+15% FBS	Phys.TM: electroporation	400 µg/ml for 4 weeks	Fountain et al. 1988
Spontaneously immortalized human skin keratinocytes (HaCaT)	EMEM+10% FBS, 100 U/ml penicillin and 50 µg/ml streptomycin	Chem.TM: the Ca^{2+}-phosphate precipitation method	800 µg/ml	Boukamp et al. 1990
Mouse murine melanoma (HFH18)	DMEM+5% CS	Phys.TM: electroporation	1000 µg/ml for 10-14 days	Armstrong et al. 1994
Human primary lung cancer cell lines	RPMI+10% FBS	Chem.TM: DMRIE-C reagent	800 µg/ml for 12-21 days	Tomizawa et al. 2001
Human cervical cancer (HeLa); Human melanoma (A375, WM9)	(not mentioned)	Phys.TM: electroporation	50 µg/ml (WM9), 500 µg/ml (HeLa, A375)	Leaman et al. 2002
Human melanoma (A2058)	DMEM+10% FCS, 2 mM glutamine, 10 mM HEPES, penicillin-streptomycin (100 IU/ml, 100 µg/ml)	Chem.TM: Lipofectamine	400 µg/ml	Sounni et al. 2002
Primary fetal mouse hypothalamus cells	DMEM+10% FBS, 10% HS, 1% penicillin-streptomycin and 20 mM D-glucose	Biol.TM: virus	400-600 µg/ml for 2-3 weeks	Belsham et al. 2004
Human colon cancer (Lovo)	RPMI+10% FBS, 100 µg/ml streptomycin and 100 µg/ml penicillin	Chem.TM: LipofectAMINE2000	1000 µg/ml for 4 weeks	Wang et al. 2007
Human melanoma (SK28, 1205Lu)	RPMI+10% FCS and antibiotics	Chem.TM: FuGENE reagent	700 µg/ml for 3 weeks	Alexaki et al. 2010
Rat epithelial cells (Fischer rat thyroid line 5; FRTL-5)	Coon's modified F-12 medium + 5% CS and 6- hormone mixture	Chem.TM: FuGENE reagent	for 2 weeks	Di Palma et al. 2013

Human melanoma (A375)	DMEM+10% FCS, 15 mM HEPES, 2 mM glutamine, 100 U/ml penicillin and streptomycin	Chem.TM: Lipofectamine 2000	500 mg/ml for 2 weeks	Wang et al. 2014
Human neuroblastoma (SH-SY5Y)	DMEM+10% FCS, 100 µg/ml penicillin-streptomycin and 1% Glutamax	Chem.TM: Lipofectin reagent	500 µg/ml for 4 weeks	Pirou et al. 2017
Human skin cancer (A431), human melanoma (SK-MEL-28)	(A431) RPMI+10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin; (SK-MEL-28) MEM+10%FBS, 1 mM sodium pyruvate, 1× non-essential amino acid, 100 U/ml penicillin and 100 µg/ml streptomycin	Chem.TM: Lipofectamine® 3000 reagent	600 µg/ml for 2 weeks	Lee et al. 2018
Mouse inner medullary collecting duct 3 (IMCD3)	DMEM-F12+10% FBS and 1% penicillin-streptomycin-kanamycin cocktail	Chem.TM: Lipofectamine® 3000 reagent	800 ng/µl for 5-6 days	Mirvis et al. 2019
Human lung cancer (A549)	RPMI+10% FBS	Chem.TM: jetPEI® solution	50 mg/ml for 2 weeks	Sadeghiyeh et al. 2019
Human melanoma (A375)	RPMI+10% FBS	Chem.TM: jetPEI® solution	460 µg/ml for 72 h	Sehati et al. 2019
Human melanoma (A375, SK-MEL-28)	DMEM+10% FBS	Chem.TM: Lipofectamine 2000 reagent	500 µg/ml for 4 weeks	Yang et al. 2019

Table 2. Sample of the self-determined IC_{50} concentration of geneticin for the used cell lines

Cell type (cell line)	Growth medium	Selection of G418 concentration	IC_{50}	Reference
melanoma (A375)	RPMI+10% FBS	MTT assay (100, 200, 400, 600, 800, 1000, 1200 µg/ml) for 72 h	460 µg/ml	Sehati et al. 2019
lung cancer (A549)	RPMI+10% FBS	MTT assay (100, 200, 400, 600, 800, 1000, 1200 µg/ml)	494.1 µg/ml	Sadeghiyeh et al. 2019

Table 3. The IC_{50} concentrations of geneticin used after cell transfection in different growth media (TOKU-e Company, information materials)

Cell type (cell line)	Growth medium	IC_{50}
colon cancer (Caco-2)	DMEM	500 µg/ml
	MEM	1000 µg/ml
hamster, Chinese ovary (CHO-K1)	DMEM	600 µg/ml
	DMEM + F12 medium	150 µg/ml
	EMEM	500 µg/ml
	Ham's F-12 nutrient mixture	1000 µg/ml
cervical cancer (HeLa)	DMEM	1000 µg/ml
	DMEM + F12 medium	600 µg/ml
	EMEM	400 µg/ml

G418 FOR ELIMINATING CELLS FROM MIXED CULTURE

Except for cells transfected with the neomycin resistance gene, other type of cells naturally resistant to geneticin are melanocytes. These slowly dividing cells (origi-nating from the neural crest) are present in the epidermis and are responsible for the production of melanin – the UV photon-absorbing pigment (Sobiepanek and Kobiela 2020). Their mitosis *in vivo* has been rarely observed, but during the melanocytes culture *in vitro* the doubling time varies from 48 hours to up to even 10 days depending on the growth conditions (De Luca et al. 1988, Hoerter et al. 2012). These cells are important for the research connected with their maturation, migration, melanin production as well as cell transformation to melanoma, the deadliest type of skin cancer (Satyamoorthy and Herlyn 2002, Sobiepanek et al. 2017).

During melanocyte isolation from skin mainly two types of contaminant cells may occur: keratinocytes (other components of the epidermis) and fibroblasts (components of the dermis) (Halaban and Alfano 1984, Sobiepanek et al. 2020). Keratinocytes can be easily separated either from melanocytes as well as from fibroblasts based on differential trypsin digestion (Ścieżyńska et al. 2019), however, separation of melano-cytes and fibroblasts causes a lot of difficulties. The first attempts were made with the addition of cholera toxin (CT) or 12-O-Tetradecanoylphorbol-13-acetate (TPA) to the culture medium, but fibroblasts remained insensitive to these components (Halaban 2005). In 1984 Halaban and Alfano showed that the antibiotic G418 at the concentration of 100 µg/ml applied for 2 days can help in receiving a pure culture of normal melanocytes. From that time, geneticin has been used frequently for these purposes; especially due to the fact that this method requires minimal efforts to obtain a good success rate (Tomonobu et al. 2019). The elimination of fibroblasts, however, may not be immediate and may require some time to observe the expected effect. Moreover, the exposure of melanocyte-fibroblast coculture to G418 has to be repeated in some cases in order to entirely eliminate fibroblasts (Halaban 2005). A few examples of the implementation of geneticin prior to fibroblast eradication from melanocyte culture are gathered in table 4. Similar approach can be included for the elimination of fibroblasts from melanoma cell culture (Chapman et al. 2009).

Table 4. Examples of the G418 usage on cell coculture to eliminate fibroblasts and receive pure melanocytes culture (***shortcuts: IBMX – isobutylmethyl xanthine, EGF – epidermal growth factor, BPE – bovine pituitary extract)

Origin of cells	Growth medium	G418 dose and incubation time	Research focus	Reference
Human neonatal foreskin	Ham F-10 medium + 10% Nu-serum, 2% FCS, penicillin-streptomycin, 48 nM TPA, 2.5 nM CT and 0.1 mM IBMX	100 µg/ml for 48 h	to establish a pure human melanocyte culture *in vitro*	Halaban and Alfano 1984
Human skin from biopsy	Dulbecco-Vogt Eagle's and Ham's F12 media (3:1) + 5% FCS, 5 µg/ml insulin, 5 µg/ml transferrin, 0.18 mM adenine, 0.4 mg/ml hydrocortisone, 1 nM CT, 20 pM triiodothyronine, 10 ng/ml EGF, 10 ng/ml PMA, 50 µg/ml BBE and 50 IU/ml penicillin/streptomycin	100 µg/ml for 2-4 days	to gain knowledge of the dependencies between melanocytes and keratinocytes cultured *in vitro*	De Luca et al. 1988
Human eyes	Ham's F12 medium + 10% FBS, 100 nM TPA, 0.1 mM IBMX and 10 ng/ml CT	100 µg/ml for 3-7 days	to establish isolation and cultivation methods of human uveal melano-cytes	Hu et al. 1993
Human scalp	EMEM + 10% FBS, 0.2 µg/ml CT, 50 nM PMA, 0.05 mg/ml gentamicin, 2.5 µg/ml fungizone and keratinocyte serum-free medium	(details not mentioned)	what is the role of amelanotic hair-follicle melanocytes in hair growth and diseases	Tobin et al. 1995
Hanwoo cattle skin from muzzle	DMEM + 2.5% FBS, 2 mM glutamine, 1 mM sodium pyruvate, 1 % non-essential amino acids, 2.5 ng/ml EGF, 25 µg/ml BPE, 10 ng/ml TPA, 100 U/ml penicillin, 100 µg/ml streptomycin	100 µg/ml for 3-4 days	investiga-ting melanin production and gene expression (MC1R, Tyr, Tyrp-1, Tyrp-2)	Amna et al. 2012
Human neonatal foreskin	Clonetics MGM-4 Melanocyte Growth Media-4 with 100 nM endothelin-3 (EDN3)	150 µg/ml for 48 h	investiga-ting melanin production, transfer and uptake	Gledhill et al. 2015

G418 FOR POTENTIAL TREATMENT OF VARIOUS DISEASES

While being an antibiotic, geneticin may be used in medicine as an antiparasitic agent, but with some limitations due to its toxicity to human ear and kidney (Vincens and Westhof 2003). The *in vivo* research of Aubrecht et al. performed on mice (with the lethal dose of 153 mg/kg) confirmed that the primary target organs of G418 toxicity are kidney and liver. This was shown by the serum biochemistry analysis (a significant increase in the parameters: blood urea nitrogen (BUN) and creatinine (CRE) – markers of the kidney functionality) and histopathological data (present signs of mild nephrotoxicity) (Aubrecht et al. 2011). In case of the *in vitro* study of normal rate kidney cells treated with 400 μg/ml of G418 for 3 days, the cells presented obvious apoptotic features: cell rounding and shrinkage as well as nuclear condensation and fragmentation. Apoptosis was activated in those cells by the caspase- 3 pathway (Jin et al. 2004). In general, G418 acts through different mechanisms depending on the number of positive charges on the antibiotic, its concentration and the organ where the toxicity develops. Known mechanisms also include: the production of hydroxyl radicals by geneticin/ metal ion complexes and the disturbance of membrane functionalities by the interaction with phospholipids and the inhibition of the phospholipases activity (Vincens and Westhof 2003).

Furthermore, in case of patients with cancer, the treatment of the occurring infection may cause a risk to the patient. An example was shown during the *in vitro* study of the human breast cancer cell (MCF-7) treatment with geneticin (10 μg/ml), which allowed the cancer cell survival (by suppressing apoptosis) despite all glucose consumption from the medium under hypoxia (1% O_2). At the same time the untreated cells after glucose consumption died. As it is well-known, in solid tumors there are regions where oxygen supply is very low (hypoxic conditions), thus the addition of G418 to treat the infection may cause a side effect of the cancer cells' resistance as well as the limitation of the action of the drugs dedicated to cancer treatment (Lee et al. 2002).

On the other hand, the anti-apoptotic activity of geneticin may be used against perinatal hypoxic-ischemic (H-I) brain injury to cause a neuroprotective effect. The *in vivo* study was performed on an animal model of Sprague-Dawley rat pups and an intraperitoneal injection of 0.1 μg/kg G418 was made before and after the rats were placed in the hypoxic chamber for a 2.5-hour period (92% N_2 and 8% O_2). In case of mice with H-I brain injury and treated with G418, a decreased number of apoptotic cells was observed as well as a decrease of the Bax/Bcl-2 expression ratio and a decrease of caspase-3 activity (Ju et al. 2008).

Administration of geneticin has proven helpful also in the treatment of patients suffering from several genetic disorders (Vincens and Westhof 2003). A genetic abnormality may be a discrete mutation not influencing our organism or of a major significance (e.g. insertion or deletion), which in consequence leads to genetic diseases. Some disorders may be inherited, while others occur randomly or because of some environmental influence. Genetic disorders are caused by the nonsense or frameshift mutation, which induces the premature termination codons (PTCs) (Miller and Pearce 2014). The most common human genetic disease is cancer, where the appearance of a PTC in a tumor suppressor gene results in the loss of the protein or the synthesis of a truncated protein unable to inhibit cell proliferation or to promote apoptosis. The known examples are mutations in *p53* and *APC* suppressor genes present in 50% of human cancers. The first application of G418 in disease-causing nonsense mutation was in 1985 (Nudelmann et al. 2010). Aminoglycosides (such as geneticin and gentamicin) induce the readthrough of PTCs by binding to ribosomes, which restores the synthesis of a full-length functional protein in the cultured *in vitro* mammalian cells and *in vivo* animal models (Floquet et al. 2011, Bidou et al. 2017). An example is the human cancer cell line containing a PTC (the non-small-cell lung cancer cell line, H1299; p53-null), for which high levels of the readthrough were obtained in the presence of G418 during the *in vitro* study. Also, the viability of cancer cells with the nonsense-mutated *p53* gene was significantly decreased after the aminoglycoside treatment (Floquet et al. 2011).

About 12% of human genetic disorders involve the PTCs (Kuschal et al. 2013), where various aminoglycosides have presented a therapeutic potential for the treatment of cystic fibrosis, Duchenne muscular dystrophy, dystrophic epidermolysis bullosa, the Werner syndrome as well as the Hurler syndrome (Heier and DiDonato 2009, Prokhorova et al. 2017). Recent

development also emphasizes the use of translational read-through inducing drugs as a strategy for treating nonsense mutation-based on retinal disorders (Nagel-Wolfrum et al. 2014, Samanta et al. 2019). Another thera-peutical focus of G418 is on the spinal muscular atrophy (SMA) disease, where it demonstrated an ability to induce the readthrough of the SMN target and to increase the SMN protein level. Moreover, geneticin in the dose of 14 mg/kg administered by the intraperitoneal injection into a SMA mouse increased its motor functions during the *in vivo* experiment (Heier and DiDonato 2009). In general, this antibiotic and the derivatives of aminoglycoside antibiotics offer a rational basis for developing new personalized strategies of treatment for various diseases.

SHORT CONCLUSION

Undeniably, geneticin is a very useful antibiotic with a wide range of application possibilities. For cell biology it allows to eliminate the slowly dividing cells from the culture. In melanocyte-fibroblast coculture the concentration of 100 μg/ml of G418 applied for 2 days can help in receiving a pure culture of normal melano-cytes. In case of molecular biology, it helps in selecting only those cells which underwent trans-fection. The applied concentration of geneticin has to be determined experimentally by using the cells of interest exposed to the treatment of broad G418 concentrations in an appropriate medium and via the MTT assay. The selected drug concentration should be approximately 25-50% higher than the minimal concentration required to kill all the cells within 7-14 days after transection. Concerning the medical field geneticin can be used against various diseases but when taking proper safety precautions. So far, it has been utilized as an antiparasitic agent, neuroprotective compound in the (H-I) brain injury or it has been used to treat patients with genetic disorders caused by nonsense or frameshift mutations.

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