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Journal of Pharmaceutical Innovation
From R&D to Market

ISSN 1872-5120
Volume 13
Number 4

J Pharm Innov (2018) 13:313-320
DOI 10.1007/s12247-018-9322-4

Springer

Springer

Journal of Pharmaceutical Innovation (2018) 13:313–320
https://doi.org/10.1007/s12247-018-9322-4

ORIGINAL ARTICLE

Fractal Aggregation of Dihydroquercetin After Lyophilization

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Published online: 5 May 2018

Abstract

Purpose Fractal geometry is a relatively young mathematical concept, which has been gradually implemented in various fields of human activity. The focus of this article lies on using this powerful tool for monitoring and forecasting the water solubility of dihydroquercetin (DHQ) and its new forms, which were obtained by lyophilization with different coformers. DHQ, also known as taxifolin, is the major flavonoid of larch wood. This compound prevents the accumulation of free radicals and is characterized by a wide range of pharmacological effects. DHQ is produced industrially and is registered in Russia as an active pharmaceutical ingredient (API).

Methods DHQ co-crystals with different coformers were generated by lyophilization. The fractal dimensions of the lyophilizates were measured by photomicrography processing with special software. The water solubility was determined according to the European Pharmacopoeia 8.0.

Results It was established that the solid phases of the DHQ lyophilizates generate specific structures that agglomerate in different fractals. A correlation was shown between the DHQ fractal dimension and its water solubility. The correlation coefficient was 0.94. The relative standard deviation was no more than 1.17%.

Conclusions This study demonstrated the ability to predict the water solubility of DHQ forms by measurement of the fractal dimensions. The fractal model can find wide application in the pharmaceutical industry as a tool for automating quality assays without substance loss.

Keywords Dihydroquercetin (taxifolin) · Fractals · Pharmaceutical analysis · Lyophilizates · Water solubility

Introduction

Most processes in the universe are determined by chaotic behavior, and the generation of fractal structures is a frequent result of such processes [3]. The development of fractal theory has cleared the way to a better understanding of the behavior of complex systems in various processes. Fractality is a powerful tool that can be used to explain a vast number of phenomena, such as the generation of a Langmuir monolayer [1] and the evolution of proteins [13]. There are some applications of fractal geometries in the food and drug industry, for example, monitoring the coffee bean roasting process [5], assaying the pH time series of continuous anaerobic bioreactors for vinasses treatment [6], controlling powder flow properties [7], and investigating the microstructures of dental nano-filled composite materials [12]. Despite its prevalence, the use of fractal analysis is in the initial stage in pharmaceutical analysis.

Currently, researchers are paying increasing attention to dihydroquercetin—2,3-dihydro-3,5,7-trihydroxy-2-(3,4-dihydroxy-phenyl)-4*H*-1-benzopyranone-4 (DHQ), also known as taxifolin (Fig. 1). DHQ is the major flavonoid of *Larix sibirica Ledeb.* and *Larix daurica Turcz.* Due to its high antioxidant capacity, DHQ suggests a wide range of pharmacological activities, combined with the absence of embryotoxicity, teratogenicity, and mutability [19, 20]. The presence of two chiral centers enables the selective interaction of DHQ and its metabolites with biological targets [24]. Some drugs (diquertin and ascovertin) were designed based on DHQ [15, 16]. DHQ is widely applied in the production of food additives. However, industrially isolated DHQ is slightly soluble in

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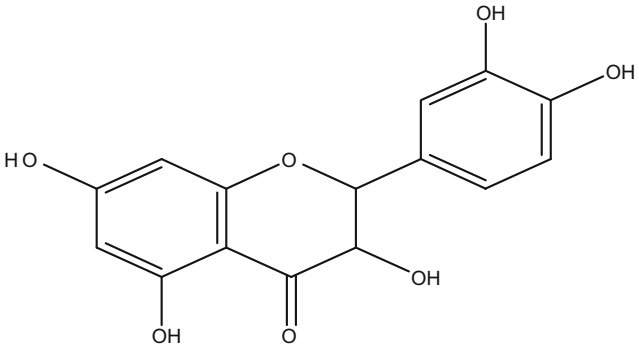

Fig. 1 Chemical structure of DHQ

water and therefore has limited bioavailability [26], which restricts the clinical use of DHQ.

There are many ways to process biologically active compounds to increase their bioavailability, such as crystal engineering [22], micronization [27], chemical modification [9], nanodispersion [21], and clathrate generation [25]. Most are based on improving the water solubility and increasing the dissolution rate. In this study, lyophilization was used to improve the water solubility of DHQ. Lyophilization, also known as freeze drying, is a process to separate an active pharmaceutical ingredient (API) from a solvent to increase its stability over time [4, 18]. No previous studies have examined lyophilizate formation by fractal geometry. Additionally, such approach would enable the use of this powerful mathematical tool for pharmaceutical needs.

The *study objective* was to investigate the potential use of fractal geometry as a rapid method for monitoring and forecasting the water solubility of DHQ forms.

Materials and Methods

Materials

Solid pharmaceutical-grade DHQ (Ametis JSC, Russia) was used as the API, and denatured ethanol (99.8%, Carl Roth GmbH, Germany) was used as the solvent in this study. The lyophilizates were generated with the following coformers: nicotinic acid (99.5%, Fluka, Germany), vanillin (99%, Sigma-Aldrich Chemie GmbH, Germany), cinnamaldehyde (99%, Acros Organics, Belgium), benzaldehyde (98 + %, Acros Organics, Belgium), and urea (99.6%, Carl Roth GmbH, Germany).

Preparation of the DHQ Stock Solution

The stock solution for the generation of each lyophilizate was prepared by the dissolution of 100 mg of DHQ in 5 mL of denatured ethanol.

Preparation of the Lyophilizates

All coformers were added to the stock solution in a 1:1 molecular ratio, and the solutions were diluted with distillated water to 5% API. Before lyophilization, the liquid samples were kept at − 78 °C for 24 h immediately after dilution to minimize ethanol loss. After this step, the flasks were connected to the laboratory freeze dryer (Alpha 1–2 LD, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), which operated under a pressure of 0.35 atm and temperature − 55 °C for 36 h. Each lyophilizate was resynthesized three times (first–third series).

Analysis of the Lyophilizates

Mass Spectrometry

Electrospray ionization mass spectrometry (ESI-MS) was applied to determine the chemical profile of the lyophilizates compared with API and coformers by using mass-spectrometer expressionL CMS (Advion, Ithaca, USA). Each sample was dissolved in a solution of methanol and water (in a volume ratio of 80:20) with 0.1% formic acid. The samples were injected by a microsyringe at a sample flow rate of 0.2 mL/min. The system was operated in both positive and negative ion mode with a resolving power with polarity switching of 50 ms and a mass range of m/z 10–1000.

Microscopy

An inverted microscope, Axiovert S 100 with a AxioCamMRc camera (Zeiss AG, Oberkochen, Germany), was used for imaging the lyophilizates under × 400 magnification. For each sample, two photomicrographs of the DHQ lyophilizates were obtained. The size of the images was 1380 × 1040 pixels. All pictures were transformed into grayscale.

Fractal Analysis

The photomicrography fragments for fractal analysis were determined manually by visual selection according to the following parameters: optimal tone and clear view of the co-crystals.

The fractal dimensions were measured by the FDim software (Laboratory for Computational Longitudinal Neuroimaging, Harvard Medical School, USA) [17]. This computer program is based on the box-counting principle, which determines the number of boxes within the fractal structure. An arbitrary grid is placed over the structure to be measured, and the number of boxes in the grid

Table 1 Water solubility of different DHQ forms

Sample		Solubility according to the European Pharmacopoeia 8.0	Range of solubility (g/ml)
DHQ (API)		Very slightly soluble	0.0001–0.001
Lyophilizates	DHQ with benzaldehyde	Sparingly soluble	0.01–0.03
	DHQ with nicotinic acid	Sparingly soluble	0.01–0.03
	DHQ with cinnamaldehyde	Soluble	0.03–0.1
	DHQ without coformers	Soluble	0.03–0.1
	DHQ with vanillin	Freely soluble	0.1–1.0
	DHQ with urea	Very soluble	> 1.0

that are filled by the fractal structure is counted. This method is based on the formula:

$$Dc = \lim_{\lambda \to 0} \frac{-\log N\ (\lambda)}{\log \lambda}$$

where Dc is the fractal dimension, N is the number of fractal elements (the number of boxes), and λ is the scaling factor, i.e., the ratio of the parameters of one box (square, information, etc.) to the parameters of the whole structure. Using the software containing this formula for photomicrography analysis, a linear relationship was obtained between log N and log λ. Dc is the tangent of the angle between the axis and this line.

Another software, Gwyddion (Czech Metrology Institute, Czech Republic) [11], which is based on the same algorithm, was used for fractal photomicrography analysis to confirm the results.

The statistical significance of equal fractal dimensions, which were measured by FDim and Gwyddion, is determined by the Mann-Whitney U test [10, 14]. The U test is a nonparametric test based on the sum of the ranks of the values in each of the two groups. The U value is calculated by the formula:

$$U = n_1 n_2 + \frac{n_x(n_x + 1)}{2} - T_x$$

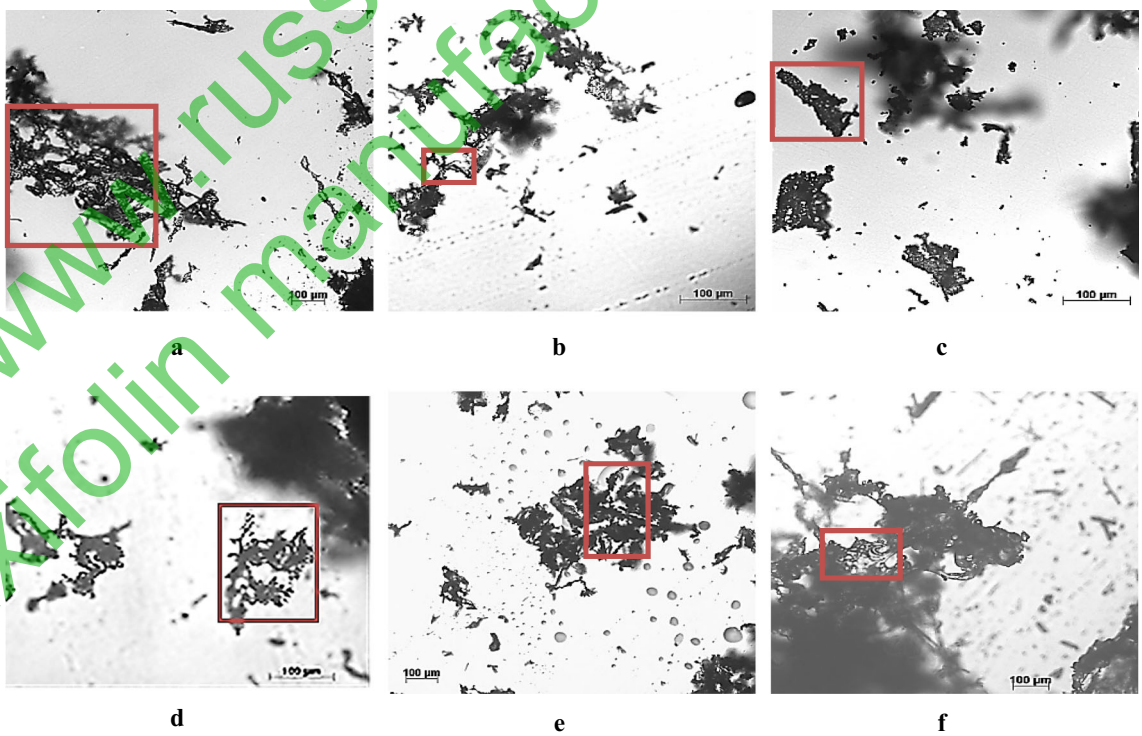

Fig. 2 Representative photomicrography of the lyophilizates: **a** DHQ without coformers, **b** DHQ with vanillin, **c** DHQ with nicotinic acid, **d** DHQ with benzaldehyde, **e** DHQ with cinnamaldehyde, and **f** DHQ with urea (area for fractal analysis is marked)

Table 2 Fractal dimension measurement

Sample	Software	*Dc*			Analytical target profile				*U* value*
		1st series	2nd series	3rd series	Expected value	Variance	Standard deviation	Relative standard deviation, %	
DHQ with benzaldehyde	FDim	2.307 2.999	2.322 2.309	2.317 2.301	2.309	0.0001	0.01	0.39	10.5
	Gwyddion	2.383 2.331	2.331 2.338	2.342 2.301	2.338	0.0007	0.03	1.13	
DHQ with nicotinic acid	FDim	2.396 2.415	2.351 2.368	2.366 2.294	2.365	0.0017	0.04	1.76	17
	Gwyddion	2.364 2.370	2.333 2.387	2.388 2.371	2.369	0.0004	0.02	0.84	
DHQ with cinnamaldehyde	FDim	2.410 2.412	2.498 2.421	2.309 2.411	2.410	0.0036	0.06	2.49	10
	Gwyddion	2.406 2.436	2.400 2.397	2.367 2.387	2.399	0.0005	0.02	0.95	
DHQ without coformers	FDim	2.441 2.455	2.427 2.435	2.423 2.424	2.434	0.0001	0.01	0.51	17
	Gwyddion	2.316 2.422	2.392 2.451	2.457 2.499	2.423	0.0041	0.06	2.63	
DHQ with vanillin	FDim	2.486 2.469	2.494 2.506	2.500 2.507	2.494	0.0002	0.01	0.58	8
	Gwyddion	2.456 2.460	2.463 2.475	2.482 2.511	2.474	0.0004	0.02	0.83	
DHQ with urea	FDim	2.594 2.601	2.568 2.590	2.591 2.580	2.589	0.0001	0.01	0.43	13
	Gwyddion	2.580 2.612	2.621 2.591	2.588 2.592	2.597	0.0003	0.02	0.64	

*There is no difference between software measurements if *U* value > 7 (statistical significance is 5%)

where n_1 is the sample size of the first sample, n_2 is the sample size of the second sample, n_x is the sample size of the largest sample, and T_x is the sum of the largest sample ranks. The result is then checked against the appropriate table.

The predictive ability of fractal analysis was estimated by leave-one-out cross-validation [14]. This method is based on the removal of each individual sample from the dataset and testing the predictive ability on the remaining samples and can be to calculate the training equation.

Solubility

The solubility of the lyophilizates was analyzed according to the European Pharmacopoeia 8.0 [2]. First, 1.0 g of substance was added to 1.0 mL of solvent, and the mixture was shaken continuously for 10 min at a temperature of 20 °C. If the substance fully dissolved than it is *very soluble*. A substance is considered dissolved if no particles can be seen when examined against light. If the substance did not fully dissolve, 1.0 mL of solvent was added to 100 mg of the substance and performed dissolution as described above. If the substance fully dissolved than it is *freely soluble*. If the substance did not fully dissolve, 2.0 mL of solvent was added and dissolution was continued. If the substance has fully dissolved than it is *soluble*. If the substance did not fully dissolve, 7.0 mL of solvent was added and dissolution was continued. If the substance fully dissolved than it is *sparingly soluble*. If the substance did not fully dissolve, 10.0 mL of solvent was added to 10.0 mg of substance and perform dissolution as described above. If the substance fully dissolved than it is *slightly soluble*. If the substance did not fully dissolve, 100 mL of solvent was added to 10.0 mg of substance and performed dissolution as described above. If the substance fully dissolved than it *is very slightly soluble*. And if the substance did not dissolve, then it is *practically insoluble*.

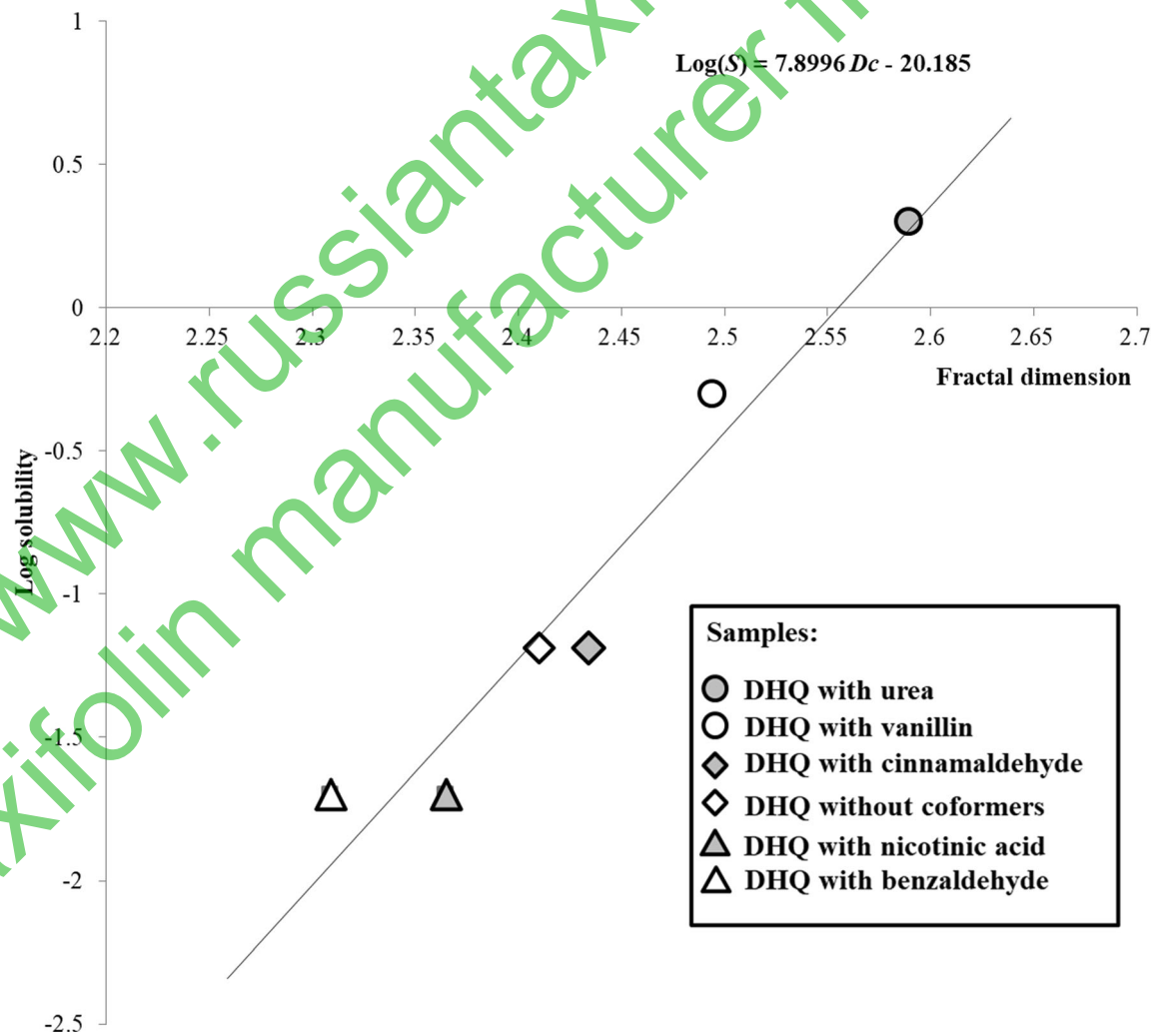

Fig. 3 Logarithmic relationship between the solubility of DHQ lyophilizates and the fractal dimension of their specific elements

Results

The strategy of coformer selection was based on the complementarity rules for functional groups in the formation of noncovalent bounds in supramolecular synthons. DHQ is a heterofunctional compound with five phenol hydroxyl groups with acidic properties and one carbonyl group with weak basic properties. These functional groups can participate in intermolecular hydrogen bond formation. According to this, there is an opportunity for heterosynthon generation between DHQ molecules and carbonyl-containing compounds (benzaldehyde, vanillin, and cinnamaldehyde) or nitrogen-containing compounds (nicotinic acid and urea).

The co-crystal nature of DHQ lyophilizates was discussed previously [23]. Mass spectrometry did not identify any product arising from chemical interactions between lyophilizate components.

During the solubility analysis, it was shown that all the lyophilizates are more soluble than the starting API batch, but the increases in sample solubility were different (Table 1).

During the microscopic analysis of the lyophilizates, it is easy to notice the absence of lyophilizates with a completely homogeneous crystal structure—the majority are in the hopper form. This can be explained by the rapid crystal growth at low lyophilization temperature. In such conditions, the solid phase cannot possibly fill the gaps. At the same time, the microscopic analysis of the representative sampling frame showed that a disordered lyophilizate geometry frequently demonstrates the generation of a specific structural element, an isosceles triangle with angles at the base between 66.18° and 76.03°, which have statistical self-similarity. Despite the superficial visual resemblance these structures, the software can distinguish them by patterns in the fractal geometry (Fig. 2a–f).

The fractal dimensions were calculated for each specific structural element by the box-counting method, which is integrated in FDim. The final values of the fractal dimensions for all co-crystals were obtained by mathematical analysis (Table 2). The relative standard deviation of all the measurements is less than 5%—an acceptable error for pharmaceutical analysis.

The statistical significance of equal fractal dimensions was determined by the Mann-Whitney *U* test, because the fractal dimensions are not binary or discrete and the number of samples cannot give a clear view if the dataset shows a normal distribution.

In the process of data analysis, a logarithmic dependence was found between the water solubility of the lyophilizate and the fractal dimension of its specific elements (Fig. 3). The correlation coefficient is 0.94. Such correlation can be explained by the physical meaning of the fractal dimensions. Dissolution depends on the surface of the API particles. An

Table 3 Leave-one-out cross-validation

Sample	Experimental *Dc**	Training linear equation without sample	Calculated *Dc*	Relative standard deviation (%)	Mean relative standard deviation (%)
DHQ with benzaldehyde	2.309	$\text{Log}(S) = 8.7974Dc - 22.456$	2.359	2.18	1.17
DHQ with nicotinic acid	2.365	$\text{Log}(S) = 7.3363Dc - 18.780$	2.328	1.55	
DHQ with cinnamaldehyde	2.410	$\text{Log}(S) = 7.6845Dc - 19.666$	2.405	0.22	
DHQ without coformers	2.434	$\text{Log}(S) = 7.7002Dc - 19.667$	2.400	1.40	
DHQ with vanillin	2.494	$\text{Log}(S) = 7.3941Dc - 19.010$	2.530	1.45	
DHQ with urea	2.597	$\text{Log}(S) = 7.6589Dc - 19.398$	2.603	0.22	

*According to FDim

increase in fractal dimension is associated with an increase in the square of the interaction with water molecules—the surface of the substance takes an intermediate value between those of 2D and 3D objects. In this case, the fractal dimension is the primary parameter used in the method for the rapid quality control of pharmaceutical substances.

We used leave-one-out cross-validation to examine the predictive ability of our fractal analysis. This statistical method is not very sensitive to different data, but it gives meaningful results if the validation set and training set are drawn from the same population. Our dataset reflects these requirements, so leave-one-out cross-validation is acceptable for prognostic score development (Table 3).

The calculated *D*c, obtained by training a linear equation (without sample, which was used as the control), did not differ with statistical significance from the *D*c generated by FDim. So our predictions were confirmed by experimental data. Thus, it was shown that there is a correlation between the water solubility of the lyophilizates and the fractal dimension of their specific elements.

Conclusions

According to the principles of Good Manufacturing Practice, a pharmaceutical process must be designed and carried out in such a way that the product quality is analyzed at the end of the process and suitable systems are used for designing, monitoring, and controlling a process with the goal of ensuring final product quality. The fractal model can find wide application in the pharmaceutical industry as a tool for automating quality assays without substance loss.

New DHQ forms with different coformers and modified water solubility were obtained by lyophilization. The correlation between the solubility of DHQ forms and the fractal dimension of their specific elements makes it possible to use this parameter for rapid quality control.

This study has some limitations. Further research is needed to determine if it is possible to use fractal analysis for other substances, though it was shown previously that the fractal dimension has a significant influence on the solubility of a substance [8]. However, this study showed the general ability to predict the water solubility of DHQ forms by measurement of the fractal dimension.

Acknowledgments We would like to thank Professor Emeritus N.A. Tyukavkina of Sechenov University for her constructive advice. We also thank Dr. M. Buchholz and Dr. D. Ramsbeck from Fraunhofer Institute for Cell Therapy and Immunology IZI for the opportunity to carry out research in the Department of Molecular Drug Biochemistry and Therapy Development and for their assistance during this work. We also thank Dr. K.V. Balakin and his team for providing help with the language in this manuscript.

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