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Safety evaluation of resveratrol as an active compound for drug-eluting cardiovascular implants

Abstract: Resveratrol is a member of stilbenoids with promising anti-atherosclerotic properties. This hallmark makes it an extremely interesting candidate for local drug delivery to damaged tissue adjacent to the implant in order to reduce implant-related complications. For the regulatory approval drug-eluting medical devices have to be thoroughly tested for safety, efficacy and interactions with the surrounding tissue, including tests for sensitization among others. Studies for sensitization help to estimate the risk for an allergic reaction upon prolonged exposure to a chemical compound. Due to increased social and regulatory demand for replacement of animal experiments by *in vitro* approaches a number of reliable predictive non-animal tests have been developed. Here, we assessed the skin sensitization potential of resveratrol by the direct peptide reactivity assay (DPRA), one of the first non-animal tests adopted by the OECD.

Keywords: stents, endothelialization, resveratrol, drug-eluting, sensitization.

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1 Introduction

Cardiovascular disease is the leading cause of death worldwide [1]. Stents and heart valves are medical devices that save lives by correcting structural and functional defects of the cardiovascular system. However, thrombosis,

neointimal hyperplasia, calcification and foreign body reaction are common post-implantation complications leading to implant failure. Drug-eluting implants gradually release a pharmaceutical compound to counteract these side effects and support the process of tissue healing [2].

Stilbenoids are polyphenolics synthesized in plants in response to stress with promising pharmaceutical properties. Resveratrol (3,4',5-trihydroxy-*trans*-stilbene, RSV) is a member of stilbenoids and has attracted attention because of its outstanding pharmacological potential including anti-atherosclerotic effect, prevention of stenosis and inflammation [3].

The evaluation of the hazard potential of chemicals represents an important part of the risk assessment. Traditionally, this is achieved with animal skin sensitization tests, such as the Local Lymph Node Assay (LLNA) on mice and the guinea pig tests (i.e. Guinea Pig Maximization Test) [4]. To date, animal welfare has become a matter of growing social and regulatory concern raising the need for the reduction of animal experiments and their replacement by *in vitro* and *in chemico* test procedures summarized as the “Three R’s principle” (Replacement, Reduction and Refinement) [5]. Several animal-free skin sensitization test methods have been developed, with DPRA (Direct Peptide Reactivity Assay, OECD 442C), KeratinoSens (OECD 442D) and the h-CLAT (the human Cell Line Activation Test, OECD 442E) being the best known of them and the first implemented by the OECD. Despite the effort put into development of non-animal skin sensitization approaches a combination of at least two *in chemico/in vitro* methods is required as equivalent substitute for the LLNA [6].

In this study we have established an *in chemico* skin sensitization method described in OECD 442C and applied it to evaluate hazard potential of resveratrol when used as active agent for drug-eluting cardiovascular devices.

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2 Materials and Methods

Chemicals and reagents

Peptides containing cysteine (Ac-RFAACAA-OHM) or lysine (Ac-RFAAKAA-OH) with a purity degree of more than 95% were obtained from JPT Peptide Technologies GmbH (Berlin, Germany). Two different acetonitrile (ACN) batches were tested (ACN ultra gradient HPLC grade Lot: 1829801862 (J.T. Baker) and ACN ROTISOLV Ultra LC-MS $\geq 99.98\%$ (Lot:1118321, Roth, Karlsruhe, Germany). Resveratrol (RSV) with a purity $>99\%$ was obtained from biomol (Hamburg, Germany). All other chemicals were obtained either from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, USA).

Stability of the peptides and resveratrol

HPLC (high performance liquid chromatography) analysis of the stability of the peptides containing cysteine or lysine was carried out in accordance with the OECD 442C guideline with the exception that a Eurospher 100 C18, 5 μm , 100 $\times$ 4 mm ID column (Knauer GmbH, Berlin, Germany) was used. Solutions of the peptides containing cysteine (Cys) and lysine (Lys) were prepared at 0.667 mM in 100 mM phosphate buffer (pH 7.5) and in 100 mM ammonium acetate buffer (pH 10.2) respectively. 100 mM stock solutions of resveratrol and cinnamic aldehyde were prepared in DMSO:ACN (10/90 v/v) and pure ACN respectively. 750 μL Cys-peptide was mixed with 200 μL ACN and 50 μL RSV representing a 1:10 ratio of the peptide to the test chemical. 750 μL Lys-peptide was mixed with 250 μL of the test chemical giving a 1:50 ratio. Peptides at 0.0167, 0.0334, 0.0668, 0.1335, 0.2670 and 0.534 mM were used for the standard calibration curve measurement. Reaction vials were incubated in the dark at 25 $^{\circ}\text{C}$ for 24 ± 2 h. All samples were prepared in triplicates and analyzed by reverse-phase HPLC using settings specified in Table 1. For the quantitative analysis areas under the peptide peak were integrated and concentrations of the respective peptide in solution were calculated from the standard calibration curve.

Chemical stability of resveratrol (RSV) was assessed via HPLC as described in Table 2. Briefly, 250 μL of a 100 mM resveratrol stock solution in DMSO/ACN was mixed with 750 μL 100 mM ammonium acetate buffer (pH 10.2) and incubated at room temperature in the dark. Samples of this solution were analyzed at 0, 20 min and 18 h time points. The system was equilibrated at 30 $^{\circ}\text{C}$ with 50% mobile

phase A (0.1% (v/v) TFA in water) and 50% phase B (0.085% (v/v) TFA in ACN) for at least 2 h prior to analysis.

Table 1: HPLC analytical conditions for the DPRA

System	Knauer
Column	Eurospher 100 C18, 5 μm , 100 $\times$ 4 mm ID column (Knauer GmbH, Berlin, Germany).
Mobile phase	A: 0.1 % TFA-water, B: 0.085% TFA-ACN
Time program:	A: 0-10 min (90-75 %), 10-11 min 10 %, 11-13 min (10 %), 13-13.5 min (10-90%), 13.5-20 min (90%).
Flow rate	0.7 ml/min
Temperature	30 $^{\circ}\text{C}$
Injection volume	30 μL
Detection at	220 nm

Table 2: HPLC analytical conditions for the assessment of resveratrol stability

System	Knauer
Column	Eurospher 100 C18, 5 μm , 100 $\times$ 4 mm ID column (Knauer GmbH, Berlin, Germany).
Mobile phase	Methanol / 0.01M Phosphate buffer pH6,8 (50/50 v/v)
Flow rate	0.8 ml/min
Temperature	30 $^{\circ}\text{C}$
Injection volume	30 μL
Detection at	306 nm

3 Results

Solubility of resveratrol

As a prerequisite for the analysis of a chemical by the DPRA test method a compound must be dissolved in an appropriate solvent at 100 mM and remain stable for at least 30 h. RSV did neither dissolve in acetonitrile, nor in water or 1:1 ACN:H₂O (1:1) at the target concentration. Although it was possible to obtain 100 mM RSV solution in isopropanol its color was light yellow and turned brown after prolonged incubation (24 h). Thus, isopropanol is not a suitable solvent and DMSO was used next to dissolve RSV. It was possible to make a 100 mM stock solution of RSV in DMSO that remained colorless after 24 h and was therefore used for subsequent testing.

Comparison of different acetonitrile sources

One of the main problems of the DPRA test method is the poor stability of the Cys-peptide in certain lots of the mobile phase reagent ACN. Here, we have tested two different sources of ACN: Ultra gradient HPLC Grade ACN (J.T.Baker Lot: 1829801862) and ROTISOLV Ultra LC-MS $\geq 99,98\%$ (Roth, Lot:1118321). While the Cys-peptide precipitated immediately in the J.T. Baker ACN, the peptide was much more stable in the ROTISOLV ACN, where some precipitate appeared after 36 h of incubation. Thus, ROTISOLV ACN was used for all further HPLC measurements.

Linearity of peptide standard solutions

In the first step of the DPRA assay the Cys-peptide und Lys-peptide standards are analyzed by HPLC. Figures 1 and 2 show the results for the respective standard calibration curve recorded 30 h after the reaction setup. As depicted in Figure 1, a strong degradation of the Cys-peptide has been observed with a tremendous impact on the calibration curve. With a correlation coefficient of 0.9704 the quality acceptance criteria could not be fulfilled and thus the determination of the remaining Cys-peptide in samples was not feasible. However, this phenomenon has not been observed for the Lys-peptide standard solutions resulting in a linear curve with a correlation coefficient of 0.9999 (Figure 2).

Figure 1: Standard curve for the Cys-peptide solutions.

Figure 2: Standard curve for the Lys-peptide solutions.

Determination of resveratrol chemical stability

RSV is known for its poor stability at high pH, upon exposure to light or high temperature. Standard conditions for testing chemicals using the DPRA assay are 25-30°C, dark, and pH 7.5 or 10.2 for Cys-peptide or Lys-peptide respectively. We therefore tested the stability of RSV in ammonium acetate buffer at pH 10.2. To this end RSV was mixed with the Lys-buffer at ratio of 1:4 and was incubated in the dark at 25°C. HPLC analysis of the mixture has shown that the retention time of RSV was strongly reduced after 18 h of incubation (Figure 3).

Figure 3: HPLC elution profile of Resveratrol upon incubation in ammonium acetate buffer (red line: 18 hrs of incubation, black line: 0 min).

4 Summary and Conclusion

The chemical instability of RSV remains a major challenge for implementation of this active reagent in clinical practice. In this study we could show that DPRA is not a suitable test method for investigating the skin sensitization potential of RSV. The key problem is the poor stability of RSV in the

alkaline environment (pH 10.2) of the ammonium acetate buffer used to dissolve the Lys-peptide. For the DPRA assay the chemical must be stable over 30 h, however the RSV peak shifted entirely from 3 to 1.5 min after 18 h of incubation in Lys-buffer. Thus, assessment of the Lys-peptide stability when exposed to RSV could not be performed. On the other hand, while RSV has been reported by others to be stable at pH 7.5, the pH of the phosphate buffer used to solubilize the Cys-peptide, the correlation coefficient of the Cys-peptide standard curve was outside the acceptance range due to insufficient stability of the peptide in the selected ACN preparation [7].

Future work will address the improvement of parameters for the current DPRA protocol (i.e. testing ACN from other source) such that the skin sensitization potential of RSV can be predicted, at least to some extent, by the Cys-peptide reactivity measurement.

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