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Research paper

Chemical and enzymatic stability of a cyclic depsipeptide, the novel, marine-derived, anti-cancer agent kahalalide F

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Kahalalide F is a cyclic depsipeptide isolated from the Hawaiian mollusk *Elysia rufescens*. This compound is under present phase I clinical investigations as an anti-tumor drug. The role of possible metabolic reactions of this drug in (pre-)clinical investigations has not yet been explored. The first results for kahalalide F in this field of research are given in this paper. The chemical degradation of kahalalide F was investigated under acid, neutral and alkaline conditions using high-performance liquid chromatography with ultraviolet detection. The half-lives at 80°C were 1.1, 20 and 8.6 h at pH 0, 1 and 7, respectively. At 26°C and pH 11, the half-life was 1.65 h. At pH 7 and 11, only one reaction product of kahalalide F was observed, kahalalide G, the hydrolyzed lactone product of kahalalide F. At pH 0 and 1, additional reaction products emerged. Metabolic conversion of kahalalide F was tested *in vitro* using three different enzyme systems based on pooled human microsomes, pooled human plasma and uridine 5′-diphosphoglucuronyl transferase, respectively. The incubated samples were analyzed using the same chromatographic technique as for the degradation samples. Biotransformations were not observed under these conditions and, therefore, it is concluded that kahalalide F is a metabolically stable drug.

Key words: Chemical stability, cyclic depsipeptide, kahalalide F, kahalalide G, metabolism.

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Introduction

Kahalalide F is chemically the largest kahalalide molecule and is also biologically the most active representative from the kahalalides.[1] These, mostly cyclic, depsipeptides originate from the Hawaiian mollusk *Elysia rufescens* and are also present in its diet, the green alga *Bryopsis pennata*. The structure (Figure 1) of the 14-membered peptide kahalalide F has been totally revealed with the linear part showing more than one conformation.[2] This compound has shown significant effects on lysosomes, resulting in large vacuoles, which is a potential mechanism of action for the antitumoral activity of the drug.[3] Other possible mechanisms may be the inhibition of *erbB2* trans-membrane tyrosine kinase and an effect on transforming growth factor-α gene expression.[4] Overall, kahalalide F shows a new, not yet totally understood mechanism of action. This has also recently been demonstrated by the lack of complete cross-resistance between kahalalide F and a number of established anti-cancer therapeutics.[5] Kahalalide F has shown anti-tumor, anti-viral, anti-fungal and slight immunosuppressive activities,[6] and its cytotoxic capacities have been most prominently shown *in vitro* against prostate (alysosome excreting organ) cancer models and also against colon cancer.[7] Activities against solid tumors have also been observed *in vivo*, again also against prostate cancer models, using doses well below the maximum tolerated dose. The promising pre-clinical results have led to the recent start of the first phase I clinical investigation for kahalalide F in patients with androgen-independent prostate tumors.

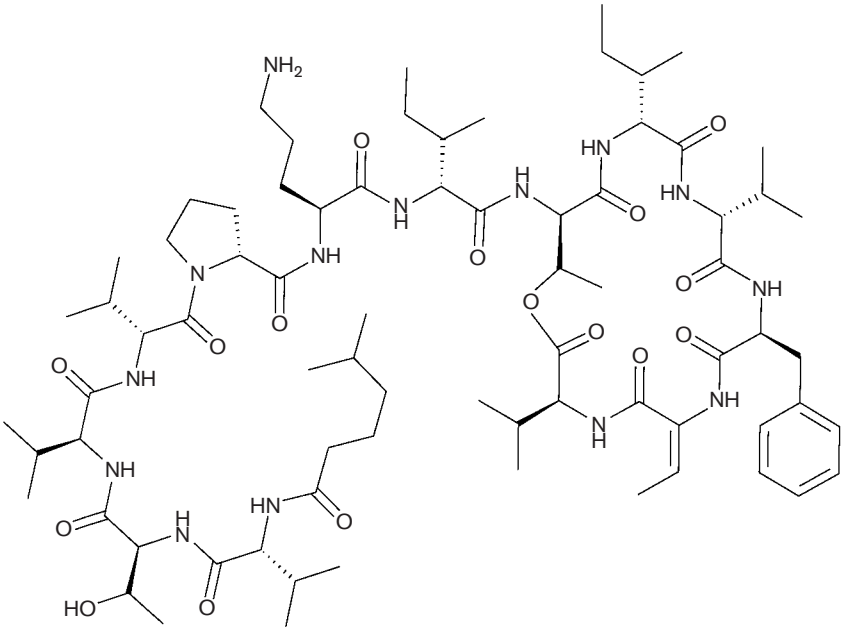

Figure 1. Chemical structure of kahalalide F.

The maximum tolerated dose of kahalalide F in female mice is 280 μg/kg.[8] Administration of this dose resulted in a peak concentration of approximately 1 μM for i.v. and 0.3 μM for i.p. injection. The drug is rapidly eliminated with a 35-min biological half-life after i.v. administration.[8] The role of possible metabolic reactions of kahalalide F in pharmacokinetic and pharmacologic studies has not yet been investigated. We therefore started investigations into potential *in vitro* biotransformations of kahalalide F using different enzymatic systems. High-performance liquid chromatographic (HPLC) analysis with ultraviolet (UV) detection was used to monitor the conversion of kahalalide F and to discover the formation of other compounds during incubations using pooled human microsomes, pooled human plasma and uridine 5′-diphosphoglucuronyl transferase, respectively. We also studied the chemical stability of kahalalide F under acid, neutral and basic conditions as degradation products might guide us in the search for potential metabolites. Coupled liquid chromatography–mass spectrometry (LC-MS) was used for the identification of degradation products.

Materials and methods

Chemicals

Kahaladide F (Figure 1) originated from PharmaMar (Madrid, Spain). Acetonitrile (Gradient grade) was provided by Biosolve (Valkenswaard, The Netherlands) and water was home-purified by reversed osmosis on a multi-laboratory scale. Pooled human microsomes [20 mg/ml protein in 250 mM sucrose; 440 pmol/mg cytochrome P450 (CYP) of total protein; fractions (5–15%) of 10 individuals; lot. 14] were supplied by Gentest (Woburn, MA) and blank, drug-free human plasma was obtained from the Bloedbank Midden Nederland (Utrecht, The Netherlands). Perchloric acid was provided by Acros (Geel, Belgium) and trifluoroacetic acid (TFA; >99%, spectrophotometric grade) by Aldrich (Milwaukee, WI). All other chemicals were of analytical grade from Merck (Darmstadt, Germany) or Sigma (St Louis, MO).

A 100-μl volume of 15 U/ml glucose-6-phosphate dehydrogenase (Type VII, from bakers yeast) in 2% (w/v) sodium hydrogencarbonate, 100 μl of 5 mg/ml $NADP^+$ sodium salt, 100 μl of 39 mg/ml disodium D-glucose-6-phosphate dihydrate and 700 μl water were mixed to obtain the $NADP^+$ regenerating system (NRS). Stock solutions of kahalalide F (2 mg/ml) were prepared in both dimethylsulfoxide (DMSO) and methanol. Human plasma was pooled using equal portions of four healthy individuals.

Equipment

Chromatographic analyses were performed on the following configuration: an AS300 autosampler, a P100 pump, a UV100 absorbance detector and an SP4400

integrator, all from Thermo Separation Products (Fremont, CA). The integrator was later replaced by a classVP chromatographic data system, version 5.03 (Shimadzu, Kyoto, Japan), installed on a Hermac personal computer (Hermac Power Systems, Scherpenzeel, The Netherlands).

Additional measurements to obtain on-line UV spectra were performed on an alternative apparatus: a Gynkotek model 300 pump (Gynkotek HPLC, Germering, Germany) was coupled to a U6K injector (Waters Chromatography, Milford, MA) and a Hewlett Packard (HP) series 1100 Photodiode array detector (Wilmington, DE). Chromatographic and spectrophotometric data were recorded and processed on a HP Vectra XA personal computer (Delfgauw, The Netherlands), equipped with the HP Chemstation chromatographic data system.

LC-MS analyses were performed on a HP1100 series liquid chromatograph (Agilent Technologies, Palo Alto, CA) connected to an API2000 triple quadrupole mass spectrometer equipped with a turbo-ionspray source (Sciex, Thornhill, ON, Canada). Data were recorded and processed using the Analyst™ 1.1 software (Sciex).

Conditions

Chromatography. Partial-loop injections (50 μl) were made on a Symmetry C_{18} column (100 × 4.6 mm, d_p=3.5 μm, average pore diameter=10 nm; Waters) with a Symmetry C_{18} pre-column (20 × 3.8 mm, d_p=5 μm; Waters). The column was used at ambient temperature, and the eluent comprised a mixture of 55% (v/v) water, 45% (v/v) acetonitrile and 0.04% (v/v) TFA. Detection was performed using UV absorption at 215 nm or alternatively using the photodiode array detector.

LC-MS. A 5-μl sample was injected on an Extend C_{18} analytical column (Agilent Technologies; 150 × 2.1 mm, d_p=5 μm). The mobile phase was a mixture of 15% (v/v) 10 mM ammonium hydroxide and 85% (v/v) acetonitrile. A flow rate of 200 μl/min was used and the column temperature was maintained at 30°C. The mass spectrometer was used in the positive ion single quadrupole mode and the MS settings are listed in Table 1.

Table 1. Experimental settings of the mass spectrometer

Parameter	Setting
Duration	10 min
Cycle time	0.028 s
Mass range	735–790 a.m.u.
Declustering potential	71 V
Focusing potential	310 V
Entrance potential	−9 V
Collision cell entrance potential	32 V
Ion spray voltage	5500 V
Curtain gas (N_2)	15 p.s.i.
Temperature	350°C
Nebulizer gas	35 p.s.i.
Turbo gas	65 p.s.i.
Ion energy Q_1	0.6 V
Channel electron multiplier	2300 V
Deflector	−200 V

Procedures

Chemical degradation. The methanolic stock solution of kahalalide F (2 mg/ml) was diluted to 40 μg/ml with water and an aliquot of this solution was then pipetted into a 1.5-ml glass autosampler vial together with an equal amount of buffer. The vial was capped and subjected to ambient temperature (26°C) in the autosampler for the solution with pH 11 and to 80°C in a heating block (dri-block DB-2A; Techne, Duxford, UK) for the others. The buffers, after mixing with the kahalalide F solution, were: (pH 11) a mixture of 1 mM sodium hydroxide and 0.1 M sodium perchlorate, (pH 7) a 0.1 M phosphate buffer, (pH 1) 0.1 M perchloric acid, and (pH 0) 1 M perchloric acid. At alkaline conditions two samples were taken at 20-min intervals. For the other conditions, 12 samples were allowed to stand in the heating block for different appropriate times and then placed in the autosampler for injection onto the column.

Microsomal experiments. A 100-μl volume of an aqueous dilution of the kahalalide F stock solution (typically 100 μg/ml) was pipetted into a polypropylene (PP) micro tube on ice and 50 μl of a 0.5 M potassium phosphate buffer (pH 7.4), 100 μl of the NRS solution and 15 μl of 20 mg/ml magnesium dichloride hexahydrate were added. After vortex mixing briefly, the tubes were conditioned at 37°C for 2 min. Next, 10 μl of the microsomal suspension was added, and the tube was vortex mixed again and incubated further at 37°C for maximal 24 h. The reaction was terminated by adding 250 μl acetonitrile and vortex mixing briefly. Finally, the proteins were removed by centrifugation for 5 min at 1300 *g*. Control experiments were performed without the substrate, without magnesium dichloride and without the microsomal suspension, respectively.

Plasma incubations. For plasma incubations, the kahalalide F stock solution was diluted to 40 μg/ml in

pooled human plasma. Then 100-μl portions of this solution were pipetted into a PP micro tube and incubated at 37°C for maximal 19 h. The reaction was terminated by adding 200 μl acetonitrile and vortex mixing briefly. The proteins were removed by centrifugation for 5 min at 1300 *g*. Control experiments were performed without the substrate and also using phosphate-buffered saline as a replacement for plasma.

Glucuronidation. Attempts to form a glucuronide of kahalalide F were performed by pipetting equal volumes (40 μl) of 0.2% Triton X-100 in water, 0.1 M magnesium dichloride, 15 mg/ml uridine 5′-diphosphoglucuronyl transferase (UDPGT, Type II, from rat liver) in 0.5 M Tris buffer (pH 7.4), 15 mg/ml uridine 5′-diphosphoglucuronic acid (UDPG) in water and 400 μg/ml kahalalide F in water into a PP microtube. After vortex mixing briefly the mixture is incubated at 37°C for maximal 21 h. The reaction was terminated by adding an equal volume of acetonitrile to an aliquot of the reaction mixture and vortex mixing briefly. The protein was removed by centrifugation for 5 min at 1300 *g*. Control experiments were performed without the enzyme, without UDPG, without Triton X-100 and without kahalalide F, respectively.

Purification of the degradation product of kahalalide F. Portions of 100 μl of a mixture resulting from a microsomal incubation were injected in the chromatograph in separate chromatographic runs. Fractions of eluate were collected in a PP micro tube at the retention time of the degradation product. Four portions were evaporated at ambient temperature using a nitrogen flow and pooled until a total volume of about 1 ml was obtained. This purified sample was used for identification using LC-MS.

Results and discussion

Kahalalide F is a depsipeptide with the ester function in the cyclic part of the molecule (lactone), and contains 13 amino acid residues and a 5-methylhexanoate (MeHex) end group (Figure 1). In order to achieve narrow symmetric peaks of Kahalalide F, several chromatographic parameters were varied during the initial method development. The tested column temperatures ranged between 22 and 65°C, and the modifier content of the eluent between 50 and 60%. Further, ammonium acetate added to the eluent (40 mM in the aqueous phase) was investigated as an alternative for TFA and a Chromspher C_8 (150 × 4.6 mm, 5 μm, Chrompack, Middelburg, The Netherlands) column was tested instead of the Symmetry C_{18} column. However, kahalalide F could not be eluted as one single Gaussian peak under all conditions. This is probably caused by the presence of different conformations of the peptide in solution.[2,6] The presence of two conformers has been confirmed by the ^{1}H- and ^{13}C-NMR signals of the MeHex function. Both conformers were also observed in kahalalide B and G, two kahalalides also containing the MeHex end group.[1] Partial chromatographic separation between the two conformations was achieved with the column temperature at 65°C. The chromatograms at 65°C and at ambient temperature are depicted in Figure 2.

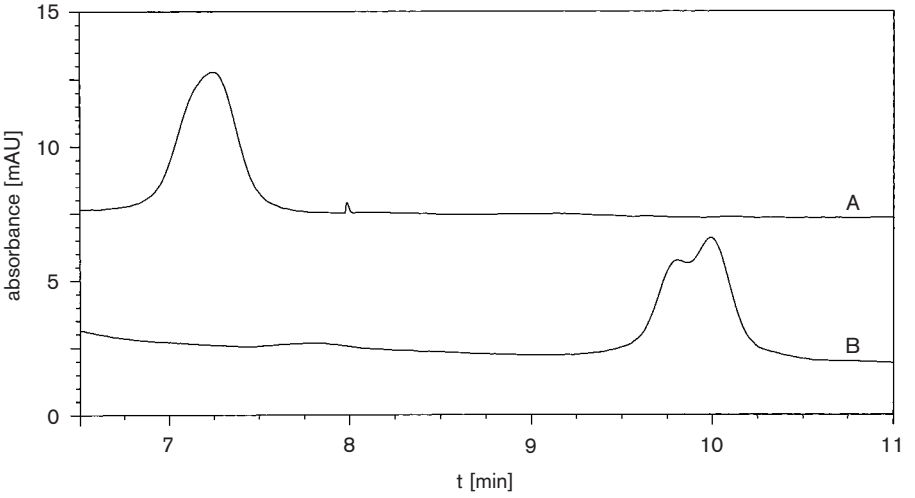

Figure 2. Chromatograms of a kahalalide F standard solution in water (5 μg/ml) at (A) ambient column temperature (standard conditions) and at (B) 65°C as the column temperature.

Chemical degradation

The half-lives of kahalalide F were 1.65 h at pH 11 (26°C), 8.6 h at pH 7, 20 h at pH 1 and 1.08 h at pH 0 (all 80°C). At pH 11 and 7, only one reaction product could be observed (Figure 3A), with a UV spectrum identical to kahalalide F (Figure 4). The mass spectrum of this degradation product, resulting from the LC-MS analysis, is shown in Figure 5. The most prominent signal $[M+H_2O+2H]^{2+}$ at 748.8 a.m.u. in this spectrum corresponds with a mass shift of +9 a.m.u. compared to the corresponding double-charged ion of kahalalide F. Additionally, several sodium and other adducts of the degradation product are present in the spectrum (Figure 5). The corresponding molecular mass shift (+18 a.m.u.) matches the expected hydrolysis of the ester function, resulting in the formation the linear peptide kahalalide G.[1,9] Further, the ester hydrolysis results in a more hydrophilic molecule with a carboxylic function that corresponds with the shorter retention time of the degradation product compared to the retention time of kahalalide F. Thus, under neutral and alkaline conditions, the ester bond is chemically clearly the weakest bond of the kahalalide F molecule.

Because the sum of the UV response of kahalalide F and G remains almost constant in the degraded

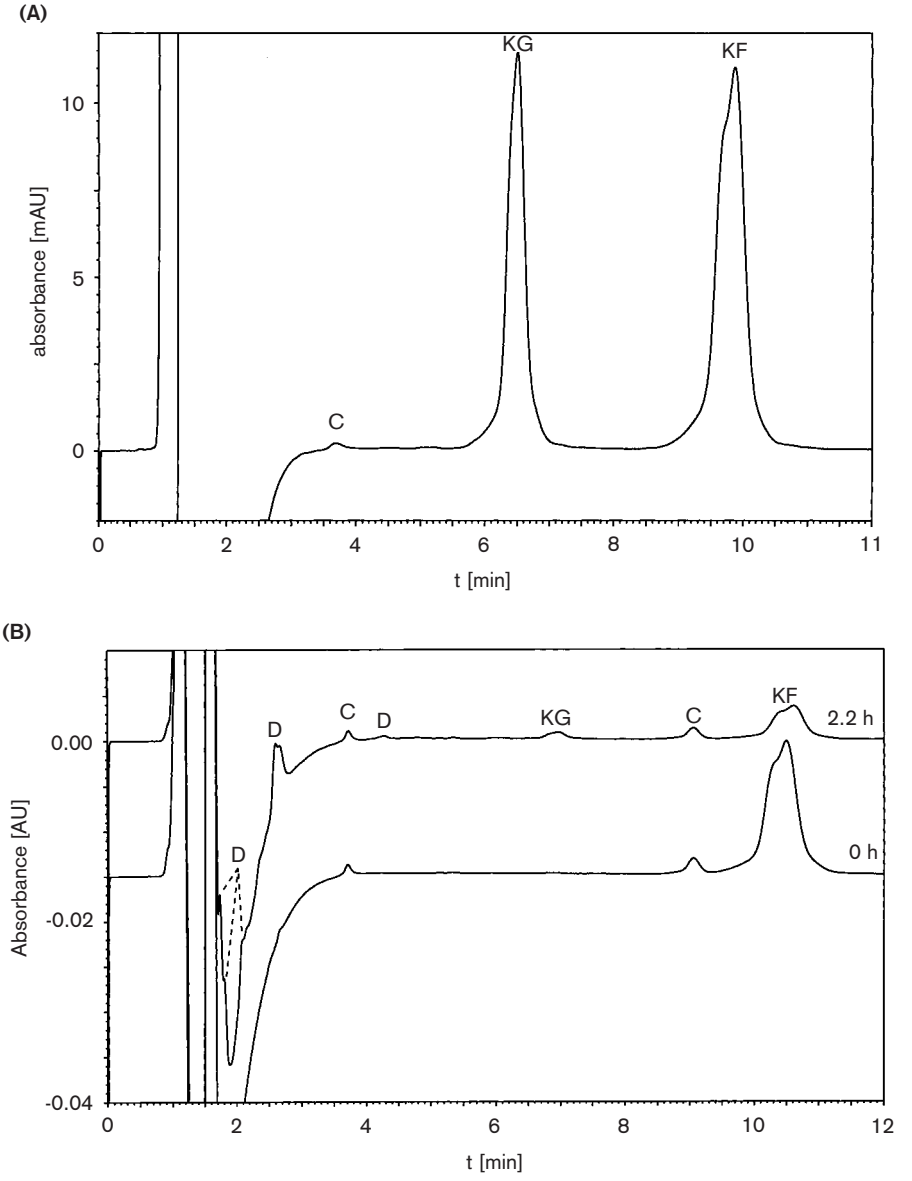

Figure 3. Chromatograms of partially degraded kahalalide F samples (20 μg/ml). (A) pH 11, 26°C, 63 min; (B) pH 0, 80°C, 0 and 2.2 h, respectively.

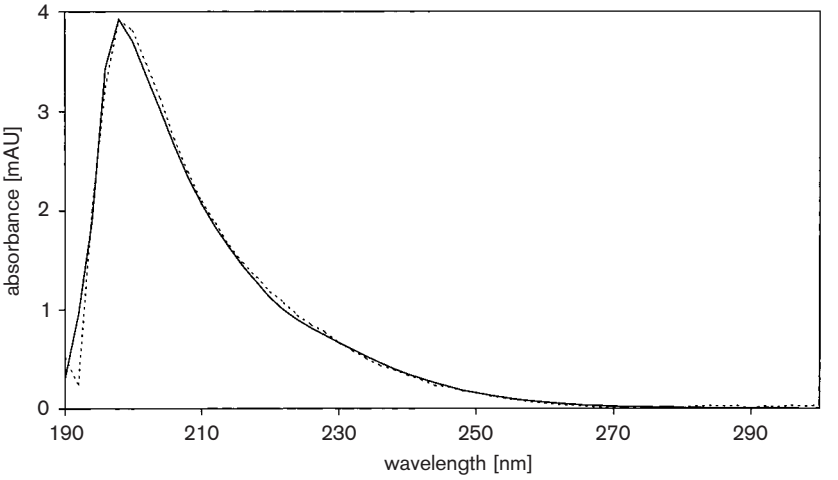

Figure 4. UV spectra of kahalalide F (———) and kahalalide G (-------).

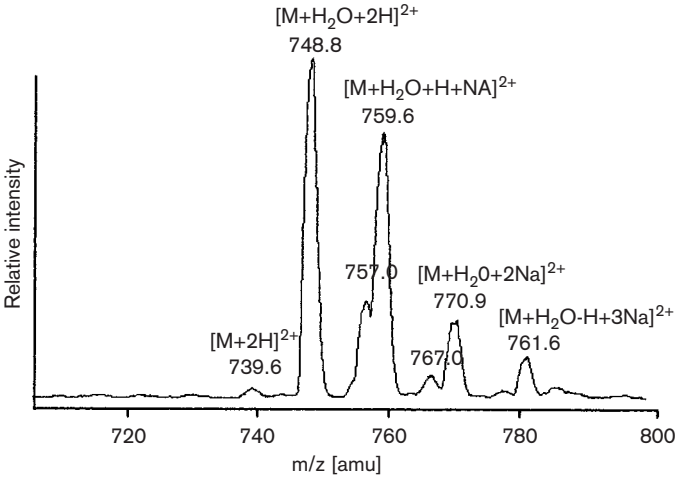

Figure 5. Mass spectrum of kahalalide G after liquid chromatographic separation (t_R=2.2 min).

samples, and because no other peaks of potential degradation product were observed, hydrolysis of the ester bond was probably the only degradation route under neutral and alkaline conditions. Under acid conditions (pH 0 and 1), however, a few significant additional products occur at short retention times in the chromatogram (Figure 3B). Only a minor amount of kahalalide G is formed under these acid conditions and the hydrolysis of the ester bond is therefore clearly not the only degradation route. Acid hydrolysis of kahalalide F has been previously investigated by Goetz *et al.*[2] Four different peptide fragments, formed in methanolic hydrochloric acid (6N) over 60 min, were observed and identified. They resulted from cleavage of the ester bond and different amide bonds of kahalalide F. These fragments may correspond to the additional degradation products under acid conditions in our experiments. Since these products are apparently only formed, in low yields, under extreme conditions, no further research was devoted to elucidate their chemical structures.

Microsomal experiments

No significant conversion of kahalalide F was observed in these experiments. Initially, however, two reaction products were observed (Figure 6). The minor one was, based on its LC and UV properties, kahalalide G, but it was also formed without the presence of the microsomal proteins. The small amount of kahalalide G can therefore be attributed to chemical degradation without a significant enzymatic contribution. The second product (X, Figure 6) appeared to be related to the DMSO from the kahalalide F stock solution.

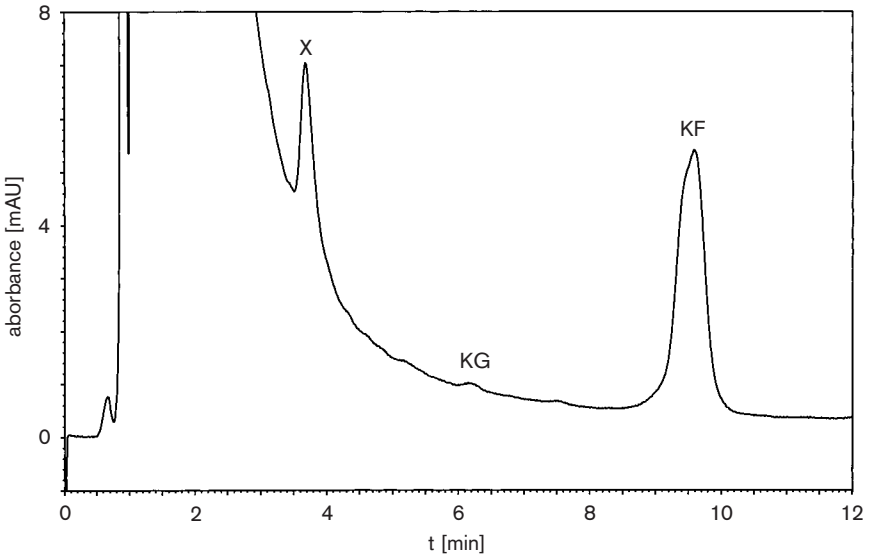

Figure 6. Chromatogram of a kahalalide F sample (40 μg/ml during incubation), diluted from the DMSO stock solution, after incubation for 23.5 h with human microsomes. (X) DMSO-related compound.

Using the methanolic stock solution of kahalalide F, this product was not formed.

The metabolic behavior of peptide-(like) drugs can be very different. Linear endogenous peptide drugs are easily truncated by hydrolytic activity, e.g. bradykinin,[10] cetrorelix[11] and peptide T.[12] On the other hand, peptide drugs with non-endogenous end groups can be relatively resistant to hydrolytic biotransformations, e.g. hexarelin,[13] MEN 11420[14] and U-71038.[15] Finally, non-peptide like structural characteristics in peptide derived drugs can undergo manifest phase I metabolic reactions like hydroxylation or *N*-demethylation, e.g. cyclosporin A,[16] saquinavir[17] and zafirukast.[18]

Kahalalide F is an endogenous-like peptide with only two, for human, exogenous structural features, the ester bond and the MeHex end group. Therefore, potential phase I metabolic reactions of kahalalide F, catalyzed by cytochrome P450 isoforms might, theoretically, lead to hydrolysis of the ester bond and also to aliphatic hydroxylation of the MeHex. These products, however, were not formed under the tested conditions. Other factors that may play a role in the *in vitro* metabolic stability of kahalalide F are sterical hindrance of the kahalalide F molecule during the approach to enzymatic active sites of the cytochrome P450 molecules and a limited enzymatic capacity. At clinical relevant concentrations, expected to be lower than the concentrations applied in the present study,[8] enzymatic activity of any cytochrome P450 isoform can therefore not be excluded. Under *in vivo* conditions, however, the drug has to be transported through the cellular membrane previous to intracellular metabolization. For a relatively large molecule such as kahalalide F, diffusion will be very slow and potential drug transporters facilitating the membrane passage of kahalalide F have not been reported yet.

Plasma incubations

The recovery of kahalalide F after precipitation of plasma proteins using acetonitrile is greater than 90%. The incubation experiments in plasma did not show any conversion of kahalalide F. Due to endogenous interference, minor products of kahalalide F, e.g. kahalalide G formed by chemical degradation, could not be distinguished. Again, esterase activity, in these experiments originating from plasma proteins, would be the first option for kahalalide F biotransformation. However, no signs of such enzymatic activity were observed after plasma incubations of kahalalide F.

Glucuronidation

Incubations with UDPGT also showed no significant conversion. Again, based on retention times and UV spectra, a small amount of kahalalide G was formed and in the presence of the enzyme the DMSO-related product was formed. No peaks of kahalalide F glucuronide or other potential metabolites of kahalalide F could be observed in the chromatograms.

The enzymatic system with UDPGT was previously successfully used in our laboratory for the glucuronidation of ecteinascidin 743, another marine-derived anti-cancer drug.[19] However, glucuronidation of the

parent kahalalide F molecule was not likely to be expected. Two potential chemical functions, a hydroxide and an amino, that may be glucuronidated are present in kahalalide F. However, both functions are not in the vicinity of electronegative groups in the molecule, as generally is in substrates suited for glucuronidation.[20]

Conclusion

Kahaladide F is chemically rather stable, alkaline conditions excluded. Degradation under neutral or alkaline conditions results in the formation of only the hydrolyzed product kahalalide G. *In vitro*, kahalalide F is a metabolically stable drug—metabolites of kahalalide F will therefore probably not contribute to toxic and anti-cancer effects in patients.

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