**Introduction**

Various herbal antiviral drugs have been developed that interfere with the viral life cycle [1]. During the first appearance of SARS about 50% of the patients in mainland China were treated successfully with Chinese herbal medicine in addition to Western medicine [2, 3]. Several viruses encode for ion-selective channels that become incorporated into the membrane of the infected cell [4–7]. Activation of such channels seems to be involved in the process of virus production and release [8–12]. Hence, inhibition of the ion channel activation will counteract virus production; this may allow the infected body to build up or strengthen its own immune system. The viral ion channel will, therefore, be a potential candidate for developing new antiviral drugs. The ORF 3a of SARS CoV encodes for an ion-permeable channel. We could previously demonstrate that micromolar concentrations of the anthraquinone emodin can inhibit the 3a channel activity with an IC50 value of 20 µM and also inhibit coronavirus release with a similar sensitivity from infected cells [13]. This indicates that the viral ion channel is an interesting target for antiviral drugs.

**Abstract**

The protein coded by the open-reading-frame 3a of SARS coronavirus has been demonstrated to form a cation-selective channel that may become expressed in the infected cell. The activity of the channel is involved in the mechanism of virus release. Drugs that inhibit the ion channel can, therefore, inhibit virus release, and they could be a source for development of novel therapeutic antiviral agents. Various drugs found in Chinese herbs that are well known as anticancer agents also have an antiviral potency. Here we tested the flavonols kaempferol, kaempferol glycosides, and acylated kaempferol glucoside derivatives with respect to their potency to block the 3a channel. We used the Xenopus oocyte with a heterologously expressed 3a protein as a model system to test the efficacy of the flavonols. Some of these drugs turned out to be potent inhibitors of the 3a channel. The most effective one was the glycoside juglanin (carrying an arabinose residue) with an IC50 value of 2.3 µM for inhibition of the 3a-mediated current. Kaempferol derivatives with rhamnose residue also seem to be quite effective. We suggest that viral ion channels, in general, may be a good target for the development of antiviral agents, and that, in particular, kaempferol glycosides are good candidates for 3a channel proteins of coronaviruses.

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**Abbreviations**

CoV: coronavirus
ORF: open reading frame
ORi: oocyte Ringer’s
G-ORi: ORi supplemented with gentamycin
NMR: nuclear magnetic resonance
SARS: severe acute respiratory syndrome
S1: test solution without Ba2+
S2: test solution with 10 mM Ba2+

**Key words**
- coronavirus
- virus release
- ion channel
- kaempferol derivatives
- flavonoids
tends preliminary data [1] to investigate the role of this class of compounds in more detail.

Results and Discussion

The flavonoids listed in Table 1 are well known for their anti-cancer activity, but also various antiviral effects have been reported [18–20]. Here we investigated these drugs with respect to their efficacy to inhibit Ba2+-sensitive current. Fig. 2a shows that 20 µM kaempferol reduced endogenous Ba2+-sensitive current. At −100 mV the current was inhibited to 0.77 ± 0.08 (p < 0.01) of the control current in the absence of the drug. The degree of inhibition was independent of voltage.

In oocytes with expressed 3a protein, Ba2+-sensitive current was larger by a factor of about 3 to 5 than in control oocytes (compare Fig. 2a and b). Kaempferol also affected this additional 3a-mediated current component (Fig. 2b). After subtraction of the endogenous contribution (Fig. 2c), the current at −100 mV was reduced to 0.82 ± 0.10 of the current component in the absence of drug; this indicated that the endogenous and the 3a-mediated components exhibited similar sensitivity to kaempferol. This is in contrast to emodin which selectively inhibited the 3a-mediated current and at 20 µM already produced more than 50% block (see [13]). The poor solubility of kaempferol in water did not allow testing a higher concentration for evaluation of an IC50 value. We therefore did not further follow up the effect of kaempferol, but rather screened for the effect of various other flavonoids.

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flavonoids. In particular, the glycosides (Table 1) are watersoluble and in addition exhibit higher bioavailability [21]. In contrast to kaempferol, the tested kaempferol glycosides hardly affected Ba²⁺-sensitive endogenous current (for juglanin see, e.g., Fig. 3a). In oocytes with an expressed 3a protein, stronger effects could be detected than with kaempferol (compare Table 1). Juglanin seemed to be the most potent kaempferol glycoside that gave complete inhibition at 20 µM; even 10 µM produced nearly complete inhibition. Therefore, we focused on this drug for a more detailed analysis. Fig. 3b illustrates the effect of two concentrations on the current-voltage dependencies of Ba²⁺-sensitive current in 3a protein-expressing oocytes. Already 2.5 µM exhibited a significant inhibition. The dependence of the 3a-mediated current component on juglanin concentration is shown in Fig. 3c. The dashed line is a fit to the data of

\[
I_{3a\text{-mediated}} = \frac{IC_{50}}{IC_{50} + [juglanin]}^{n}
\]

At a concentration of about 2.3 µM juglanin, 50% inhibition (IC₅₀) was obtained. Hence juglanin is about one order of magnitude more potent to block 3a-protein channel than emodine [13]. With an even higher IC₅₀ value of 200 µM, emodine was shown to inhibit interaction between virus and host cell, which was considered to be a potent mechanism in herbal treatment of SARS [22]. The higher sensitivity of the 3a channel makes this protein an even more interesting target for drug development.

Two other tested kaempferol glycosides, tiliroside and afzelin, were less potent than juglanin but were nevertheless as effective as emodine. Tiliroside at 20 µM produced a block to 0.48 ± 0.09 (Table 1 and Fig. 3d); at the same concentration, juglanin completely blocked the 3a-mediated current (Table 1 and Fig. 3d). A similar degree of inhibition as with 20 µM kaempferol was obtained with only 10 µM of afzelin (inhibition to 0.83 ± 0.01) compared to the current in the absence of drug (Table 1 and Fig. 3d)

In a series of experiments, we also tested the acylated kaempferol derivatives kaempferol-3-O-(2,6-di-p-coumaroyl)-glucoside and kaempferol-3-O-(3,4-diacetyl-2,6-di-p-coumaroyl)-glucoside, which all had an additional p-coumaroyl group (see

| Drug Purity (%) | Concentration (µM) | Remaining current (relative to control) | n  | p   |
|----------------|-------------------|-----------------------------------------|----|-----|
| Kaempferol glycosides | Kaempferol > 97 | 20 | 0.82 ± 0.01 | 7  | < 0.01 |
|                       | Juglanin 98 | 20 | Complete inhibition | 5  | < 0.01 |
|                       |                | 10 | 0.01 ± 0.06 |     |     |
|                       | Tiliroside > 95 | 20 | 0.48 ± 0.09 | 5  | < 0.01 |
|                       | Afzelin 98 | 10 | 0.83 ± 0.01 | 5  | < 0.01 |
|                       | Kaempferol-3-O-(2,6-di-p-coumaroyl)-glucoside > 95 | 20 (40) | No effect | 8  | sns |
|                       | Kaempferol-3-O-(3,4-diacetyl-2,6-di-p-coumaroyl)-glucoside > 95 | 20 (40) | No effect | 8  | sns |
|                       | Kaempferol-3-O-α-rhamnopyranosyl (1 → 2) [α-rhamnopyranosyl(1 → 6)]-β-glucopyranoside > 95 | 20 | 0.68 ± 0.11 | 4  | < 0.05 |
| Other flavonoids | Quercetin > 95 | 10 | 0.91 ± 0.10 | 8  | sns |
|                       | Naringenin 98 | 20 | 0.93 ± 0.05 | 4  | sns |
|                       | Genistein > 96 | 20 | 0.91 ± 0.15 | 5  | sns |

Fig. 2 Effect of kaempferol on current-voltage (IV) curves of Ba²⁺-sensitive current. Open squares describe the current-voltage dependencies in the absence and filled squares in the presence of 20 µM kaempferol. a Endogenous currents, b currents of cells with heterologously expressed 3a protein, and c the 3a-protein-mediated current component (endogenous current subtracted). Data represent averages of n = 4 to 7 experiments ± SEM.

Table 1 Effect of drugs on a 3a-mediated current. Current remaining in the presence of the respective drug concentration is expressed as ratio compared to control current in the absence of drug; values are given as mean ±SEM, based on n measurements. The values were determined from currents at −100 mV. Two of the kaempferol glycosides were, in addition to 20 µM, also tested at 40 µM. Statistical difference from the control value was determined by t-test and is given by the p value; sns stands for statistically not significant (p > 0.2).
At 20 µM, both derivatives showed no effect on Ba²⁺-sensitive current. Even at concentrations up to 40 µM (Table 1), no significant inhibition could be detected. On the other hand, in a few orientating experiments, we found that the kaempferol triglycoside kaempferol-3-O-α-rhamnopyranosyl (1→2)[α-rhamnopyranosyl(1→6)]-β-glucopyranoside exhibited about 30% inhibition at 20 µM (Fig. 1); this was similar to the effect of afzelin which was also applied at 20 µM in two of these experiments. Interestingly, both drugs are characterized by rhamnose residues (see Fig. 1).

As another flavonol, we tested the effect of quercetin, which was reported to also act as an effective drug against virus infections including SARS CoV [23]. We found that the 3α-mediated current was not significantly affected by 10 µM quercetin (see Table 1); concentrations even up to 50 µM hardly affected the 3α-mediated current. Also the quercetin derivative with an arabinofuranoside, acelin, was without any effect.

The flavanone naringenin and the isoflavone genistein are also known for their antiviral potency (see, e.g., [24–26]), but neither naringenin nor genistein exhibited any significant modulation of the 3α-mediated current (see Table 1).

Though the flavonols naringenin and acelin, the flavanone naringenin, and the isoflavone genistein do not affect the activity of the 3α protein, the flavonol kaempferol exhibits a clear inhibition of the 3α-mediated current: the kaempferol glycosides are even more potent inhibitors thus suggesting an importance of sugar residues. The most potent drug was the kaempferol glycoside juglanin with an arabinose residue. Interestingly, the kaempferol glycoside afzelin and the triglycoside with rhamnose residues seem also to be quite effective. In addition to the higher effectiveness of the flavonoid glycosides to inhibit the 3α protein ion channel, they show higher solubility in water with higher bioavailability [19, 21].

Though flavonoid glycosides may be absorbed in the small intestine, biodegradation will limit their therapeutical application, and levels in plasma are probably below 1 µM [21]. An important task for developing new antiviral drugs will therefore have to focus on improving bioactivity [27]; several strategies have been tackled for increasing bioavailability including drug delivery and metabolic stability (compare [19, 21, 27, 28]).

Activity of the 3α protein results in ion channel gating which allows small cations to cross the membrane. Although the channel shows selectivity for K⁺, also Na⁺ can penetrate with slightly lower permeability [11]. As a consequence, activity of channel openings will lead to membrane depolarization, and activation of L-type Ca²⁺ channels [29] to an elevation of intracellular Ca²⁺ [30]. This could account for the 3α-protein-dependent release of CoV from infected cells via exocytosis. Indeed, inhibition of 3α channel activity blocks virus release; this could be demonstrated by suppression of 3α expression as well as pharmacological inhibition of the 3α channel [11, 13]. This reduction in virus production offers the body the chance to adjust its immune system to counteract the viral attack. Inhibition of ion channels encoded by other viruses could also be demonstrated to inhibit the respective virus production [31–33].

As a conclusion, we suggest that emodin and kaempferol could form the basis for the development of new antiviral drugs with higher bioavailability. In particular, the glycosides of kaempferol seem to be highly potent candidates for development as anti-coronaviral agents. The fact that these drugs not only block the 3α channel, thus counteracting virus production, but that they also interfere with other steps of the viral life cycle [20] emphasises the importance of multi-target drugs.

**Materials and Methods**

**Expression of 3α protein in Xenopus oocytes** To investigate effects of kaempferol and its derivatives on the 3α protein of SARS-CoV, we used the Xenopus oocyte for heterologous expression and applied voltage-clamp techniques (for details see [11, 13]). Females of the clawed toad *Xenopus laevis* (Maosheng Bio-Technology Com.) were anaesthetized with tricaine (1 g/L H₂O, MS222; Sandoz) or in ice water. Parts of the ova-
ry were removed and treated with 0.3 units/mL Liberase (Roche) or with 1 mg/mL Collagenase (Sigma) for 3 to 4 h to remove enveloping tissue and to obtain isolated oocytes. The entire procedure follows standard protocols including care of laboratory animals that have been established according to German Animal Protection Law. For expression of 3a protein, oocytes of stage V or VI [34] were selected and injected with 20 or 30 ng cRNA for 3a protein (for details see [11]) (at 1 ng/nL) two to three days before the experiments; uninjected oocytes served as controls. The cells were stored for 2 days at 19°C in oocyte Ringer’s-like solution (G-ORi, see solutions). Experiments were performed at room temperature (24–26°C).

Solutions
Standard ORi solution contained: 90 mM NaCl, 2 mM KCl, 2 mM CaCl2, and 5 mM Hapes (adjusted to pH 7.4 with Tris). For cell incubation, the ORi was supplemented with 70 µg/L gentamycin (G-ORi). Since the 3a protein channel showed high permeability to K+ [11], the test solution without Ba2+ (S1) contained: 100 mM KCl, 2 mM MgCl2, and 5 mM Hapes (pH 7.4); because the Xenopus oocytes express endogenous Ca2+-activated K+ and Cl− channels, Ca2+ was omitted from the bath solutions (but replaced by Mg2+) to reduce these background currents. Test solution S2 contained 10 mM BaCl2 in addition. The change in osmolarity due to addition of BaCl2 did not affect the membrane currents. The difference between the current measured in S1 and S2 was considered as the Ba2+-sensitive current component. Both solutions, S1 and S2, contained some DMSO (see below).

All stock solutions of drugs were made up in DMSO. Kaempferol was purchased from Sigma-Aldrich; two probes of the kaempferol glucosides (juglanin, kaempferol-3-O-α-L-rhamnoside) were kindly provided by Prof. X. Hao and Dr. Y. Wang, Kunming, China, or bought from BioBioPha. Kaempferol acylated glucosides were previously isolated from polar extracts from the leaves of the plant Quercus ilex L. [35]; the kaempferol triglycoside was an isolate from Viola odorata L. [36]. Isolation was carried out mainly by column chromatography on Sephadex LH-20 and silica gel. The structure of the compounds was established by NMR experiments. The purity was checked by NMR and high-performance liquid chromatography with diode array detector and was over 95%. Quercetin and naringenin were purchased from Sigma-Aldrich, and genistein from Sinopropharm Chemical Reagent Co., Ltd. The purities of all drugs are listed in Table 1.

Voltage-clamp experiments
We applied conventional two-electrode voltage clamp using Turbo TEC-03 with CellWorks software (NPI Electronic) to measure the current mediated by SARS-3a protein. This method allowed directly monitoring modulations of the ion channel function under various conditions including inhibition by drugs. Previously, we had successfully applied this method to show that emodin (purity > 95%) inhibits ion flow through the 3a protein ion channel [13]. To determine steady-state-current–voltage dependencies (IV curves), membrane currents were averaged during the last 20 ms of 200 ms, rectangular voltage pulses from −150 to +30 mV in 10-mV increments; the pulses were applied from a holding potential of −60 mV. To avoid changes at the bath electrodes due to changes in Cl− activity, the electrodes were uncoupled from the bath via ORi-filled channels. Current mediated by the 3a protein can be blocked by Ba2+ [11]. Therefore, we determined Ba2+-sensitive current as the difference of steady-state current in the presence and absence of 10 mM BaCl2 (see Solutions). Since oocytes which did not express 3a protein also exhibited some Ba2+-sensitive current contribution, this endogenous component was determined in uninjected control cells and used for subtraction from total Ba2+-sensitive current of the injected oocytes from the same batch. The difference was considered to represent 3a-mediated current.

To correct for possible drift with time, Ba2+-sensitive current was calculated according to:

\[
I_{Ba\text{-sensitive}} = \frac{I_{S1 before} - I_{S1 after} - I_{S2}}{2}
\]

or

\[
I_{Ba\text{-sensitive}} = \frac{I_{S1} - I_{S2 before} - I_{S2 after}}{2}
\]

IS1 and IS2 stand for current measurements in the absence and presence of the Ba2+, respectively, the subscripts before and after refer to measurements before and after the measurement with the respective other solution. For a typical experiment, either of the following sequences of solutions was used for perfusing the chamber with the oocyte:

S1 → S2 → S1 → S1+ → S2+ → S1+ → S1 → S2 → S1
S2 → S1 → S2 → S2+ → S1+ → S2+ → S2 → S1 → S2.

The + sign indicates solutions with the respective drug.

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Conflict of Interest

All authors declare that there are no conflicts of interest.

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