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Clementine juice has the potential for drug interactions – In vitro comparison with grapefruit and mandarin juice

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ABSTRACT

Adverse drug interactions due to grapefruit juice are well known prompting warnings even in drug labels. Similar issues have not been reported for clementines and available data is scarce, despite of genetic descent. We observed substantially increased tacrolimus trough concentrations in a renal transplant patient consuming high clementine amounts and, thus, scrutinised the effects of clementine juice on drug metabolism and drug transporters in vitro and compared it to the effects of mandarin and grapefruit juice. All citrus juices profoundly induced several drug transporters and drug metabolising enzymes, whereas the effects of grapefruit juice were most pronounced (e.g. 156-fold and 34-fold induction of cytochrome P450 (CYP) 3A4 mRNA by grapefruit juice and clementine juice, respectively). However, the juices also inhibited e.g. CYP3A4, raising the question which effect prevails in vivo. Using an enzymatic activity assay, we demonstrated that at least in vitro CYP3A4 inhibition prevails for both grapefruit and clementine juice, whereas for CYP1A2 induction appears to predominate. Thus, inhibition of CYP3A4 is presumably the underlying reason for the observed increase in the concentrations of the CYP3A4 substrate tacrolimus in the patient. Taken together, our data indicate that clementine juice as well as grapefruit juice and to a lesser extent also mandarin juice can induce several important drug metabolising enzymes and drug transporters, but also inhibit some of these proteins. Our data indicate that clementine juice similar to grapefruit juice bears the potential for profound interactions with drugs potentially leading to adverse drug effects e.g. through over-exposure to CYP3A4 substrates.

1. Introduction

In November 2013, a 27-year old female with a functional living kidney allograft who was well adjusted since July 2013 to an immunosuppressive therapeutic regime comprising total daily oral doses of 4.5 mg tacrolimus, 1440 mg mycophenolate, and 4 mg methylprednisolone suddenly presented with a significantly increased tacrolimus whole blood trough level of 17.4 μg/ml during routine follow-up, which was ~2.5-fold increased compared to a previously measured concentration with the same dosing regimen. Recent medical history and physical examination were unremarkable and a check for drug interactions was inconspicuous. Only one particular life style change, the consumption of large amounts (>1 kg/d) of clementines during autumn months, was detected. The patient was advised to stop clementine intake and tacrolimus levels checked 14 days later had returned to a trough level (7.8 μg/ml) close to the target concentration range of 3–7 ng/ml. Upon re-challenge with clementines, tacrolimus levels again increased to 19.6 μg/ml within another 14 days. With a positive de-challenge and re-challenge, a definite causal relationship was established according to the WHO-UMC causality scale and a probable relationship (6 points) was determined through the Naranjo algorithm. In consequence, the patient was advised to refrain from excessive clementine intake at home. Surprised by the observation, we investigated the possible underlying pharmacokinetic mechanisms of this phenomenon in vitro.

The taxonomy of citrus fruits is complex and the nomenclature inconsistent. According to Mabberley, there are only three edible species of origin: the citron (*Citrus medical*), the pomelo (*Citrus maxima*), and the mandarin (*Citrus reticulata*) (Mabberley, 1997). Sweet oranges (*Citrus sinensis*) represent crossbreeds of pomelo and mandarin, clementines (*Citrus clementina*) are crossbreeds of sweet orange and mandarin, and grapefruits (*Citrus paradisi*) are hybrids of pomelo and sweet orange (Nicolosi et al., 2000; Barrett and Rhodes, 1976).

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According to genetic analyses, grapefruits share 70.4% identity with pomelos and only 16.7% with clementines (Garcia-Lor et al., 2013).

To the best of our knowledge, there has been no interaction reported so far between clementines or clementine juice with drugs. Only for mandarin juice there are a few reports indicating a lack of interaction with CYP3A4 substrates such as cyclosporine or midazolam (Sorkhi et al., 2007, 2008; Backman et al., 2000). In contrast, for grapefruit juice numerous interactions with drugs are known (Seden et al., 2010), including grapefruit juice-mediated increases of tacrolimus bioavailability (Liu et al., 2009; Peynaud et al., 2007). Given the fact that clementines are genetically different from grapefruit and mandarins, they might exhibit a fundamentally different interaction profile.

Because tacrolimus pharmacokinetics is relevantly influenced by drug transporters such as P-glycoprotein (P-gp, ABCB1) (Iwasaki, 2007) and the cytochrome P450 (CYP) isozymes 3A4 and CYP3A5 and thus the respective inhibitors, inducers (Iwasaki, 2007), or genetic polymorphisms (Provenzani et al., 2013), we hypothesised that inhibition of CYP3A4 and/or P-gp by clementines might be the underlying reason for the increased tacrolimus trough levels. Consequently, we scrutinised the effect of clementine juice on the expression and activity of CYP3A4 and P-gp and also included other important drug metabolising enzymes and drug transporters and compared it to the effects of mandarin juice and the partly well-known effects of grapefruit juice. The citrus juices used were also analysed for their flavonoid and furanocoumarin content by liquid chromatography coupled to tandem mass-spectrometry (LC-MS-MS).

2. Materials and methods

2.1. Materials

Cell culture media, phosphate buffered saline, (PBS), supplements, anti-β-actin (Clone AC-74), aprotinin, and the GenElute™ Mammalian Total RNA Miniprep Kit were purchased from Sigma-Aldrich (Taufkirchen, Germany). Dimethyl sulfoxide (DMSO), crystal violet, sodium dodecyl sulphate (SDS), rifampicin, tris-hydroxymethyl-aminomethane (TRIS), dithiothreitol (DTT), and Tween®20 were from AppliChem (Darmstadt, Germany). Foetal calf serum (FCS) was obtained from Biochrom (Berlin, Germany). Rhodamine123 and the antibody against human P-glycoprotein (P-gp) clone C219 were from Calbiochem (Darmstadt, Germany), the antibodies against human CYP1A2 (clone D-3) and human CYP3A4 (clone C-17) and the secondary donkey anti-goat antibody were from Santa Cruz (Heidelberg, Germany), the secondary anti-mouse antibody was from GE Healthcare (Freiburg, Germany), and Rotiphorese® Gel 30 was obtained from Carl Roth GmbH (Karlsruhe, Germany). 2,3,7,8-Tetrachlordibenzo-*p*-dioxin (TCDD, dioxin) was purchased from LGC Standards GmbH (Wesel, Germany). Pheophorbide A (PhA) was from Frontier Scientific Europe (Carnforth, UK). Calcein acetoxymethyl ester (calcein-AM) was obtained from Invitrogen (Karlsruhe, Germany). 8-Fluorescein-cAMP (8-FcA) was purchased from BIOLOG Life Science Institute (Bremen, Germany). Pefabloc was obtained from Serva (Heidelberg, Germany), bromphenol blue, leupeptin, and pepstatin were from Biomol (Hamburg, Germany). The RevertAid™ H Minus First Strand cDNA Synthesis Kit was obtained from Fermentas (St. Leon-Rot, Germany). The Absolute QPCR SYBR Green Mix was supplied by Abgene (Hamburg, Germany) and the QuantiTect® Primer Assay by Qiagen (Hilden, Germany). Primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany). The SuperSignal®West Pico Chemiluminescent Substrate Kit and the BCA® Protein Assay Kit and were purchased from Pierce (Rockford, USA). The nitrocellulose membranes (Optitran BA-S 85) from Schleicher & Schuell BioScience (Dassel, Germany). The P450-Glo™ CYP1A2 Induction/Inhibition Assay, the P450-Glo CYP1A2 Screening System Dual-Glo™, the Steady-Glo™ Luciferase Assay Systems, the P450-Glo™ CYP3A4 Assay with Luciferin-IPA, the pGL4.21 vector, the pGL4.74 [hRluc/TK] renilla vector, and FuGENE® HD Transfection Reagent were obtained from Promega Corporation (Madison, USA). The *NR1I2* (NM_003889) Human cDNA TrueClone® (pCMV6-XL4 vector containing the cDNA of the PXR gene *NR1I2*) was obtained from OriGene (Rockville, USA). Gaze (pore size 150 μm) was obtained from Neolab (Heidelberg, Germany). LY335979 was a kind gift of Eli Lilly Company (Bad Homburg, Germany). HPLC-grade acetonitrile and water were supplied by Sigma-Aldrich (St. Louis, MO, USA), dimethylformamide (DMF) by Carlo Erba (Milano, Italy). Hesperidin, eriocitrin, narirutin, didymin, poncirin, sinensetin, nobiletin, tangeretin, naringin, neohesperidin, bergamottin and epoxybergamottin were supplied from Extrasynthèse (Genay, France). 6,8-di-*C*-Glu-apigenin and lucenin-2 4′-methyl ether were separated from *Citrus limetta* (Barreca et al., 2011), chrysoeriol 7-*O*-neohesperidoside from *Citrus bergamia* (Barreca et al., 2016), and they were used as standards. The Iso-Disc P-34, 3 mm diameter PTFE membrane (0.45 μm pore size) was obtained from Supelco (Bellefonte, PA, USA). Grapefruit juice (*Citrus paradisi*, not-from concentrate) was purchased from Aldi Süd GmbH & Co KG and mandarin juice (*Citrus reticulata*, not-from concentrate) was from Völkel GmbH (Höhbeck, Germany).

2.2. Preparation of fresh clementine juice

Spanish clementines (*Citrus clementina*, var. clemenules) of the same lot the patient had consumed were peeled and homogenised with a blender. The mixture was filtered with a sieve and the filtrate centrifuged at 9000 × *g* for 5 min. The supernatant was then filtered with fine gaze and centrifuged again at 9000 × *g* for 5 min and subsequently adjusted to pH 7.0 with 10 M NaOH, and frozen at −80 °C as aliquots. To minimise oxidative loss of ingredients, only freshly thawed aliquots were used.

Grapefruit juice and mandarin juice were also adjusted to pH 7.0 and frozen in aliquots at −80 °C.

2.3. Identification and quantification of flavonoids and furanocoumarins

2.3.1. Sample preparation

Prior to analysis, the juices (10.0 ml) were mixed with DMF (10.0 ml) and the mixture was centrifuged for 5 min at 2300 × *g*. The supernatant liquid was then filtered through an Iso-Disc P-34, 3 mm diameter PTFE membrane, 0.45 μm pore size.

2.3.2. LC-MS-MS analysis of flavonoids

LC-MS-MS analyses of juice samples were carried out with a ThermoQuest Model LCQ-Duo equipped with a diode array spectrophotometer and an ion trap mass spectrometer with an electrospray ionisation source (ESI). Separation of each compound was performed on a 150 mm × 4.6 mm i.d., Kinetex® 5 μm XB-C18, supplied by Phenomenex (Torrance, USA). The column was placed in a column oven set at 30 °C. The injection loop was 20 μl, and the flow-rate was 0.5 ml/min. The mobile phase consisted of a linear gradient of acetonitrile in H_2O as follows: 5–20% (0–15 min), 20–30% (15–20 min), 30–100% (20–35 min), 100% (35–40 min), 100–5% (40–45 min), and 5% (45–55 min). UV spectra were recorded between 200 and 450 nm, and simultaneous detection by diode array was performed at 278 and 325 nm. Operating parameters of the mass spectrometer were set as follows: capillary temperature 250 °C; spray needle voltage set at 4.50 kV; ES capillary voltage +3 and −47 V for positive and negative polarity, respectively; tube lens offset 0 and −25 V for positive and negative polarity, respectively. Nitrogen was used as a sheath gas with a flow of 50 arbitrary units. Mass analysis was carried out in full-scan mode in the 80–900 amu range, both in positive and negative mode. MS-MS spectra were obtained using applied collision energy of 20–30% of instrument maximum. A source fragmentation of 20 V as a collision energy was used in MS and MS-MS analysis when required. Each sample was tested three times and gave superimposable chromatograms. Identification of the compounds was performed by their retention time, UV spectra, MS