Tonantzitlolone is a Nanomolar Potency Activator of TRPC1/4/5 Channels

Hussein N Rubaiy[1], Melanie J Ludlow[1], Karsten Siems[2], Katherine Norman[3], Richard Foster[3] Dietmar Wolf[2], John A. Beutler[4], David J Beech[1]*

[1]School of Medicine, University of Leeds, Leeds, LS2 9JT. [2]AnalytiCon Discovery GmbH, D-14473 Potsdam, Germany. [3]School of Chemistry, University of Leeds, Leeds, LS2 9JT. [4]Molecular Targets Program, Center for Cancer Research, National Cancer Institute, Frederick, MD 21702, USA.

*Author for correspondence: Professor David J Beech, Leeds Institute of Cardiovascular and Metabolic Medicine, School of Medicine, LIGHT Building, Clarendon Way, University of Leeds, Leeds LS2 9JT, UK. Telephone +44 (0) 113 343 4323. Email d.j.beech@leeds.ac.uk.

BACKGROUND AND PURPOSE
The diterpene ester tonantzitlonone (TZL) is a natural product which displays cytotoxicity towards certain types of cancer cell such as renal cell carcinoma cells. The effect is similar to that of (-)-Englerin A (EA) and so, although it is chemically distinct, we investigated whether TZL also targets transient receptor potential canonical (TRPC) channels of the TRPC1, TRPC4 and TRPC5 type (TRPC1/4/5 channels).

EXPERIMENTAL APPROACH
Renal cell carcinoma A498 cells natively expressing TRPC1 and TRPC4, modified HEK 293 cells over expressing TRPC4, TRPC5, TRPC4-TRPC1 or TRPC5-TRPC1 concatemer, TRPC3 or TRPM2 or CHO cells over expressing TRPV4 were studied by intracellular Ca^{2+} measurement or whole-cell or excised membrane patch-clamp electrophysiology.

KEY RESULTS
TZL evoked intracellular Ca^{2+} elevation in A498 cells, similar to that evoked by EA. TZL activated overexpressed channels with concentration for 50% activation (EC_{50}) at 123 nM (TRPC4), 83 nM (TRPC5), 140 nM (TRPC4-TRPC1) and 61 nM (TRPC5-TRPC1). Effects of TZL were reversible on wash-out and potently inhibited by the TRPC1/4/5 inhibitor Pico145. TZL activated TRPC5 channels when bath-applied to excised outside-out but not inside-out patches. TZL failed to activate endogenous store-operated Ca^{2+} entry in HEK 293 cells or overexpressed TRPC3, TRPV4 or TRPM2 channels.

CONCLUSIONS AND IMPLICATIONS
TZL is a novel potent agonist for TRPC1/4/5 channels which should be useful for testing the functionality of this type of ion channel and understanding how TRPC1/4/5 agonists achieve selective cytotoxicity against certain types of cancer cell.

Abbreviations
TRPC, Transient Receptor Potential Canonical; Tonantzitlonone, TZL; EA, (-)-englerin A; HEK 293 cells; human embryonic kidney 293 cells; A498 cells, human renal cell carcinoma cell line 498;

Introduction

Tonantzitlolone (TZL) (Figure 1) is a natural product from plants which include Stillingia sanguinolenta Müll. Arg. (Euphorbiaceae) (Jasper et al., 2005; Busch et al., 2008; Busch et al., 2016). TZL has attracted attention because it displayed nanomolar cytotoxicity against certain types of human cancer cell including renal, ovarian and breast cancer cells (Sourbier et al., 2015). Sixty cancer cells were tested in this NCI-60 cytotoxicity screen and many were resistant to TZL until high micromolar concentrations, suggesting the possibility for selectivity towards subtypes of cancer cell (Sourbier et al., 2015). Intriguingly, the profile of TZL in this screen was strikingly similar to that of EA, which is chemically distinct but also a natural product (Ratnayake et al., 2009; Sourbier et al., 2015; Wu et al., 2017). In both cases the target has been suggested to be PKCθ – specifically the activation of PKCθ (Sourbier et al., 2013; Sourbier et al., 2015). However, the potency of TZL and EA at PKCθ is apparently less than the potency in cytotoxicity assays (Sourbier et al., 2013; Sourbier et al., 2015). We therefore considered that another higher affinity target might exist.

In the case of EA, such a target is known to be Ca^{2+} and Na^+-permeable ion channels generated by the tetrameric assembly of transient receptor potential canonical 1, 4 or 5 proteins (TRPC1, 4 or 5) (Akbulut et al., 2015; Carson et al., 2015; Caropreso et al., 2016; Muraki et al., 2017; Rubaiy et al., 2018). We refer to these channels as TRPC1/4/5 channels but they may be formed as homomers of TRPC4 or TRPC5 or heteromers of TRPC4 or TRPC5 with TRPC1 (Abramowitz et al., 2009; Beech, 2013; Gaunt et al., 2016; Ludlow et al., 2017; Muraki et al., 2017; Rubaiy, 2017; Rubaiy et al., 2017b). TRPC1 appears to be largely non-functional on its own but it profoundly alters the properties of TRPC4/5-containing channels; including the ion selectivity, voltage-dependence and pharmacology (Storch et al., 2012; Beech, 2013; Dietrich et al., 2014; Rubaiy et al., 2017b). The composition of native channels is not known with certainty but data from A498 renal cell carcinoma cells and other cancer cells are consistent with these cells expressing heteromeric channels comprising TRPC1 and TRPC4 proteins (TRPC1/4 channels) (Akbulut et al., 2015; Muraki et al., 2017). EA potently activates these TRPC1/4 channels of A498 cells and other overexpressed or native TRPC1/4/5 channels (Akbulut et al., 2015). It acts in the nanomolar concentration range and appears to be highly selective for the channels at these concentrations (Akbulut et al., 2015; Carson et al., 2015). In this study we investigated whether TZL might similarly activate TRPC1/4/5 channels.

Methods

Chemicals and reagents
Compounds EA and Pico145 were prepared according to the procedure stated in Rubaiy et al (Rubaiy et al., 2017a; Rubaiy et al., 2017b). Pico145 was stored as a 10 mM stock at -20 °C. All commercial chemicals utilized in this work were acquired from Sigma-Aldrich, except TZL which was prepared at AnalytiCon Discovery GmbH. All chemicals were dissolved in 100% DMSO as stock solutions.

Cell culture and expression systems

HEK 293 cells stably expressing tetracycline-regulated human TRPC3, TRPC4, TRPC5, or TRPM2 were described previously.(Rubaiy et al., 2017b). All cell lines were grown at 37 °C in a 5% CO_2 incubator and culture media supplemented with fetal bovine serum (FBS 10%), penicillin (50 units/ml), and streptomycin (0.5 mg/ml) (Sigma-Aldrich). The modified HEK 293 cells were kept in Dulbecco's modified Eagle's medium/F-12 GlutaMAX together with selection antibiotics blasticidin (5 µg/ml) and Zeocin (400 µg/ml) (Invitrogen). 24 hours before experiments, 1 µg/ml tetracycline was added to the media to induce expression of channels in these modified HEK 293 cells. Chinese hamster ovary (CHO) K1 cells stably expressing human TRPV4 were used in the study of TRPV4 and they were maintained in Ham's F-12 (Gibco) in the presence of 1 mg/ml G418 (Sigma). TRPC4-TRPC1 and TRPC5-TRPC1 concatemers were generated as described previously (Rubaiy et al., 2017b).

Intracellular Ca^{2+} measurement

HEK cells were seeded at 90% confluence into 96-well clear-bottomed poly-D-lysine-coated black plates (Corning Life Sciences) 24 hr before experimentation. To monitor changes in intracellular ionized Ca2+ concentration, Fura-2 Ca^{2+} indicator dye was applied. One hour ahead of performing the experiments, the cells were incubated with Fura-2-AM (2 µM) in standard bath solution (SBS) at 37 °C along with 0.01% pluronic acid. The SBS contains 135 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 8 mM glucose, and 10 mM Hepes (pH titrated to 7.4 using NaOH). The cells were subsequently washed twice with SBS before adding C31 or ML204 for 30 min prior to recording Ca^{2+} measurements. The recording of Fura-2 fluorescence was performed using a 96-well fluorescence plate reader and the excitation wavelengths of 340 – 380 nm (FlexStation II384, Molecular Devices, Sunnyvale, CA). The ratio of the fluorescence (F) emission intensities for the two excitation wavelengths was indicated by Ca^{2+}. The measurements were made at room temperature (21 ± 3 °C).

Patch-clamp recording

Conventional whole-cell configuration patch-clamp recordings were performed under voltage clamp at room temperature using 2-4 MΩ patch pipettes fabricated from borosilicate glass capillaries with an outside diameter of 1 mm and an inside diameter of 0.58 mm (Harvard Apparatus). The patch clamp currents were recorded using an Axopatch 200B amplifier, digitized by a Digidata 1440 and recorded to a computer using pCLAMP10 (Molecular Devices). The data was filtered at 1 kHz and analysed offline using Clampfit 10.2 software (Molecular Devices) and Origin 9.1 software (OriginLab, Northampton, MA). The bath solution consisted of SBS and the pipette solution (intracellular solution) contained 145 mM CsCl, 2 mM $MgCl_2$, 10 mM Hepes, 1 mM EGTA (free acid), 5 mM ATP (sodium salt) and 0.1 mM NaGTP (sodium salt), titrated to pH 7.2 with CsOH. Cells were plated on glass cloverslips 24 hrs prior to the experiments at a low density of 20-30% and induced with tetracycline (1 µg/ml).

Data Analysis

The results were analysed using Origin 9.1 software. All data are presented as mean ± s.e.mean. Two-Sample t-Test was used for statistical comparison. P < 0.05 was considered statistically significant and P < 0.0001 is indicated as ***. The concentration for half maximal effect (EC_{50}) were achieved by nonlinear curve fit of the Hill equation (Hill1 in Origin software). Independent experiment repetitions and within experiment replicates are indicated by n and N, where n specifies

the number of independent repeated experiments of the same type and N specifies the number of replicates within a single repeat (e.g. replicate wells studied within a 96-well plate). All experiments were repeated independently at least 5 times (i.e. $\geq$n=5).

Results

Structure of tonantzitlolone (TZL)
The chemical structure of tonantzitlolone (TZL) is shown in Figure 1 and chemical validation and analytically pure material according to NMR and LC-MS analysis are provided in the supplementary information (Figure S1-S3).

TZL evokes Ca^{2+} entry in A498 cells
We first compared the ability of 100 nM EA and 100 nM TZL to activate Ca^{2+} entry in A498 cells, which natively express TRPC1/4 heteromeric channels (Akbulut et al., 2015). Strikingly, both compounds caused similar activation within 200-300 s, suggesting that TZL might indeed be a potent activator of these channels (Figure 2A, B).

TZL is a nanomolar activator of overexpressed TRPC1/4/5 channels
To test the hypothesis more directly we used an overexpression system in which heteromeric human TRPC1/4 channels were reconstituted in a modified human embryonic kidney 293 cell line (HEK 293 cells). To ensure study of the heteromers, we used concatemers of TRPC4 with TRPC1 which were stably-incorporated in HEK 293 cells for tetracycline-inducible expression (Ludlow et al., 2017; Rubaiy et al., 2017b). Figure 3A shows typical Ca^{2+} measurement data from a 96-well plate revealing concentration-dependent activation by TZL (3 – 3000 nM). Averaging data across 6 independent experiments and fitting of the Hill equation to the data suggested an EC_{50} of 140.6 $\pm$ 21.5 nM (Figure 3B). Similarly, we studied homomeric human TRPC4 channels overexpressed in HEK 293 cells and observed similar effects (Figure 3C, D). The EC_{50} was 123.1 $\pm$ 8.6 nM (Figure 3D). The data suggest that TZL is a potent agonist at homomeric TRPC4 and heteromeric TRPC1/4 channels.

We next investigated TRPC5 channels, again overexpressing them in modified HEK 293 cells (Figure 3E, F). Here again TZL was an agonist, with an EC_{50} in this case of 83.6 $\pm$ 9.0 nM (Figure 3E, F). Similarly, heteromeric concatemer TRPC1/5 channels (Naylor et al., 2016; Ludlow et al., 2017; Rubaiy et al., 2017b) were activated, revealing the lowest EC_{50} of 61.6 $\pm$ 10.6 nM (Figure 3G, H). The data suggest that TZL is a potent agonist at homomeric TRPC5 and heteromeric TRPC1/5 channels, with slightly better potency at these channels compared with TRPC4 or TRPC1/4 channels.

Pico145 inhibits TZL-evoked Ca^{2+} entry
To further characterize the TZL response we tested its sensitivity to Pico145 (Figure 4A, B). Pico145 is a potent small-molecule inhibitor of TRPC1/4/5 channels. It completely blocks TRPC5 channels at 30 nM concentration (Rubaiy et al., 2017a; Rubaiy et al., 2017b). Consistent with TZL activating TRPC5 channels, the TZL-evoked Ca^{2+} response was prevented by 30 nM Pico145 (Figure 4A, B).

TZL activates channels in whole-cell patch-clamp recordings

We also measured the channel activity using the independent technique of whole-cell patch-clamp in which 1000 nM TZL evoked a large current in TRPC5-expressing HEK 293 cells (Figure 4C). Importantly the response had the characteristic deck chair-like shape of the TRPC5 current-voltage relationship (IV) (Figure 4D). The effect of TZL was reversible on wash-out (Figure 4C). It was also completely blocked by 30 nM Pico145 (Figure 4C). Concentration-response curves were constructed by whole-cell patch-clamp (Figure 4E, F), revealing TZL EC_{50}s of 76 nM and 64 nM at +100 and -100 mV respectively (Figure 4G, H). The data suggest that TZL is a very efficacious and potent activator of TRPC5 channels.

TZL activates channels via an extracellular site

To investigate whether TZL might act on the channels relatively directly via an intracellular or extracellular site we performed excised membrane patch recordings from HEK 293 cells overexpressing TRPC5 (Figure 5). Using inside-out patches, bath application of TZL (1 µM) to the intracellular surface failed to activate TRPC5 channels (Figure 5A, B). By contrast, by using outside patches, bath application of 1 µM TZL to the extracellular surface was found to activate large currents repeatedly with the characteristic TRPC5 IV and sensitivity to Pico145 (Figure 5C, D). The data suggest that TZL activates the channels directly or via a closely associated mechanism which does not require intracellular organelles or signaling components (i.e. it is a direct or membrane-delimited effect). Moreover, the data suggest that the site of action of TZL is accessible only from the extracellular surface of the membrane.

TZL has specificity for TRPC1/4/5 channels

To investigate the specificity of TZL for TRPC1/4/5 channels we tested if it activates other members of the TRP super-family. We studied another member of the TRPC family (TRPC3), a member of the TRPV family (TRPV4) and a member of the TRPM family (TRPM2). Even at 1 µM, TZL failed to activate TRPC3, TRPV4 or TRPM2 (Figure 6A-C). In each case, positive control agonists of these channels were effective, confirming that the channels were expressed and capable of activation in our experimental conditions: 1-oleoyl-2-acetyl-snglycerol (OAG, 100 µM) for TRPC3, 4-phorbol 12,13-didecanoate (4-PDD, 5 µM) and hydrogen peroxide (H_2O_2, 1 mM) for TRPM2 (Figure 6A-C). HEK 293 cells express native Orai1 store-operated Ca^{2+} entry channels which generate Ca^{2+} elevation at least as large as that mediated by overexpressed TRPC3 (Figure 6A cf (Rubaiy et al., 2017b)). Therefore the data of Figure 6A and 6C suggest that TZL was also not an activator of Orai1 channels.

Discussion and conclusions

Based on this study we suggest that TZL is a highly efficacious, potent and relatively specific activator of TRPC1/4/5 channels. TZL activated homomeric TRPC4 and TRPC5 channels as well as heteromeric channels involving TRPC1. Its effect was reversible on wash-out and the TZL-activated channels were inhibited by a potent known inhibitor of the channels, Pico145. The effect of TZL occurred in excised membrane patches, suggesting that it did not require intracellular organelles or messengers and was therefore a membrane-delimited effect. There was effect when TZL was applied to the outer face of the membrane, suggesting an extracellular binding site or a site which is accessible via the extracellular leaflet of the lipid bilayer.

The effect of TZL was at least superficially similar to that of EA. Nevertheless, TZL is chemically distinct from EA. It could therefore present important new opportunities because unacceptable in vivo toxicity of EA in healthy rodents has been identified as a potential barrier to its development towards therapeutics (Carson et al., 2015). Moreover, EA exhibits severe metabolic instability (Carson et al., 2015) which is difficult to overcome without loss of efficacy. TZL might be a route to surmounting these challenges because it is chemically distinct from EA yet with similar potency and efficacy at TRPC1/4/5 channels.

We anticipate that TZL has a distinct binding site on or near to the channels but the nature of this binding site is currently unknown. The slope of the concentration-response curves approached unity and so a single binding site on or near to each channel is suggested. We surmise that the site is extracellular or accessible only via the extracellular leaflet of the bilayer because there was channel activation when TZL was bath-applied to excised outside-out but not inside-out patches. We cannot completely exclude that the inside-out configuration somehow rendered the channels insensitive to TZL, preventing an effect via the intracellular surface. Conceivably, TZL could penetrate the membrane sufficiently to access an intracellular site in intact cells and therefore activate the channels via an intracellular site, but such a mechanism is unlikely to explain the robust effect seen in outside-out patches. An intracellular target for TZL has been suggested in kinesin-5 but only at high micromolar concentrations (Pfeffer et al., 2016), so this effect is unlikely to be related to the TZL effect on TRPC1/4/5 channels. Overall the data support the idea of a binding site which is extracellular or dominantly accessible from the extracellular surface. Whether this binding site is physically on the channel protein is unknown. Recent insight into TRPC3 and TRPC4 structures obtained by cryo-EM methodology (Fan et al., 2018; Vinayagam et al., 2018) potentially allow predictions about binding sites which might exist on the channel proteins for TZL. Such predictions would need to be thoroughly tested and they would ideally be complemented by co-structural data for ligand and channel together.

Our studies were triggered by findings in the cancer field (Sourbier et al., 2015) and may enable progress towards better understanding of how selective cancer cell death is achieved through agents like TZL and EA and TRPC1/4/5 channel activation. However there are also broader implications of our findings outside the cancer field because of the suggested roles of TRPC1/4/5 channels in pathophysiological conditions which include epilepsy, anxiety, pain and cardiac remodeling (Westlund et al., 2014; Camacho Londono et al., 2015; Gaunt et al., 2016; Just et al., 2018). While antagonists would be wanted for therapeutic purposes in such conditions, specific agonists such as TZL are important for testing functionality of the channels and for testing the effectiveness of inhibition in in vitro and in vivo preclinical assays. Agonists are also important for high throughput screening assays where robust channel activation is a requisite.

Conflict of interest
The authors declare that they have no conflicts of interest with regard to the contents of this article.

Author contributions

HNR designed experiments, performed the cell culture and Ca^{2+} measurement which initially identified the sensitivity of TZL, patch-clamp experiments, analyzed data, and generated figures. MJL generated the TRPC1/4 and TRPC1/5 concatemers and associated cell lines. DW, JAB, KS, KN, and RF performed or advised on chemical purification, synthesis or analysis. DJB initiated the project, generated research funds and ideas, led and coordinated the project and interpreted data. HNR and DJB co-wrote the manuscript. All authors commented on the manuscript.

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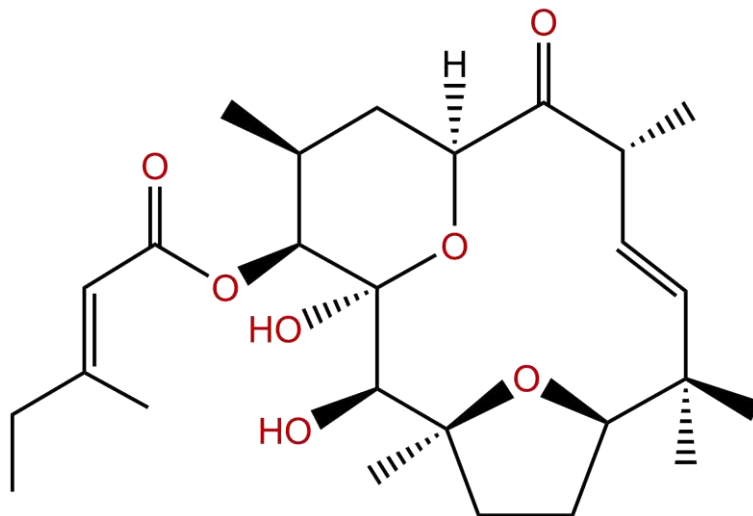
Figure 1. Chemical structure of Tonantzitlolone (TZL).
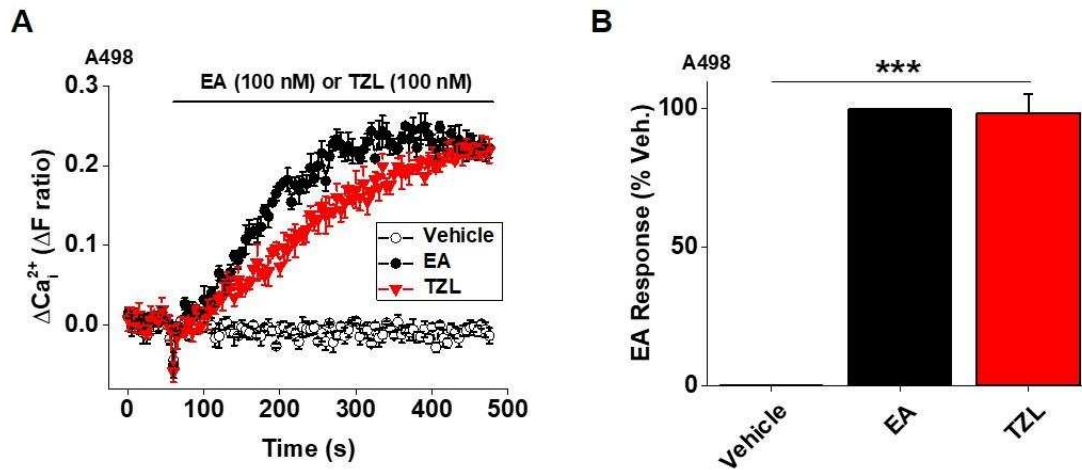
Figure 2. TZL causes intracellular Ca^{2+} elevation in A498 cells. (A) Representative intracellular Ca^{2+} measurement data from a single 96-well plate showing the effect of the vehicle control (white), 100 nM TZL (red) or 100 nM (-)-englerin A (EA) (black) (n=1 independent experiment and N=3 replicate wells each). (B) For experiments of the type shown in (A), mean ± s.e.mean TZL data normalized to the amplitude of EA response (n=6 independent experiments). Responses were measured 450-480 s after starting the application of the compound. *** indicates P < 0.0001.
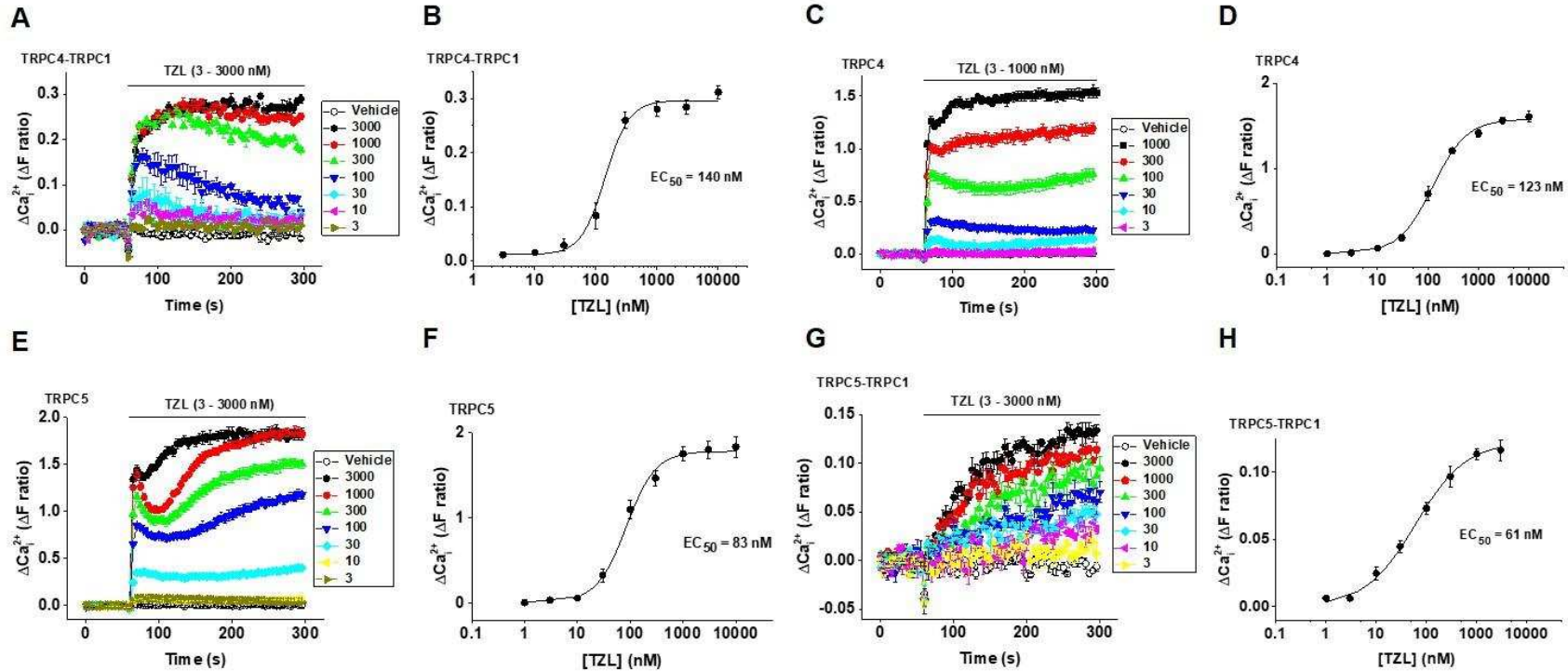
Figure 3. TZL activates intracellular Ca^{2+} elevation in TRPC1/4-, TRPC4-, TRPC5- and TRPC1/5- overexpressing HEK 293 cells. (A) Representative traces from one independent experiment on TRPC1/4 cells exposed to increasing concentrations of TZL (3-3000 nM) (N=4 replicate wells for each trace). (B) For experiments of the type shown in (A), mean ± s.e.mean data fitted with the Hill equation to determine the concentration required for 50% effect (EC_{50}) (n/N = 6/24 i.e. n=6 independent experiments and N=4 replicates per independent experiment). (C) Representative traces from TRPC4 cells exposed to increasing concentrations of TZL (3-1000 nM) (N=4 each). (D) For experiments of the type shown in (C), mean ± s.e.mean data fitted with the Hill equation to determine the EC_{50} (n/N=6/18). (E) Representative traces from TRPC5 cells exposed to increasing concentrations of TZL (3-3000 nM) (N=4 each). (F) For experiments of the type shown in (E), mean ± s.e.mean data fitted with the Hill equation to determine the EC_{50} (n/N = 6/24). (G) Representative traces from TRPC1/5 cells exposed to increasing concentrations of TZL (3-1000 nM) (N = 4 each). (H) For experiments of the type shown in (G), mean ± s.e.mean data fitted with the Hill equation to determine the EC_{50} (n/N=6/24).
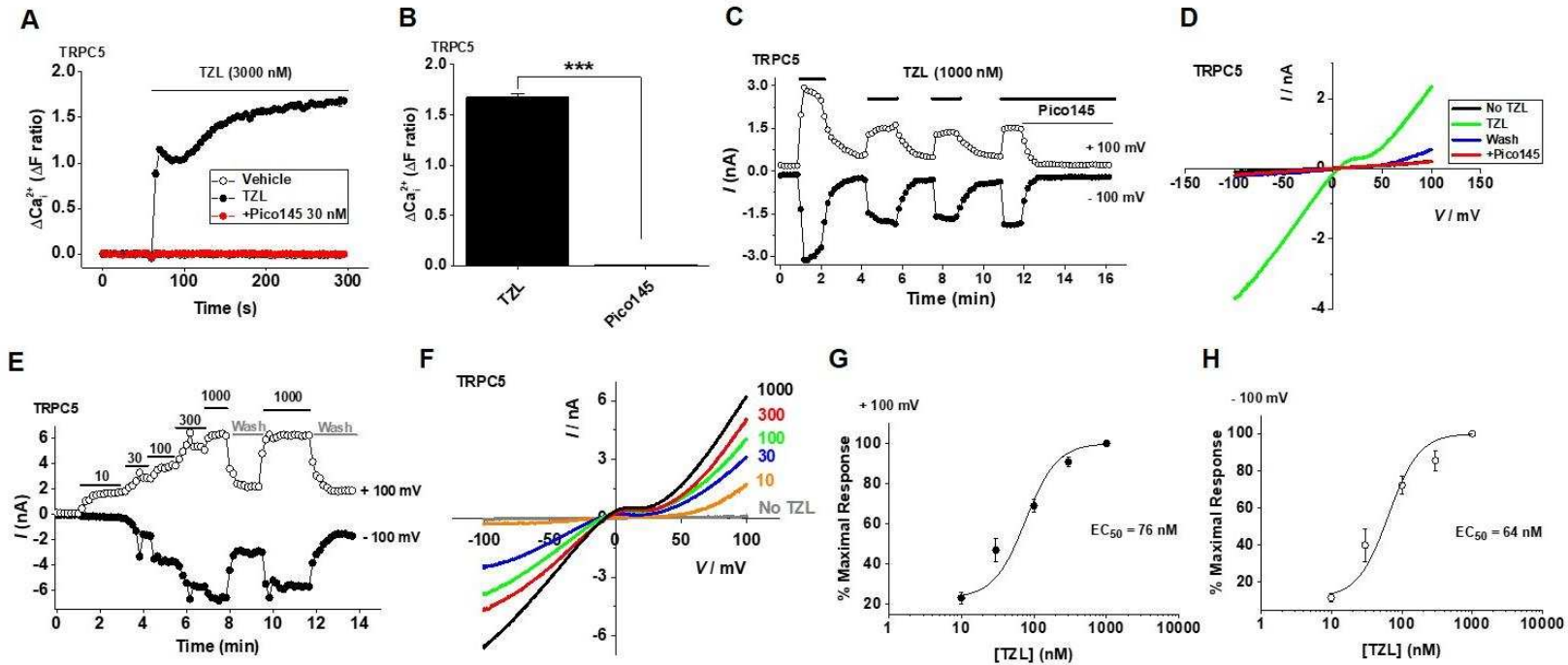
Figure 4. TZL-activated TRPC5 responses are inhibited by Pico145 and similar by whole-cell patch-clamp recording. Data are for HEK 293 cells overexpressing TRPC5. (A) Representative traces for one independent experiment on intracellular Ca^{2+} showing responses to 3 µM TZL in the absence (black) and presence (red) of 30 nM Pico145 (N=4 replicate wells each). (B) For experiments of the type shown in (A), mean ± s.e.mean data (n/N=6/24). *** indicates P < 0.0001. (C) Representative whole-cell patch-clamp recording showing the effect of bath-applied 1 µM TZL and 30 nM Pico145 as indicated by horizontal bars. Currents were sampled at -100 mV and +100 mV during ramp changes in voltage from -100 to +100 mV. (D) For experiments of the type shown in (C), example current-voltage relationships (IVs) for before TZL application (No TZL, black), at the maximum response to TZL (TZL, green), after wash-out of TZL (Wash, blue) and after Pico145 was applied in addition to TZL (+Pico145, red). Representative of 6 independent experiments. (E) Representative whole-cell patch-clamp data showing currents sampled at -100 mV and +100 mV during ramp changes in voltages from -100 to +100 mV. (F) For experiments of the type shown in (E), example IVs for before TZL application (No TZL, grey) and in response to increasing concentrations of TZL (10-1000 nM). Representative of 5 independent experiments. (G) For experiments of the type shown in (E, F), mean ± s.e.mean concentration-response data for +100 mV (G) and -100 mV (H) fitted with the Hill equation to yield EC_{50} values and slopes of 76.5 ± 9.2 nM and 0.89 (G) and 64.5 ± 7.3 nM and 0.82 (H).

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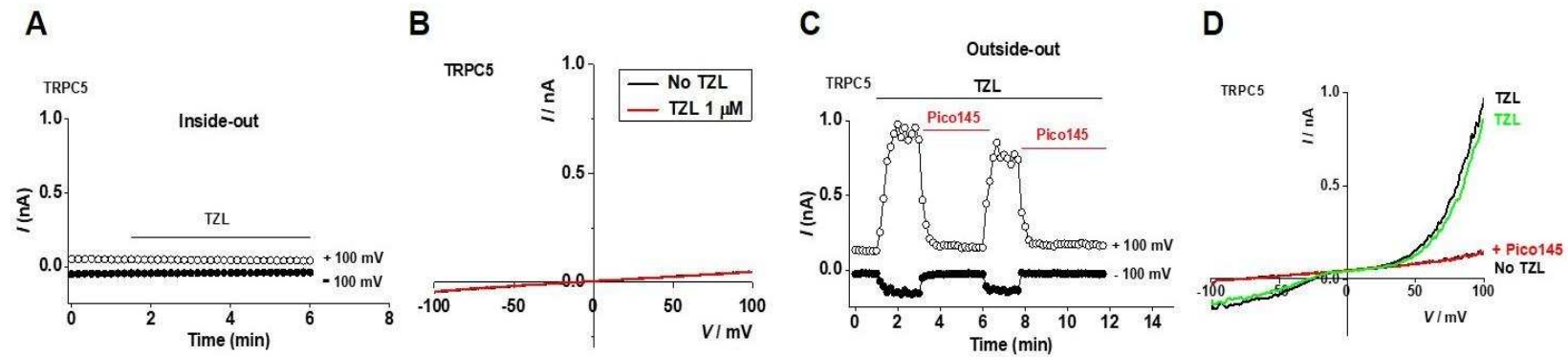
Figure 5. Bath-applied TZL activates TRPC5 in excised outside-out but not inside-out patches. Data are for HEK 293 cells overexpressing TRPC5. (A, B) Inside-out patch recording in which 1 µM TZL was bath-applied as indicated by the horizontal bar, showing a typical time-series recording (A) and IVs from this recording (B). Representative of 7 independent recordings (i.e. n=7). (C, D) Outside-out patch recording in which 1 µM TZL and 30 nM Pico145 were bath-applied as indicated by the horizontal bars, showing a typical time-series recording (C) and IVs from this recording (D). Representative of 6 independent recordings (i.e. n=6).
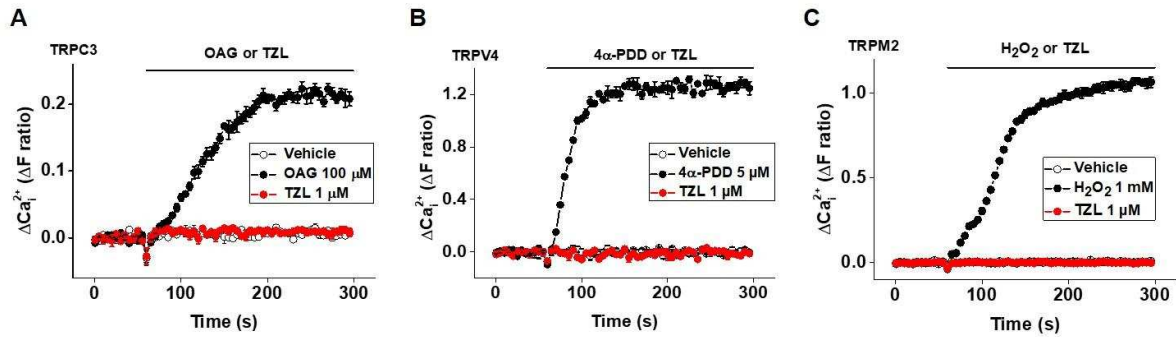
Figure 6. TZL does not activate native store-operated Ca^{2+} entry channels or overexpressed TRPC3, TRPV4 or TRPM2 channels. Intracellular Ca^{2+} measurements were made from cell lines stably expressing TRPC3 (A), TRPV4 (B) or TRPM2 (C), showing representative independent experiments from n=6 each. Data are presented as mean ± s.e.mean. (A) TRPC3 channels were activated by 100 µM 1-oleoyl-2-acetyl-snglycerol (OAG, black) but not 1 µM TZL (red) (N=4 replicate wells each). (B) TRPV4 channels were activated by 5 µM 4-phorbol 12, 13-didecanoate (4-PDD, black) but not 1 µM TZL (red) (N=5 replicate wells each). (C) TRPM2 channels were activated by 1 mM hydrogen peroxide (H_2O_2, black) but not 1 µM TZL (red) (N=5 replicate wells each).
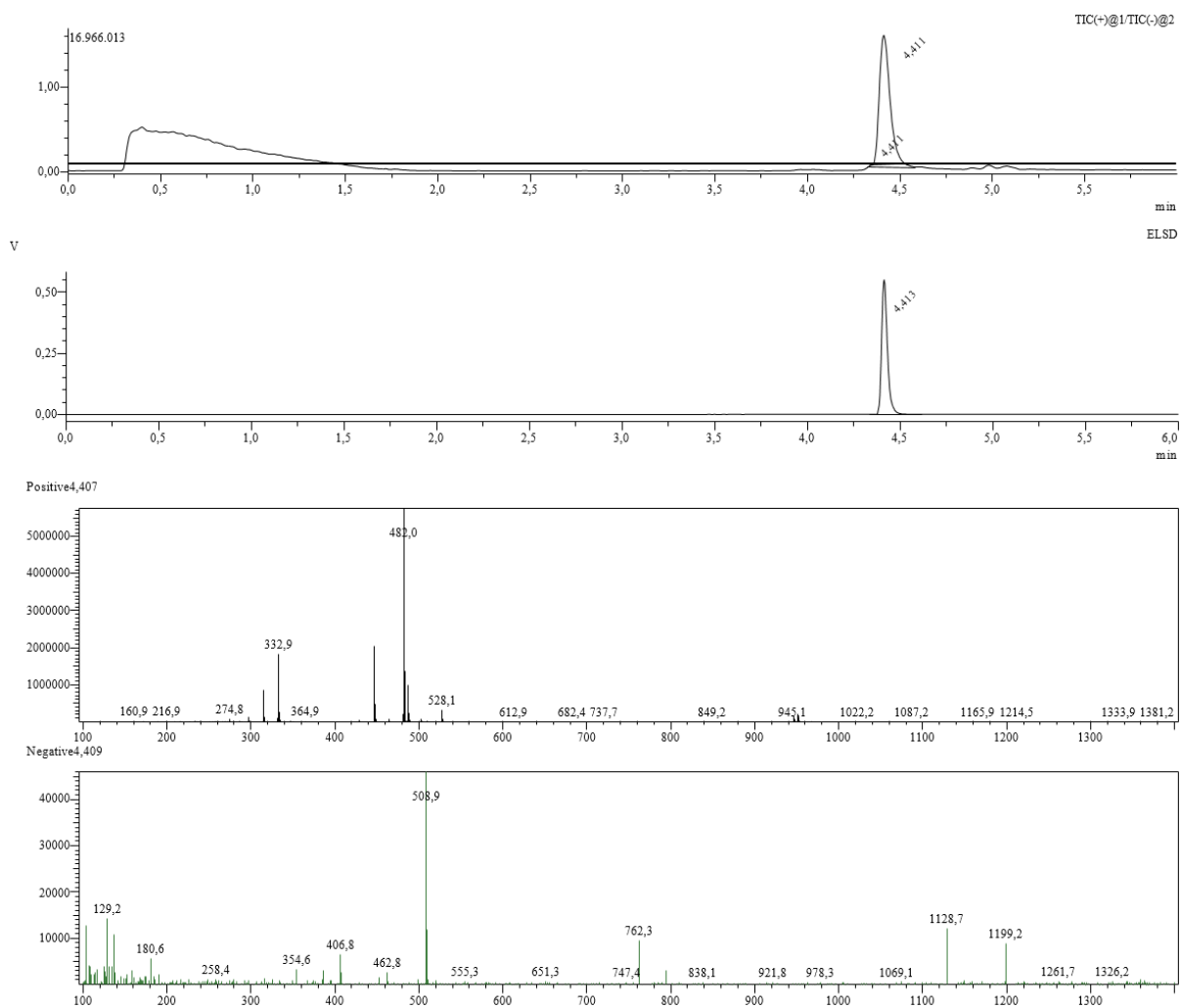
Figure S1. LC/ESI-MS-ELSD spectrum of TZL. The NMR spectroscopy did not show any impurities of >0.5% (light scattering detection).
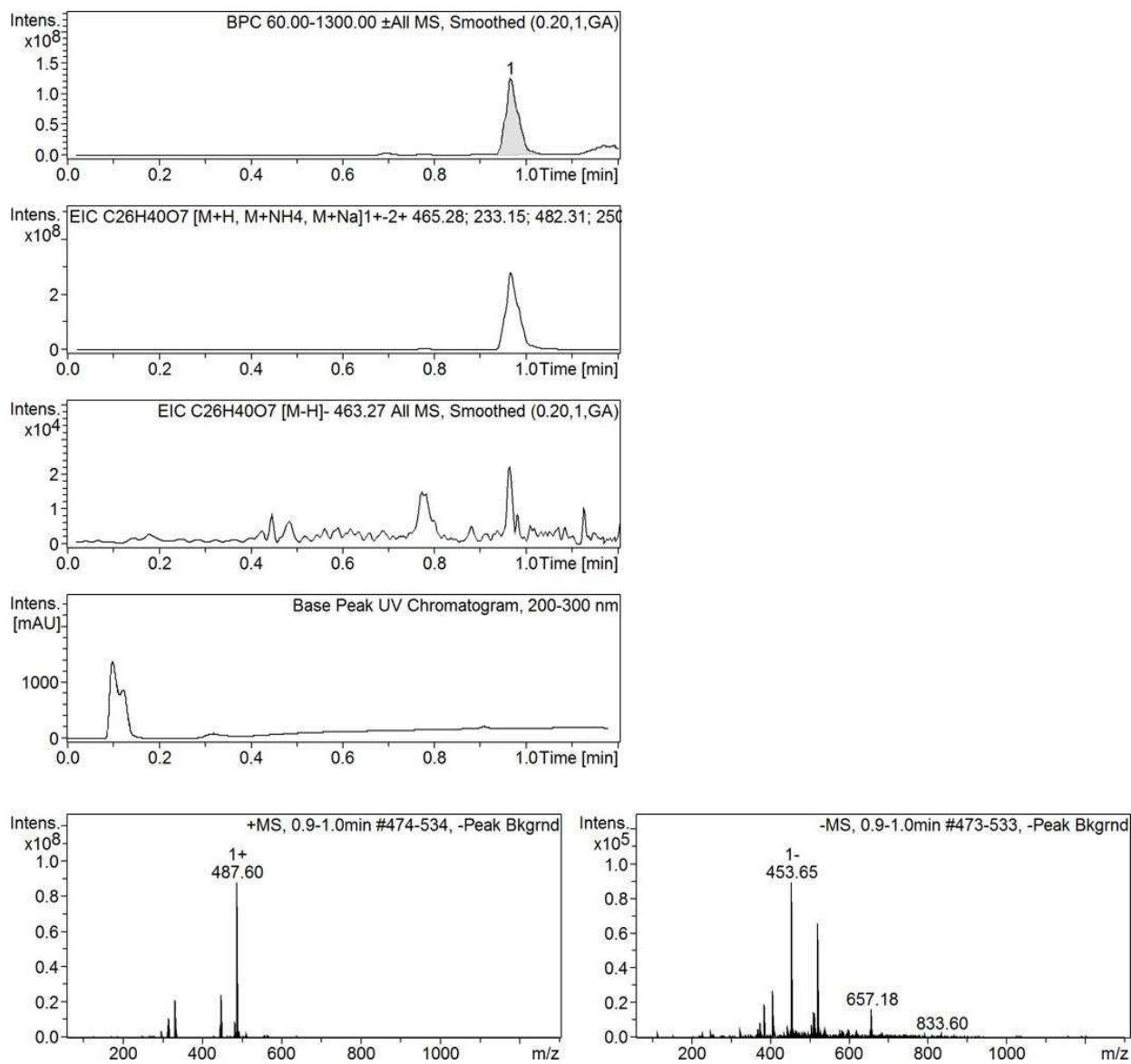
Figure S2. ESI--LCMS spectrum of TZL.
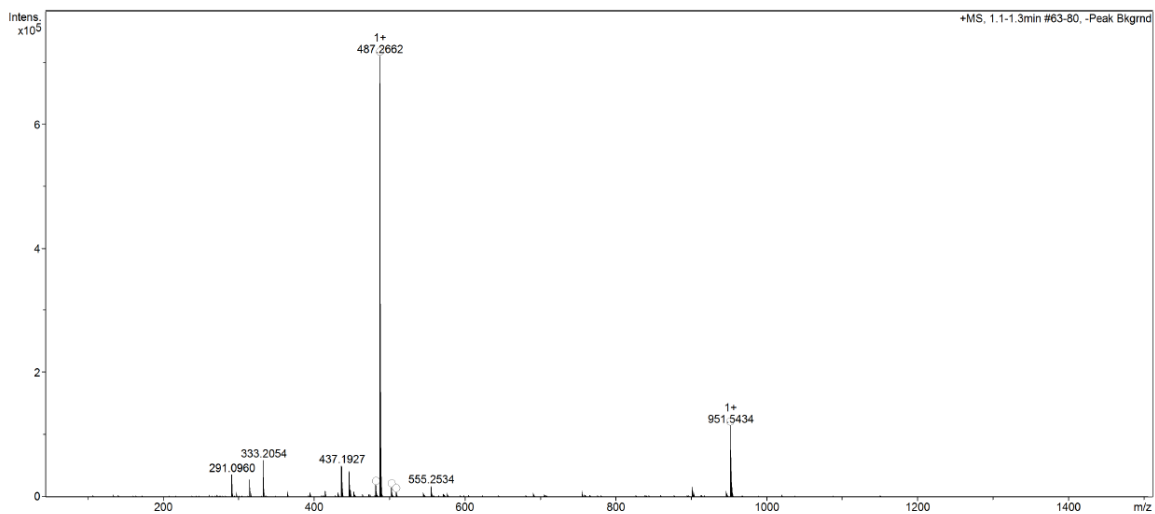
Figure S3. ESI-HRMS spectrum of TZL, where 487.2662 corresponds to the m/z for the $[M+Na]^+$ adduct (theoretical mass = 487.2666 for $[M+Na]^+$)