Antitumor Agents Hot Paper

DOI: 10.1002/anie.201411511

(−)-Englerin A is a Potent and Selective Activator of TRPC4 and TRPC5 Calcium Channels**

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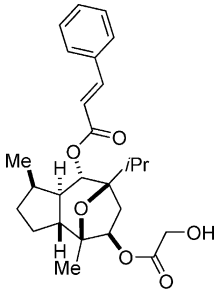
Abstract: *Current therapies for common types of cancer such as renal cell cancer are often ineffective and unspecific, and novel pharmacological targets and approaches are in high demand. Here we show the unexpected possibility for the rapid and selective killing of renal cancer cells through activation of calcium-permeable nonselective transient receptor potential canonical (TRPC) calcium channels by the sesquiterpene (−)-englerin A. This compound was found to be a highly efficient, fast-acting, potent, selective, and direct stimulator of TRPC4 and TRPC5 channels. TRPC4/5 activation through a high-affinity extracellular (−)-englerin A binding site may open up novel opportunities for drug discovery aimed at renal cancer.*

Renal cell carcinoma (RCC) is a frequently occurring and particularly challenging malignancy,[1] as it is often diagnosed with poor prognosis at the metastatic stage. Only a few noncurative treatments with serious side effects are available for RCC.[2] Therefore, the discovery of innovative approaches for the treatment of RCC and small-molecule modulators of their function is of major current interest. Highly potent natural products may enable the identification of novel targets for drug discovery.[3]

The sesquiterpene (−)-englerin A[4,5] (**1**) is a very potent and selective inhibitor of renal cancer cell growth compared to cancer cell lines of different origin and, even more remarkably, compared to normal kidney cells. Sourbier et al. recently suggested that englerin A directly activates PKCθ, which results in phosphorylation of insulin receptor substrate 1 (IRS1) and the transcription factor HSF1 to simultaneously cause insulin resistance and glucose dependence.[4d]



(-)-Englerin A

While investigating the bioactivity of (−)-englerin A and derivatives with enhanced potency,[4b] to our surprise we determined that the renal cancer cell line A498, which is most sensitive for (−)-englerin A among the cell lines investigated by Ratnayake et al.,[4a,c] does not express PKCθ (see Figure S1 in the Supporting Information). Thus, at least in this cell line, the natural product must target a different protein. In addition, in time-resolved investigations we found that the cytotoxicity induced by (−)-englerin A is already manifested within minutes (see Figure S2 and Movies S1 and S2 in the Supporting Information). This finding excludes regulation of gene transcription, which typically occurs on the time scale of hours, as the primary mode of action and is also indicative of a different primary target.

Here we report that (−)-englerin A ((−)-EA) is a selective and potent activator of the transient receptor potential Ca^{2+} channel C4 (TRPC4) in RCC cells and that (−)-englerin A induces cell death by elevated Ca^{2+} influx and Ca^{2+} cell overload.

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[**] The research was supported by grants from the Wellcome Trust, the Medical Research Council, the Max Planck Gesellschaft, the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ERC Grant agreement no. 268309, and the Alexander von Humboldt Foundation. M.S.A. was supported by a Daniel Turnberg Travel Fellowship from the Academy of Medical Sciences. We thank Judith Ritchie for comments on the manuscript.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201411511.

Initial attempts to identify the cellular target of (−)-EA by means of affinity-based chemical proteomics experiments remained fruitless, which indicated that the cellular target of (−)-engerin A may be a low-abundance protein and/or a membrane protein.

Low-abundance G-protein-coupled receptors (GPCRs) and ion channels usually respond to stimuli within milliseconds to seconds, and typically cannot be identified by established chemical proteomics methods. The investigation of several prototypic GPCRs and ion channels as potential (−)-EA targets revealed only occasional weak inhibition (see Tables S1 and S2 in the Supporting Information). However, a report linking transient receptor potential canonical channel 4 (TRPC4) to renal cell carcinoma[6] led us to pursue functional studies of this protein. This hypothesis was underscored by gene expression analysis of the NCI60 cell line panel that had revealed a highest degree of expression of TRPC4 in A498 cells among the NCI60 cell lines.[14] TRPC4 is one of six members of the membrane-spanning TRPC family of human proteins, a subset of the TRP superfamily[7] which assembles as homo- or heterotetramers to form Ca^{2+}-perme-

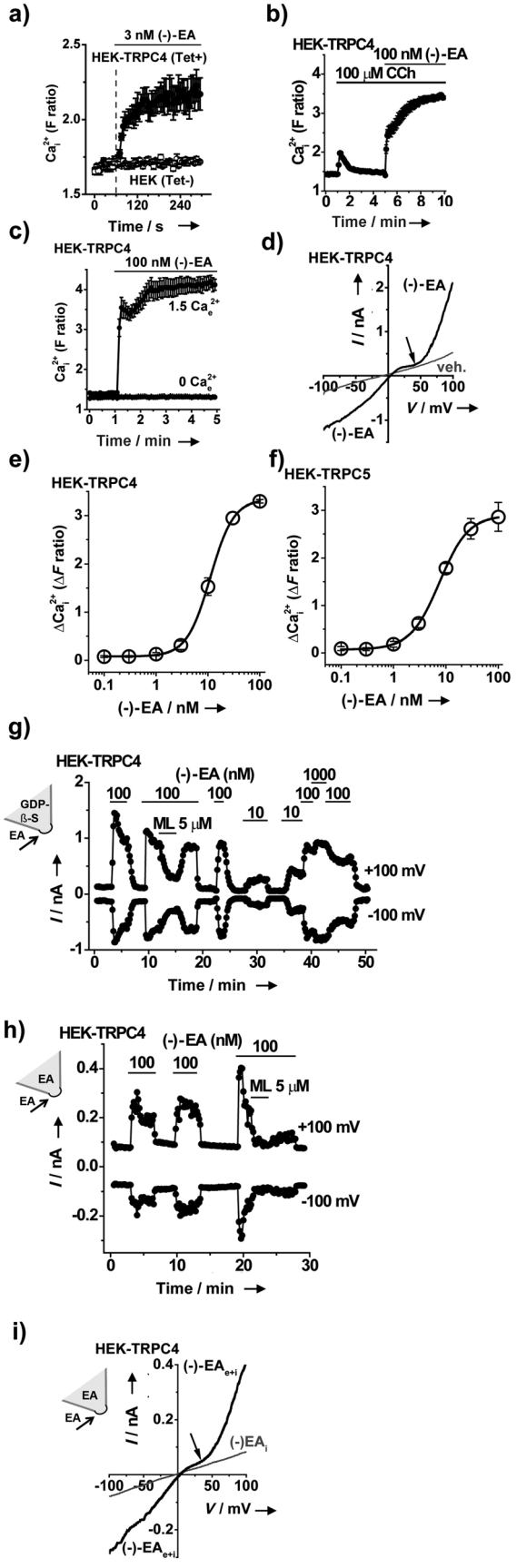

Figure 1. a–c, e–f) Measurements of the free intracellular calcium ion (Ca^{2+}_i) concentration shown as the fura-2 fluorescence (*F*) ratio or change (Δ) in this ratio. Extracellular Ca^{2+} was 1.5 mM unless otherwise indicated. a) Genetically modified HEK 293 cells not induced (HEK (Tet-)) or induced using tetracycline (Tet+) to overexpress TRPC4 (HEK-TRPC4). After 1 min of recording, 3 nM (−)-EA was applied to the extracellular medium as indicated by the vertical dashed line ($N = 5$ each). Representative of $n = 4$. (n = number of independent experiments, N = number of replicates within a single independent experiment.) b–e) Genetically modified HEK 293 cells induced to overexpress TRPC4 (HEK-TRPC4). b) Extracellular application of 100 µM carbachol (CCh) and then, in addition, 100 nM (−)-EA ($N = 6$ each). Representative of $n = 3$. c) Application of 100 nM (−)-EA in the presence (1.5 Ca^{2+}_e) or absence (0 Ca^{2+}_e) of extracellular 1.5 mM Ca^{2+} ($N = 6$ each). Representative of $n = 3$. d) Whole-cell voltage-clamp recording of membrane current (*I*) from a single cell during ramp changes in membrane voltage (*V*) from −100 to +100 mV, shown during the application of an extracellular vehicle (dimethyl sulfoxide and pluronic acid) and then 100 nM (−)-EA. The arrow points to the seatlike inflection in the *I*–*V* curve. Typical of $n = 12$ (4 with standard pipette solution, 8 with aspartate solution). e) Concentration–response data for (−)-EA ($n/N = 4/18$–19). The fitted curve is a Hill Equation indicating the 50% maximum effect (EC_{50}) at 11.2 nM. f) As for (e) except the cells were genetically modified HEK 293 cells induced to over-express TRPC5 (HEK-TRPC5) ($n/N = 3$–4/15–20). The fitted curve is a Hill equation indicating an EC50 of 7.59 nM. g–i) Ionic currents across outside-out membrane patches from genetically modified HEK 293 cells induced to overexpress TRPC4 (HEK-TRPC4). g) As indicated by the inset diagram, 1 mM guanosine 5′-[β-thio]diphosphate (GDP-β-S) was in the patch pipette and (−)-EA (EA) was bath-applied to the extracellular surface of the membrane (indicated by horizontal bars above the experimental traces). ML204 (ML) was also bath-applied. The vehicle (dimethyl sulfoxide and pluronic acid) was kept constant throughout the recording. Ramp changes in membrane voltage from −100 to +100 mV were applied every 10 s, and the currents sampled at −100 and +100 mV are displayed. Typical of $n = 4$. h) As for (g) except, as indicated by the inset diagram, GDP-β-S (and ATP) were omitted from the patch pipette and the pipette contained 100 nM (−)-EA. Typical of $n = 3$. i) From the experiment shown in (h), full current traces during two ramp changes in voltage, one before ((−)-EA_i) and the other after bath-application of (−)-EA ((−)-EA_{e+i}). The arrow points to the seatlike inflection in the *I*–*V* curve.

able nonselective cationic channels.[8] Despite much effort towards understanding TRPC4 channels, the absence of highly potent, selective, and efficacious modulators[8b] has been a major limitation to progress.

In HEK 293 cells over-expressing human TRPC4 (see Figure S3a in the Supporting Information), 3 nM (−)-EA evoked sustained elevation of the intracellular Ca^{2+} concentration within 1 min (Figure 1a). At the higher concentration of 100 nM, it caused an even larger increase in the Ca^{2+} concentration, which contrasted markedly with the small or undetectable responses to previously reported TRPC4 activators (Figure 1b and see Figure S4a,b in the Supporting Information). The response to 100 nM (−)-EA was abolished by removal of extracellular Ca^{2+} and unaffected by prior depletion of intracellular Ca^{2+} stores, thus suggesting mediation by Ca^{2+} entry rather than Ca^{2+} release (Figure 1c and Figure S4 in the Supporting Information). (+)-Englerin A[4b] was ineffective at elevating Ca^{2+} in TRPC4-expressing cells even at the higher concentration of 1 μM (see Figure S4c in the Supporting Information), which shows there is stereoisomeric specificity. In support of TRPC4 channel activation by (−)-EA, whole-cell voltage-clamp recordings revealed large (−)-EA-evoked currents with a seatlike inflection in the current–voltage relationship (*I–V*; Figure 1d), a fingerprint of TRPC4 and closely related channels.[9] The concentration of (−)-EA required for 50% activation (EC_{50}) was 11.2 nM (Figure 1e). We tested if (−)-EA affects other TRP channels, starting with TRPC4's closest relative, TRPC5. There was striking activation of Ca^{2+} entry in HEK 293 cells overexpressing TRPC5, and the potency was again impressive ($EC_{50} = 7.6$ nM; Figure 1f and see Figure S3b in the Supporting Information). In contrast, cells overexpressing TRPC6, TRPM2, or TRPV4 (see Figures S3c and S5 in the Supporting Information) lacked responses to (−)-EA. The data suggest (−)-EA to be a potent, efficacious, and selective activator of TRPC4 and TRPC5 channels.

TRPC4/5 channels are promiscuously stimulated by agonists acting via GPCRs.[8a,9a,10] We therefore strongly blocked all G-protein activity by exposing the intracellular face of excised membranes to a high concentration of the stable guanosine diphosphate analogue GDP-β-S and recorded the TRPC4 channel currents. The application of 100 nM (−)-EA to the extracellular surface of membranes rapidly and strongly activated hundreds of TRPC4 channels, leading to macroscopic currents that were so large that they resembled whole-cell currents (Figure 1g). Currents returned to the baseline level quickly after washing out the (−)-EA and a second similar response was readily evoked when (−)-EA application was repeated (Figure 1g). The evoked current was suppressed reversibly by 5 μM of the reported TRPC4 inhibitor ML204[11] (Figure 1g). 10 nM (−)-EA was also effective at evoking currents in the excised membrane patch and the concentration dependence was similar to that seen in Ca^{2+} measurement studies (Figure 1g). The data suggest that (−)-EA does not act via G-protein signaling.

We then recorded at excised membrane patches but in the absence of any nucleotides or other regulatory cofactors and with the intracellular face of the channels exposed to 100 nM (−)-EA from the outset. Although there were constitutive currents (Figure 1h) they did not exhibit the *I–V* characteristics of TRPC4 channels (Figure 1i), which suggests TRPC4-independent background currents and that (−)-EA did not activate TRPC4 channels at an intracellular site. In contrast the subsequent application of 100 nM (−)-EA to the extracellular surface of the same patch repeatedly activated currents that were ML204-sensitive (Figure 1h) and exhibited the TRPC4 seatlike inflection in the *I–V* curve (Figure 1i). The data suggest that (−)-EA activates TRPC4 channels directly at a site exposed externally or accessible only at the external leaflet of the bilayer.

(−)-EA evoked intracellular Ca^{2+} elevations in A498 cells as well with an EC_{50} value of 10 nM (Figure 2a,b), whereas 1 μM (+)-englerin A was ineffective (Figure 2c). Moreover the Ca^{2+} elevations were ML204-sensitive, consistent with Ca^{2+} entry involving TRPC4 (Figure 2d) and supported by the finding that TRPC4 but not TRPC5 is detected in A498 cells (see Figure S6 in the Supporting Information). In whole-cell voltage-clamp recordings, 100 nM (−)-EA evoked ML204-sensitive current (Figure 2e), but the characteristic seatlike inflection of the TRPC4 *I–V* was missing (Figure 2f). We hypothesized that the different shape was due to native coexpression of the broadly expressed TRPC1 protein, which does not produce channels on its own but forms heteromers with TRPC4 and removes the seatlike inflection in the *I–V* graph.[9c,12] We therefore coexpressed TRPC4 with TRPC1 in HEK 293 cells to generate overexpressed heteromers, which almost perfectly reproduced the response of native A498 channels to (−)-EA (Figure 2g and h). The data suggest that (−)-EA also activates TRPC1/4 channels and that these heteromeric channels are the ones activated by (−)-EA in A498 cells.

Since (−)-EA does not cause the death of all types of cancer cells, we explored the (−)-EA-resistant colorectal adenocarcinoma cell line HT29.[4a] HT-29 cells were confirmed as resistant to (−)-EA-induced cell death (see Figure S7a in the Supporting Information) and then studied them by whole-cell patch-clamp recording. No (−)-EA-activated current or ML204 sensitivity was detected (see Figure S7b in the Supporting Information). The data suggest that (−)-EA-activated current occurs only in cell types with susceptibility to (−)-EA-induced cell death.

The relationship of (−)-EA-activated TRPC4/5 channels to cell viability was investigated by attempting to confer (−)-EA-induced death on HEK 293 cells, which are normally resistant.[4b] We compared HEK 293 cells overexpressing TRPC4, TRPC5, or TRPM2. (−)-EA had no effect on HEK 293 cells overexpressing TRPM2, but potently suppressed HEK 293 cell viability when TRPC4 or TRPC5 was overexpressed (Figure 3a,b, see also Figure S8 and Movies S3–S6 in the Supporting Information). To determine whether Ca^{2+} influx is the cause of cell death, we preincubated A498 cells with increasing concentrations of the cell-impermeable Ca^{2+} chelator EGTA to reduce the amount of free Ca^{2+} in the culture medium prior to addition of 100 nM (−)-EA. Interestingly, the (−)-EA-mediated reduction of cell viability was dose-dependently rescued by EGTA (Figure 3c). In addition, lowering the Ca^{2+} concentration in the culturing medium from 1.8 mM to 180 μM resulted in a 10-fold

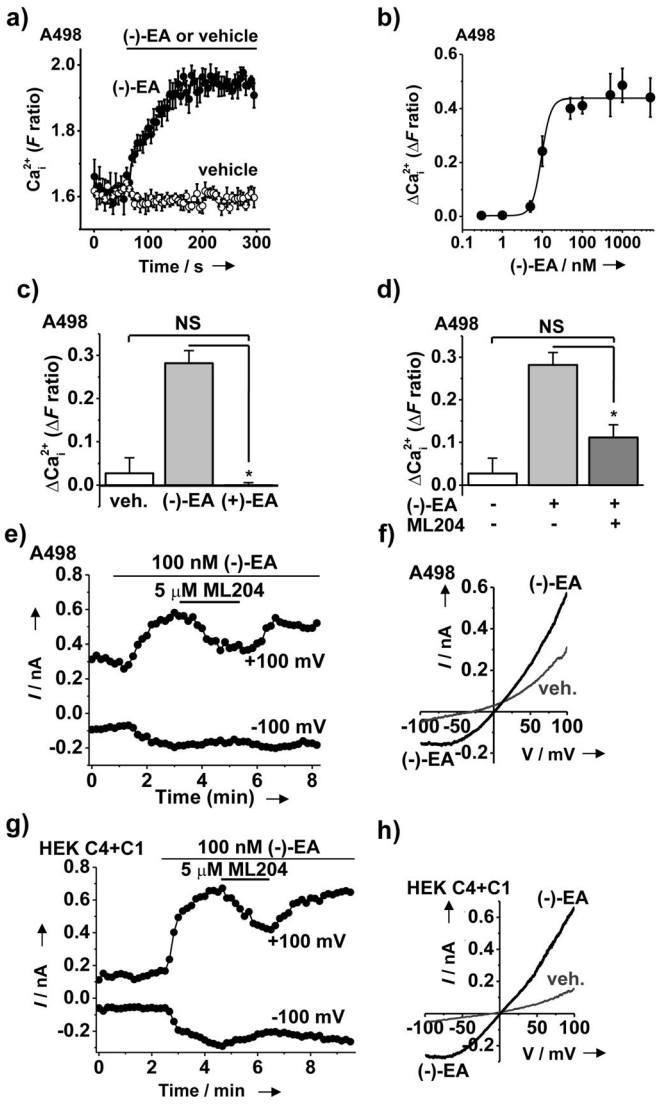

Figure 2. a–d) Measurements of the free intracellular calcium ion (Ca^{2+}_i) concentration in A498 cells shown as the fura-2 fluorescence (F) ratio or change (Δ) in this ratio. a) Example effect of extracellular application of (−)-EA or its vehicle control ($n/N = 1/5$ each). b) Concentration–response data ($n/N = 3/45$) with a fitted Hill Equation indicating an EC_{50} value of 9.5 nM. c) Mean responses after 4 min exposure to vehicle, 1 μM (−)-EA, or 1 μM (+)-EA ($n/N = 4/23$ each). d) Mean responses after 4 min exposure to vehicle, 1 μM (−)-EA, or 1 μM (−)-EA in the presence of 5 μM ML204 ($n/N = 4/23$ each). e) Whole-cell voltage-clamp recording of membrane current from a single A498 cell during ramp changes in membrane voltage from −100 to +100 mV applied every 10 s. Only current sampled at −100 and +100 mV is displayed. 100 nM (−)-EA and 5 μM ML204 were bath-applied as indicated by the horizontal bars. Representative from $n = 11$ (standard pipette solution) and $n = 5$ (aspartate pipette solution). f) From the experiment shown in (e) full current traces during two ramp changes in voltage, one during the initial application of vehicle (veh.; dimethyl sulfoxide and pluronic acid) and the other after the application of (−)-EA and before ML204. g,h) As for (e,f) except with genetically modified HEK 293 cells induced to overexpress TRPC4 and transiently express TRPC1 (HEK C4 + C1). Representative from $n = 3$ (standard pipette solution).

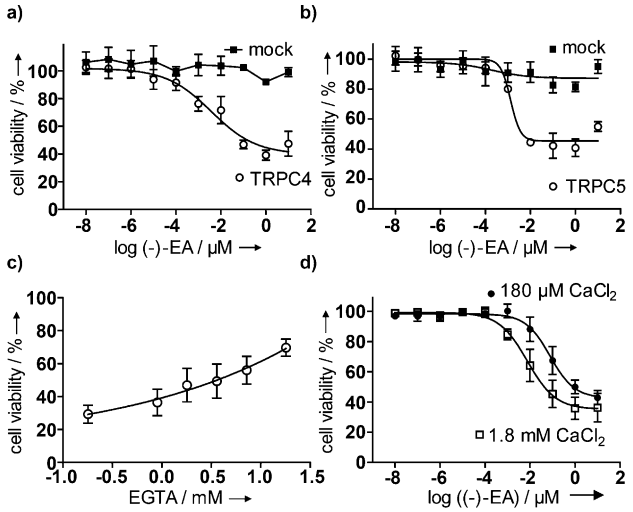

Figure 3. a,b) HEK293 cells were transiently transfected with plasmids for ectopic expression of TRPC4 (a) or TRPC5 (b). 8 h later, the cells were replated and allowed to grow for 48 h. Cells were treated with different concentrations of (−)-EA for 4 h prior to determination of cell viability using the WST-1 reagent. Data are mean values ($N = 4$) ± s.d. and are normalized to cells treated with DMSO. Data were fitted using a four-parameter Hill Equation and are representative of three independent experiments. c) Different concentrations of ethyleneglycol tetraacetic acid (EGTA) and 100 nM (−)-EA were added to A498 cells prior to subsequent determination of cell viability. Data are shown as mean values ± s.d. ($n = 3$, $N = 4$). d) A498 cells were incubated for 24 h in medium containing 180 μM $CaCl_2$ or 1.8 mM $CaCl_2$ prior to the addition of different concentrations of (−)-EA followed by determination of cell viability. Data are shown as mean values ± s.d. ($n = 3$, $N = 4$) and were fitted using a four-parameter Hill Equation, and indicate $IC_{50} = 77.7$ nM in the presence of 180 μM $CaCl_2$ and 7.5 nM in the presence of 1.8 mM $CaCl_2$.

higher IC_{50} value for inhibition of cell viability (Figure 3d), which indicates that Ca^{2+} influx and thus Ca^{2+} cell overload are the cause for the devastating influence of (−)-EA in this cell line. These results are in agreement with the finding that overexpression of constitutively active mutants of TRPC4 or TRPC5 confers Ca^{2+}-dependent death on HEK 293 cells.[13] The data suggest that activation of TRPC4/5 channels is a mechanism for (−)-EA-induced cell death caused by Ca^{2+} overload.

TRPC channels have remained enigmatic despite much investigation.[8,10] Progress towards understanding the channels has been handicapped by the absence of selective highly efficacious activators and lack of potent or selective small-molecule inhibitors.[8b] (−)-EA or analogues of it now open up entirely novel opportunities for studies aimed at better understanding the biology of TRPC4/5 channels.

Moreover, the results suggest an unanticipated opportunity for addressing the problem of renal cell carcinoma through small-molecule activation of TRPC4 or TRPC4-containing channels.

Received: November 28, 2014
Published online: February 23, 2015

Keywords: antitumor agents · calcium ions · ion channels · natural products

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