Novel indolic AMPK modulators induce vasodilatation through activation of the AMPK–eNOS–NO pathway

Marta Sanz-Gómez1,2,5, Elnaz Aledavood3,5, Marina Beroiz-Salaverri2, Elena Vega-Martín1, Marta Gil-Ortega4, Jose Cumella2, Concepción Pérez2, Francisco Javier Luque3, Carolina Estarellas3, María S. Fernández-Alfonso1, & Ana Castro2

Endothelial adenosine monophosphate-activated protein kinase (AMPK) plays a critical role in the regulation of vascular tone through stimulating nitric oxide (NO) release in endothelial cells. Since obesity leads to endothelial dysfunction and AMPK dysregulation, AMPK activation might be an important strategy to restore vascular function in cardiometabolic alterations. Here, we report the identification of a novel AMPK modulator, the indolic derivative IND6, which shows affinity for AMPKα1β1γ1, the primary AMPK isoform in human EA.Hy926 endothelial cells. IND6 shows inhibitory action of the enzymatic activity in vitro, but increases the levels of p-Thr174AMPK, p-Ser1177eNOS and p-Ser79ACC in EA.Hy926. This paradoxical finding might be explained by the ability of IND6 to act as a mixed-type inhibitor, but also to promote the enzyme activation by adopting two distinct binding modes at the ADaM site. Moreover, functional assays reveal that IND6 increased the eNOS-dependent production of NO and elicited a concentration-dependent vasodilatation of endothelium-intact rat aorta due to AMPK and eNOS activation, demonstrating a functional activation of the AMPK–eNOS–NO endothelial pathway. This kinase inhibition profile, combined with the paradoxical AMPK activation in cells and arteries, suggests that these new chemical entities may constitute a valuable starting point for the development of new AMPK modulators with therapeutic potential for the treatment of vascular complications associated with obesity.

Abbreviations

2T-DAF Thiazolofluorescein (2T-DAF)
2-DAF 4,5-Diaminofluorescein-2
2-DG 2-Deoxyglucose
ACC Acetyl-CoA carboxylase
Ach Acetylcholine
ADaM Allosteric drug and metabolite binding site
ADP Adenosine diphosphate
AICAR 5-Aminomimidazole-4-carboxamide riboside
Akt Protein kinase B
AMP 5′-Adenosine monophosphate
AMPK Protein kinase activated by 5′-adenosine monophosphate
ANOVA Analysis of variance
ATP 5′-Adenosine triphosphate
αAID Self-inhibiting domain of the alpha subunit of AMPKα

1Instituto Pluridisciplinar and Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain. 2Instituto de Química Médica, IQM-CSIC, Madrid, Spain. 3Departament de Nutrició, Ciencies de la Alimentació y Gastronomía, Facultat de Farmàcia y Ciencies de la Alimentació, Instituto de Biomedicina (IBUB) e Instituto de Química Teòrica i Comptutacional (ICTQUBUB), Universitat de Barcelona, Campus Terrassa, Santa Coloma de Gramenet, Spain. 4Departamento de Ciencias Farmacéuticas y de la Salud, Facultad de Farmacia, Universidad San Pablo-CEU, Madrid, Spain. 5These authors contributed equally: Marta Sanz-Gómez and Elnaz Aledavood. 6Email: cestarellas@ub.edu; marisolf@ucm.es; acastro@iqm.csic.es
Obesity prevalence has increased over the past decades and is now a major public health problem worldwide. It is associated with an enhanced risk of developing cardiometabolic diseases such as hypertension, insulin resistance, type 2 diabetes mellitus, coronary artery disease, myocardial infarction, heart failure, and stroke. Obesity involves changes in body composition as a consequence of an energetic imbalance in which caloric intake is higher than energy expenditure. The AMP-activated protein kinase (AMPK) is a sensor of cellular energy status that is responsible for maintaining the energy balance after depletion of energy stores, switching off ATP-consuming anabolic pathways.

Endothelial AMPK plays a key role in the regulation of vascular function through the activation of the PI3K-Akt-endothelial nitric oxide synthase (eNOS) pathway and stimulation of nitric oxide (NO) release in endothelial cells. Obesity leads to AMPK dysregulation and endothelial dysfunction, which is the first step in the progression of cardiovascular disease. We have shown that caloric restriction in young Zucker fa/fa rats has cardiovascular benefits by reducing endothelial dysfunction through AMPK–PI3K–Akt–eNOS activation associated to a reduction in blood pressure, plasma triglyceride levels, and cardiac hypertrophy. AMPK activation might be thus an important strategy to restore vascular function in cardiometabolic alterations.

AMPK is a heterotrimeric Ser/Thr kinase of 1188 amino acids (~132 kDa), which is ubiquitously distributed. It is formed by three subunits: α (α1 and α2), β (β1 and β2) and γ (γ1, γ2 and γ3), which combine to give 12 different isoforms. AMPKα is the catalytic subunit and contains a conventional kinase domain (αKD) located at the N-terminus of the protein, and a C-terminal domain required for interaction with the AMPKβ subunit. The C-terminal region of AMPKα subunit forms a globular domain around which the C-terminal region of the AMPKβ subunit is wrapped. The extreme terminus of the AMPKβ subunit then forms an interaction with the AMPKγ subunit, so that the AMPKβ subunit acts as the scaffold that bridges α and γ subunits. A carbohydrate-binding module (CBM), located within the central region of the AMPKβ subunit, forms a binding site for allosteric activators, termed the allosteric drug and metabolite (ADaM) binding pocket. The AMPKγ subunit contains four repeats in tandem of a structural module called cystathionine β-synthase (CBS) motif. Every pair of CBS repeats provides binding sites for the regulatory adenine nucleotides AXP (X = M, D, T). The range of AMPK modulators has gradually expanded over the last years, covering from AMP mimetics, such as AICAR and C2, to ADP mimetics, such as O304, which is able to protect against pThr172 or pThr174 dephosphorylation in AMPKα2 and AMPKα1 isoforms, respectively, without allosteric activation of AMPK.

On top, AMPK activator drugs such as A769661, 991, PF-739, etc., directly activate AMPK targeting the ADaM site. Strikingly, other AMPK modulators with unexpected mechanisms of action have been recently described. MT47-100 is an allosteric AMPKβ2 inhibitor that simultaneously activates AMPKβ1,
whereas SU665615 paradoxically activates AMPK signaling by directly binding at the catalytic site. All these results reveal the complex regulation of this kinase, but at the same time offer the opportunity to be exploited in the search for drugs with novel mechanisms of action. In this work, we describe novel indolic compounds as modulators of endothelial AMPK. For this purpose, the binding mode of these compounds has been assessed by combining molecular dynamics (MD) simulations, enzymatic and Surface Plasmon Resonance (SPR) assays, together with functional activation studies targeting AMPK, eNOS and Acetyl CoA Carboxylase (ACC) phosphorylation, as well as assessing NO production in human endothelial cells (EA. Hy926) and through vascular function of rat thoracic aorta. Our findings offer new possibilities for regulating endothelial AMPK, as well as exploring the therapeutic implications of this novel mechanism of action.

Results
Identification of IND6 as AMPKαβγ1 modulator. Within our drug discovery program focused on the search for novel AMPK modulators\(^1\), we combined surface plasmon resonance (SPR) assays with enzymatic activity studies with the aim to evaluate the ability of the indole derivatives (IND6, IND7, IND8, IND11) to bind to AMPKαβγ1 (Table 1). 

| Compound | % Inhibition ± SD at 30 μM\(^a\) | Binding 100 μM (RU)\(^b\) |
|----------|---------------------------------|---------------------------|
| IND6     | 62.00 ± 2.77                    | 13.3                      |
| IND7     | 39.42 ± 3.03                    | 13.6                      |
| IND8     | 52.38 ± 2.51                    | 14.6                      |
| IND11    | 23.46 ± 2.48                    | 0.9                       |

Table 1. AMPKαβγ1 inhibitory activity and binding results measured by SPR (RU) of indole derivatives.

\(^a\)Data is the mean ± standard deviation (SD) of two independent experiments. Positive control: A-769662: % activation ± SD at 30 μM: 394 ± 76. \(^b\)Binding (100 μM): Positive binding control: A-769662 (RU 31.7); Negative binding control: β-cyclodextrin (RU 1.6). Data is the mean ± SD of 1–6 independent experiments.

Scheme 1. (i) iodosuccinimide, DMF, 0 °C, 1 h; (ii) toluene:ethanol:water:1,4-dioxane (1:3:6:10), R-B(OH)\(_2\), Pd(PPh\(_3\))\(_4\), K\(_2\)CO\(_3\), 85 °C, 18 h; (iii) K\(_2\)CO\(_3\), EtOH, 85 °C, 18 h; (iv) KOH, EtOH, 100 °C, 18 h.

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In parallel, the effect of compounds IND6, IND7, IND8, and IND11 on the enzymatic activity of AMPK was examined through a luminescent assay with the recombinant AMPK isoform α1β1γ1. This assay evaluates the enzymatic activity of AMPK to phosphorylate the SAMS peptide substrate, using A-769662 as positive control. All compounds in the series were found to reduce the activity of AMPK α1β1γ1 at 30 µM and therefore may be initially considered inhibitors (Table 1). At this single concentration the percentage of inhibition ranges from 39 to 62% for IND6, IND7 and IND8, and it is less pronounced for IND11 (23%). Based on these results, IND6 was selected as representative compound to carry out a detailed evaluation of the biological effect on the enzyme activity.

**Competition assays of the indolic compound IND6.** To investigate the inhibitory mechanism of IND6, dose–response assays were performed using different ATP concentrations (from 20 to 1000 µM) and two inhibitor concentrations (10 and 20 µM). The Lineweaver–Burk plot of enzyme kinetics is depicted in Fig. 1. The results suggest that IND6 acts as a mixed-type inhibitor, as noted by the increase in the $K_s$ for ATP and the decrease in $V_{max}$ with increasing concentration of IND6. Let us note that this modality of enzyme inhibition was also reported previously for SBI-020696517. Representation of the relationship between $K_m/V_{max}$ and the concentration of IND6 led to inhibition constants $K_i$ and $K_i'$ of 6.9 and 27.1 µM, respectively. These values are 27- and 30-fold higher than the $K_i$ and $K_i'$ values determined for SBI-0206965, respectively ($K_i = 0.26$ µM; $K_i' = 0.89$ µM). Overall, although both IND6 and SBI-0206965 exhibit a mixed-type inhibition, which conceptually combines both competitive and uncompetitive inhibition, these results suggest that the inhibitory activity may reflect different mechanisms of action.

To further explore the IND6 binding mode, SPR studies were performed at increasing concentrations (from 10 to 100 µM) of either SBI-0206965 or IND6 (Fig. 2). The sensorgrams showed a progressive increase in the binding, which was much larger for SBI-0206965 in agreement with its stronger inhibitory potency (Fig. 2A,B). In a separate assay, IND6 was injected in the presence of SBI-0206965, both at a 100 µM concentration. Under these conditions, the sensorgrams showed an additive effect between SBI-0206965 and IND6 (Fig. 2C), suggesting that binding to AMPK might involve distinct binding sites. When the injection of SBI-0206965 and IND6 was performed in the presence of a high ATP concentration (200 µM) (Fig. 2D), SBI-0206965 binding was notably reduced, whereas IND6 binding was less sensible to the presence of ATP.

**IND6 promotes the phosphorylation and activation of AMPK and downstream targets in a concentration-independent manner.** In order to examine the effect of IND6 in AMPK phosphorylation and some of its targets, such as eNOS and ACC, human endothelial cells of the EA Hy.926 line were treated with IND6 at different concentrations (0.01, 0.1, 1, 10 and 100 µM). Untreated cells were used as control (CT). AICAR (at 5 mM), a known AMPK canonic activator, and 2-deoxyglucose (2-DG, at 1 mM), a caloric restriction mimetic, were used as positive controls. AICAR, once inside the cell, is phosphorylated by adenosine kinases and is converted to ZMP, an AMP mimetic, which binds the CBS sites in γ-AMPK18. On the other hand, 2-DG
is a competitive inhibitor of glucose metabolism since it is phosphorylated by hexokinase to DG-PO₄, which is trapped in the cell unable to undergo further metabolism.

**IND6** significantly increased the level of p-Thr₁₇₄AMPK at concentrations of 0.01, 0.1, 1 and 10 µM in a concentration-independent manner (Fig. 3A,B). The effect was comparable to the enhancement elicited by AICAR at 5 mM and 2-DG at 1 mM. Since the increased phosphorylation of AMPK is in contrast with the mixed-inhibitor features observed in the enzymatic assays, we determined levels of p-Ser₇⁹ACC and p-Ser₁₁⁷eNOS, which are well-known targets of AMPK in the endothelium. **IND6** significantly increased the levels of both p-Ser⁷⁹ACC/tubulin and p-Ser₁₁⁷eNOS/tubulin at concentrations of 0.01, 0.1, 1 and 10 µM in a concentration-independent manner (Fig. 3A,C,D), suggesting that **IND6** promotes a functional activation of AMPK, this effect being nevertheless less apparent at the highest concentration of 100 µM.

**Binding mode of IND6 to AMPK.** Due to the mixed-type inhibition of **IND6**, we investigated the binding to the ATP-binding site using Molecular Dynamics (MD) simulations. Four independent simulations were run for the AMPKαβγ complex with **IND6** and with SBI-020695, which was used as reference system. The X-ray structure of the AMPK–SBI-020695 complex (PDB entry 6BX6) revealed that SBI-020695 occupies a pocket located between the N- and C-lobes and the hinge region of the enzyme, overlapping with the binding site of compound C, which is a competitive inhibitor of AMPK. The results obtained from the different MD simulations showed a consistent picture, where SBI-020695 remains stably bound in the ATP-binding pocket in all simulations (Fig. 4). In particular, binding is assisted by two hydrogen bonds between SBI-020695 and the main chain of αVal₉₈, with distances (averaged for the four MD simulations) of 3.2 ± 0.2 Å and 2.9 ± 0.1 Å between the pyrimidine nitrogen and exocyclic nitrogen of the inhibitor and the amide NH and carbonyl oxygen of αVal₉₈, respectively. Furthermore, the ligand is enclosed in the hydrophobic pocket shaped by residues αLeu₂₄, αVal₃₂, αIle₇₉, αMet₉₅, αLeu₁₄₈ and αAla₁₅₈.

The competitive binding mode of **IND6** to the ATP-binding pocket was guided by the superposition with both staurosporine, SBI-020695 and compound C, taking advantage of their X-ray structures (PDB entries 4ZHX, 4CFE, 4CFF, 6BX6 and 3AQV; see Supplementary Material), which revealed the formation of hydrogen bonds between these compounds with the hinge region of the kinase, particularly involving residues αVal₉₆ and αGlu₉₄ (αVal₉₈ and αGlu₉₆ in AMPKα₁). The MD simulations performed for the AMPK–**IND6** complex revealed larger fluctuations of the ligand in the binding pocket compared to SBI-020695 (Fig. 4A). This trait can be attributed to the flexibility of the benzyl moiety as well as to the non-planarity of the central benzene ring relative to the indole ring, enhancing also the fluctuations of the P loop (Fig. 4B). This is also reflected in the hydrogen bond distances formed between the indole NH group of **IND6** with the carbonyl oxygen of αVal₉₆ (average distance of 3.4 ± 1.1 Å), and the carboxylate oxygen of **IND6** with the NH group of αGlu₉₆ (average distance of 3.7 ± 0.9 Å), which are larger than those formed by SBI-020695. Overall, these traits agree with the 27-fold lower potency of **IND6** relative to SBI-020695 (see above).
Figure 3. IND6 increases p-AMPK, p-ACC, and p-eNOS levels in a concentration-independent manner. (A) Representative immunoblots of p-Ser79ACC, p-Ser1177eNOS, p-Thr174AMPK and tubulin of EA Hy926 human endothelial cells after the treatments (60 min for AICAR and IND6 and 15 min for 2-DG). (p-Thr174AMPK CT band was cropped from channel 9 of the gel to channel 1). (B) Bar chart representation of the densitometry of the immunosensing bands (WB) expressed as the percentage of p-Thr174AMPK/tubulin with respect to the control group. (C) Bar chart representation of the densitometry of the immunosensing bands (WB) expressed as the percentage of p-Ser79ACC/tubulin with respect to the control group. (D) Bar chart representation of the densitometry of the immunosensing bands (WB) expressed as the percentage of p-Ser1177eNOS/tubulin with respect to the control group. *p < 0.05; **p < 0.01. n = 4–5. Data are presented as means ± SEM.

Figure 4. Representation of the binding mode of SBI-020695 and IND6 to the ATP-binding site of AMPK obtained from Molecular Dynamics simulations. (A) Superposition of the X-ray crystallographic structure of AMPK bound to SBI-020695 and a representative snapshot taken from the MD simulations. (B) Representative snapshot of the MD simulation run for the AMPK–IND6 complex. The P-loop is highlighted in yellow. SBI-020695 and IND6 are shown in sticks. C atoms colored in cyan and orange shown the X-ray and final position of the ligand in MD simulation, respectively. Hydrogen atoms have been removed for the sake of clarity.
Additional MD simulations were also performed to examine the binding of IND6 to the ADaM site, which mediates the activation effect played by several small molecules, such as A-769662. The ligand was oriented taking advantage of the close alignment exhibited by activators such as A-769662, 991 and SC4 (see Supplementary Material). These studies showed that IND6 may adopt two distinct binding modes (Fig. 5). In one case, IND6 is deeply bound into the hydrophobic cavity of the ADaM site, and the carboxylate group forms salt bridge interactions with the protonated amino groups of αLys31 and αLys33 (average distances of 3.0 ± 0.4 and 3.5 ± 0.9 Å). It is worth noting that the top of the P-loop points to the N-terminus of the αC-helix, leaving the ATP-binding site accessible for the binding of ATP. Indeed, a significant fraction of the conformations sampled by the P-loop superpose well with the conformations adopted in the ternary complex formed by AMPK bound to A-769662 and ATP (Fig. 6). This suggests that IND6 might mimic the role of A-769662 in this binding mode.

In the other binding mode, IND6 protrudes from the ADaM site toward the αC-helix, sitting on the top of the P-loop (Fig. 5B). This binding mode is assisted by electrostatic interactions between the carboxylate group and the protonated residues αLys31 and αLys53 (average distances of 4.9 ± 0.9 and 5.3 ± 1.1 Å), and a cation-π interaction between βArg83 and the benzyloxy ring of IND6 (average distance of 4.0 ± 0.5 Å). Remarkably, this binding mode imposes a structural distortion of the P-loop, which occludes the ATP-binding site (Figs. 5B and 6), making it unable to accommodate ATP. Therefore, this binding mode might explain the non-competitive mechanism of the mixed-type inhibition.
IND6 promotes eNOS-dependent NO production in human endothelial cells with a major potency than AICAR does. To determine whether the increase in p-Thr174 AMPK and p-Ser1177 eNOS elicited by IND6 translates to an increment of NO production, NO levels were determined in human endothelial cells EA.Hy926 in presence of 1 and 5 µM IND6. Untreated cells were used as a control (CT). Cells treated with DMSO 0.01% (maximum concentration of solvent for IND6 (5 µM)), AICAR (5 mM), IND6 (1 and 5 µM) and IND6 (1 and 5 µM) + L-NAME (100 µM), (n = 3–5). (B) Bar chart of the quantification of the intensity of green fluorescence (530 nm) by densitometry represented as the percentage with respect to the control cells. **p < 0.01 vs. CT and ****p < 0.0001 vs. CT; n = 3–5. (C) Bar chart of the quantification of the intensity of green fluorescence (530 nm) by densitometry represented as the percentage with respect to the control cells. $$$$p < 0.0001 vs. IND6 1 µM and ####p < 0.0001 vs. IND6 5 µM; n = 3–5. The data are presented as means ± SEM.

Figure 7. IND6 promotes an increase in DAF-2T fluorescence in human endothelial cells. (A) Representative fluorescence microscopy images of NO fluorescent indicator (DAF-2D incubation, first column left and right, green) and DAPI (second column left and right, blue) in cultured EA.Hy926 endothelial cells in basal conditions (CT) and after 60 min treatment with DMSO 0.01%, eNOS inhibitor L-NAME (100 µM), AMPK activator AICAR (5 mM), IND6 (1 and 5 µM) and IND6 (1 and 5 µM) + L-NAME (100 µM), (n = 3–5). (B) Bar chart of the quantification of the intensity of green fluorescence (530 nm) by densitometry represented as the percentage with respect to the control cells.
IND6 promotes a concentration-dependent vasodilation in rat aorta that is mediated by the AMPK–eNOS–NO pathway. To confirm the activation of the AMPK–eNOS–NO pathway by IND6, vascular function was assessed in endothelium-intact thoracic aorta of Wistar rats. A vasodilatory response to IND6 was observed in a concentration-dependent manner (10^{-9}–10^{-4} M) in artery segments precontracted with a single dose of NA 10^{-7} M (Fig. 8A). Relaxation elicited by IND6 was significantly reduced in artery segments preincubated with SBI-0206965 (10^{-4} M) or L-NAME (10^{-4} M) (Fig. 8B), which are inhibitors of AMPK and eNOS, respectively. Furthermore, concentration–response curves were also performed with A-769662 (10^{-9}–10^{-4} M) or AICAR (10^{-5}–8 \times 10^{-3} M) to compare their effect with the one promoted by IND6 (Fig. 8C). Since the three of them elicited a concentration-dependent vasodilation, their pharmacological efficacy (Emax) and potency (EC50) were calculated. Although the three compounds had a similar Emax, IND6 resulted as potent as A-769662 but significantly more potent than AICAR (Table 2).

Discussion

The results presented in this study point out that the novel indolic derivatives appear to act as paradoxical activators of the endothelial AMPK α1β1γ1, although they exhibit a mixed-type inhibition in the enzymatic assays. Dysregulation in the AMPK signaling pathway in over-nutrition and obesity contributes to the development of metabolic disorders and endothelial dysfunction, which is considered the first step in the progression of cardiovascular disease. Reduced endothelial AMPK phosphorylation leads to down-regulation of the PI3K-Akt-eNOS pathway together with low rates of NO synthesis. Contrarily, activation of endothelial AMPK restores impaired endothelial function and normalizes systolic blood pressure through the stimulation of the PI3K-Akt-eNOS pathway. In this context, identification of new chemical entities that can activate endothelial AMPK could be of significant interest for the treatment of obesity-related disorders. Starting with the use of SPR techniques, we selected IND6, which exhibits one of the best affinity values against recombinant AMPKα1β1γ1 (RU 13.2, 100 µM). In parallel, the AMPK α1β1γ1 enzymatic activity was assessed by means of a luminescent assay, which revealed inhibitory activity values in the micromolar range against all tested compounds (Table 1). Hence, IND6 was subjected to enzymatic kinetic analysis to examine its competition with ATP. We varied both ATP and IND6 concentrations with a constant concentration of the peptide substrate used in the enzymatic reaction. The double reciprocal plot of data (Fig. 1) indicated that IND6 behaves as a mixed-type AMPK inhibitor. A similar inhibition mode has been recently reported for SBI-0206965, which was described as type IIb AMPK inhibitor. Furthermore, SPR sensorgrams showed that IND6 and SBI-0206965 presented an additive effect on the binding to AMPK, suggesting that they may bind at different binding sites. Moreover, when we performed the same experiments in the presence of a high ATP concentration (200 µM), there was a significant reduction in the ability of SBI-0206965 to bind to AMPK, while the affinity of IND6 for AMPK remained at large extent.

Table 2. Efficacy (Emax) and potency (EC50) of IND6, A-769662 and AICAR as vasodilators in rat aorta measured with organ bath vascular function. **p-value < 0.001 vs. AICAR; n = 6 rats.
unchanged. All these results suggest that IN6 and SBI-020696 may bind the ATP-binding site, thus leading to competitive inhibition of the enzyme, but also suggest that IN6 may regulate the AMPK activity through binding to an additional pocket.

The biological effect of IN6 may be explained by the distinct binding modes observed for IN6 in the ADaM site, and the drastic influence exerted on the structural conformation of the P-loop. Thus, the similar arrangements observed for the P-loop when IN6 is deeply inserted into the ADaM site and in the X-ray structure of the AMPK bound to A-769662 suggest that IN6 may mimic the activating role attributed to this latter compound. Nevertheless, the structural distortion of the P-loop caused by the alternative binding mode, where IN6 protrudes from the ADaM site, might explain the non-competitive component of the mixed-type inhibition, in conjunction with the direct competition exerted by IN6 upon binding to the ATP-binding site. At this point, let us remark that the adoption of two partially overlapping binding modes at the ADaM site may lower the apparent IC50 of IN6, a suitable parameter to assess the chemical inhibition of AMPK. However, the IC50 of IN6 is one and three orders of magnitude higher than A-769662 and SBI-0206965, respectively.

Despite the mixed-type inhibition, cellular assays showed that IN6 promotes AMPK phosphorylation (% p-AMPK/tubulin vs CT) in the human endothelial cell line, EA Hy.926, which expresses the α1β1 isoform. This suggests a paradoxical activation of AMPK similar to the one described for the indolic compound SU6656, which seems to promote AMPK’s LKB1 dependent phosphorylation20,21. In any case, the activation of AMPK by IN6 is functional since it translates to ACC, an ubiquitous AMPK target22, as well as to eNOS and NO release. The inhibition elicited by SBI-0206965 on the concentration-dependent relaxation induced by IN6 in arterial rings confirms specific activation of vascular AMPK, whereas its inhibition by L-NAME confirms activation of endothelial eNOS. This is in accordance with the AMPK-dependent eNOS activation described in endothelial cells23, as well as in arteries associated to a reduction in blood pressure, plasma triglyceride levels, and cardiac hypertrophy24. Activation of eNOS by AMPK is a well-described pathway, although an attenuated NO production in response to AMPK activation has also been reported25.

The efficacy of IN6 to promote AMPK stimulation is comparable to AICAR (5 mM), 2-DG (1 mM) or A-769662 (0.1 mM). However, the potency of IN6 is in the micromolar range, one and three orders of magnitude higher than A-769662 and AICAR, respectively, as clearly observed in the relaxation experiments. To note, the apparent discrepancy between the concentrations of IN6 tested in the in vitro assays with recombinant proteins (SPR or inhibition studies) and the active concentrations observed in cell culture, which are much lower than in the former case. This could be explained by the synergistic effect of the intracellular machinery, which leads to signal amplification26.

In summary, IN6 binding profile provides a basis to rationalize the activating behavior of IN6 in EA Hy.926 cells and arteries by increasing AMPK activity in a functional manner, as demonstrated by the increment in both ACC and eNOS phosphorylation, two well-known targets of p-Thr172/174AMPK20,21. Moreover, this study shows that IN6 increases NO levels in both endothelial cells and arteries, demonstrating again a functional activation of AMPK. Our findings provide evidence that IN6 holds potential as treatment of vascular complications associated with obesity, where intracellular ATP levels are high due to the energy surplus and AMPK activity is reduced27. AMPK activation by IN6 might be an important strategy to restore vascular function in cardiometabolic alterations.

Methods
Chemistry. All reagents were of commercial quality. Solvents were dried and purified by standard methods. Analytical TLC was performed on aluminum sheets coated with a 0.2 mm layer of silica gel 60 F254. Silica gel 60 (230–400 mesh) was used for flash chromatography. Analytical HPLC–MS was performed on Waters equipment coupled to a single quadrupole ESI–MS (Waters Micromass ZQ 2000) using a reverse-phase SunFire C18 4.6 × 50 mm column (3.5 μm) at a flow rate of 1 mL/min and by using a diode array UV detector. Mixtures of CH3CN and H2O were used as mobile phase (gradient of 15–95% of acetonitrile in water). MS (ES, positive mode): 344 (M+H)+. Mp 194–196 °C. 1H NMR (300 MHz, DMSO-d6) δ 12.78 (s, 1H), 11.73 (s, 1H), 7.55–7.22 (m, 10H), 7.12–7.01 (m, 3H), 5.16 (s, 2H). 13C NMR (75 MHz, DMSO-d6) δ 122.1; 120.9; 120.5; 114.4; 112.9; 69.6. HRMS (EI+) m/z ([M]+) calcd for C22H17NO3 343.12175; found 343.12179.

Identification of the AMPK isoform expressed by human endothelial cells EA Hy.926 by proteomics. In order to choose the AMPK isoform to carry out the kinase assay and affinity studies, we determined the isoform mainly expressed in EA Hy.926 cells. Cell lysates were analyzed in the proteomics unit of the UCM (CAI Técnicas Biológicas). For this purpose, protein precipitation and digestion with trypsin gel was carried out. Then, LC–MS/MS (Q-EXACTIVE) analysis of triptic peptides was performed (massive analysis: Shot-
gun and directed analysis: Parallel Reaction Monitoring, PRM), obtaining an m/z MS–MS spectrum that was used for the subsequent database search and identification based on the results. After comparing the information obtained with the data dumped in the databases, it was concluded that the most abundant isoform of AMPK in these cells is AMPKα1β1 with 99.99% accuracy. Although the identification of the subunit γ was not conclusive, we chose the recombinant isoform AMPKα1β1γ1 for the affinity and kinase assay studies.

### Binding studies by surface plasmon resonance (SPR)

SPR experiments were performed at 25 °C with a Biacore X-100 apparatus (Biacore, GE) in HBS-EP (10 mM Hepes, 150 mM NaCl, 3 mM EDTA), with 2% de DMSO, 0.05% Tween 20 and 200 µM ATP when was required, at 25 °C. The protein AMPK was immobilized on a CM5 sensor chip (Biacore, GE) following standard amine coupling method. The carbamoylmethyl dextran surface of the flow cell 2 was activated with a 7-min injection of 1:1 ratio of 0.4 M EDC and 0.1 M NHS. The protein was coupled to the surface with a 300 s injection at different dilutions at 40 µg/mL in 10 mM sodium acetate, pH 5.0. The unreacted N-hydroxysuccinimide esters were quenched by a 7-min injection of 0.1 M ethanolamine–HCl (pH 8.0). The levels of immobilization were around 1000 RUs. Flow cell 1 treated as a flow cell 2 (amine coupling procedure) without protein was used as a reference. Prior to use 10 mM stock solutions of compounds were diluted several times until 100 µM final concentration in the running buffer. No regeneration was needed. For competition measurements between AMPK and different compounds the concentrations used in the mixture were 100 µM for each one. Sensorgrams data were double-referenced and solvent corrected using the Bioevaluation X-100 software (Biacore, GE).

### Kinase assay

The AMPK (α1/β1/γ1) Kinase Enzyme System from Promega (Catalog number V1921) was used to screen AMPK inhibitors following the ADP-Glo™ Kinase Assay (Catalog number V9021). The assays were performed in 96-well plates (final volume 20 µL), the assay buffer contains 40 mM Tris, 7.5, 20 mM MgCl2, 0.1 mg/mL BSA and 50 µM DTT. 4 µL of inhibitor was added to each well (final concentration of DMSO did not exceed 1%), followed by 8 µL of enzyme (30 ng), after 5 min incubation at R/T, 8 µL of ATP (150 µM final concentration) and SamStide (0.2 µg/µL) were added and incubate 60 min at room temperature, then ADP-Glo™ reagent (20 µL) was added allowing to incubate for 40 min at room temperature. Behind the incubation, the kinase detection agent (40 µL) was added and incubated for 30 min at room temperature. Finally, the luminescence was recorded using a FLUO star Optima (BMG Labtechnologies GmbH, Offenburg, Germany) multimode reader. The inhibition activities were calculated based on the maximum activity, measured in the absence of inhibitor.

For competition assays, the experiments were carried out at four different concentrations of ATP (20–1000 µM) in the absence or presence of the inhibitors, at two concentrations. The results were presented as double reciprocal Lineweaver–Burk plots (1/V vs 1/[ATP]).

### Molecular modelling simulations

Molecular Dynamics (MD) simulations were used to examine the binding mode of SBI-020695 and IND6 to AMPK, considering the binding to both the ATP-binding site and the ADaM site, which is implicated in the enzyme activation by small molecules, such as A-769662. The X-ray structure of AMPK in the PDB entry 6C9J [30], which consists of chains α1, β1 and γ1, was utilized to build up the protein, whereas the ligand (SBI, IND6) was parameterized using the GAFF force field in conjunction with restrained electrostatic potential-fitted (RESP) partial atomic charges derived from B3LYP/6-31G(d) [31] calculations. The two simulated systems were immersed in an octahedral box of TIP3P water molecules. The final systems contained around 370 residues, the ligand, around 26,000 water molecules, and one/two Na+ atom for the complexes with SBI/IND6, which were added to maintain the neutrality of the simulated systems.

Simulations were performed using the AMBER18 package [32] and the Amber ff99SB-ildn force field [33] for the protein, whereas the ligand (SBI, IND6) was parameterized using the GAFF force field in conjunction with restrained electrostatic potential-fitted (RESP) partial atomic charges derived from B3LYP/6-31G(d) [31] calculations. The two simulated systems were immersed in an octahedral box of TIP3P water molecules. The final systems contained around 370 residues, the ligand, around 26,000 water molecules, and one/two Na+ atom for the complexes with SBI/IND6, which were added to maintain the neutrality of the simulated systems.

Simulations were done in the NPT ensemble for equilibration and NVT for MD productions. The simulations for SBI and IND6 were performed for 4 independent replicas. The minimization of the two systems was performed refining the position of hydrogen atoms in the protein (2000 cycles of steepest descent algorithm followed by 8000 cycles of conjugate gradient), and subsequently of the whole system (4000 cycles for steepest descent and 1000 cycles of conjugate gradient). Then, the temperature of the system was gradually increased from 100 to 300 K in 5 steps (50 ps each) using the NVT ensemble, followed by an additional 5 ns step performed in the NPT ensemble to equilibrate the density of the system. In this process, restraints were imposed to avoid artefactual changes in the hydrogen bonds between the ligand with Val98, as well as between pThr174 and Arg140. Production MD simulations were run for 250 ns per replica, leading to a total simulation time of 1.0 µs per ligand. Restraints were gradually eliminated during the first 100 ns in order to avoid changes in the ligand binding mode due to structural fluctuations in the ATP-binding pocket, and the analysis of the trajectories was performed on the snapshots sampled in the last 150 ns unrestrained MD simulation.
Human endothelial cell cultures and treatments. Cell culture studies were performed on a human endothelium cell line (EA.Hy926). Briefly, cells were seeded in 75 cm² flasks with Dulbecco’s modification of Eagle’s High Glucose Medium (DMEM, Biowest), 10% fetal bovine serum (FBS, Biowest), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Biowest) and kept at 37 °C in a humid atmosphere and 5% CO₂. Once subconfluent (70–80%) subcultures were carried out using trypsin–EDTA (0.25%, Sigma) and used between passages 3 and 7. Cells were incubated for 1 h with either the AMPK activator AICAR (5 mM), IND6 (0.01, 0.1, 1 and 100 µM); or 10 min with 2-DG (1 mM). There were also control cells (CT) without treatment. All the treatments were kept at 37 °C in a humid atmosphere and 5% CO₂.

Determination of the phospho-proteins by WB. The expression of the phosphorylated forms of: AMPKα in the residue Thr174 (pThr174-AMPKα; 62 kDa), ACC in the residue Ser79 (pSer79-ACC; 265 kDa), eNOS in the residue Ser 1177 (p-Ser1177eNOS; 133 kDa) and tubulin (55 kDa) were determined by WB (as described in Supplementary Material) in EA.Hy926 cells lysates. Cells were seeded in 6-well plates (Sarstedt) with a density of 120,000 cells/well. After 48 h, once subconfluent, they were treated with modulating compounds (AICAR at 5 mM, 2-deoxyglucose at 1 mM, and IND6 at 0.01, 0.1, 1 and 100 µM) and in control untreated cells (CT). All the treatments were kept at 37 °C in a humid atmosphere and 5% CO₂ for 1 h, except of the 2-deoxyglucose, which were maintained only during 10 min.

For the detection of all the proteins, acrylamide gels at 7% [H₂O (5.1 mL); 1.5 M Tris–HCl pH = 8.8 (2.5 mL); SDS at 20% (50 µL) were used, acrylamide/bisacrylamide 30% (2.3 mL); ammonium persulphate 10% (50 µL), TEMED (5 µL)]. Polyclonal rabbit antibodies against the pSer79-ACC, pThr174-AMPK (1:500, Cell Signaling Technology) and pSer1177-eNOS (1:500, EMD Millipore Corporation) were used as primary antibodies and an anti-rabbit antibody (IgG) marked with peroxidase (1:2000, Santa Cruz Biotechnology) was used as secondary antibody. Tubulin was detected with a monoclonal mouse antibody (1:5000, Abcam) as primary antibody and an anti-mouse antibody (IgG) marked with peroxidase (1:10,000, GE Healthcare) as secondary. Quantification was carried out by establishing the relationship between the phosphorylated form of the different proteins and tubulin (p-protein/tubulin) based on the concentration of the modulating compounds administered, in addition to the negative control, on which no treatment was performed. It is worth to be mentioned that in order to obtain the maximum efficiency of the technique, WB gels were cut before the transference following the molecular weight markers depending on where the proteins were expected to appear.

Detection of nitric oxide (NO) by fluorescence microscopy. EA. Hy926 cells were seeded on 8-well plates (Sarstedt) at a density of 6000 cells per well and allowed to grow in DMEM until a 60–70% confluence was reached. The medium was then aspirated and the cells for NO detection were incubated for 1 h with no treatment (control, CT), with DMSO 0.01% (maximum solvent concentration achieved with IND6 5 µM), AICAR 5 mM as a control of activation of NO production, IND6 1 µM and IND6 5 µM. Once the incubation time had elapsed, the medium was aspirated and the cells were incubated with 4.5-diaminofluorescein diacetate (Molecular Probes) (DAF-2DA 10⁻⁵ M) for 30 min in the dark, at 37 °C in a humid atmosphere and 5% CO₂ in DMEM. The DAF-2DA is a non-fluorescent permeable probe capable of diffusing through the cell membrane. Inside the cell it is degraded by esterases to 4.5-diaminofluorescein-2 (2-DAF), which when reacting with intracellular NO gives rise to thiazolofluorescein (DAF-2T), capable of emitting green fluorescence (excitation wavelength 488 nm and emission wavelength 530 nm), so that the intensity of fluorescence emitted will be proportional to the production of NO in the cells. To assess eNOS involvement in DAF-2T fluorescence increase, endothelial cells were also incubated 30 min with the eNOS inhibitor L-NAME (100 µM); or 10 min with 2-DG (1 mM). There were also control cells (CT) without treatment. All the treatments were kept at 37 °C in a humid atmosphere and 5% CO₂.

Animals. Eight-week-old male Wistar rats were housed under controlled dark–light cycles (12 h:12 h from 8:00 to 20:00) and temperature (25 °C) conditions with standard food and water ad libitum. Animals were housed individually for two weeks. Then, they were anesthetized with ketamine (Rompun, Bayer; 0.8 mg/100 g) and xylazine (Imalgene, Merial; 0.4 mg/100 g) and sacrificed by exsanguination. Thoracic aorta was dissected from the aorta it is degraded by esterases to 4.5-diaminofluorescein-2 (2-DAF), which when reacting with intracellular NO gives rise to thiazolofluorescein (DAF-2T), capable of emitting green fluorescence (excitation wavelength 488 nm and emission wavelength 530 nm), so that the intensity of fluorescence emitted will be proportional to the production of NO in the cells. To assess eNOS involvement in DAF-2T fluorescence increase, endothelial cells were also incubated 30 min with the eNOS inhibitor L-NAME (100 µM); or 10 min with 2-DG (1 mM). There were also control cells (CT) without treatment. All the treatments were kept at 37 °C in a humid atmosphere and 5% CO₂.

Vascular reactivity in the thoracic aorta artery. Vascular function studies were performed on the thoracic aorta, as previously described. Once isolated, it was placed in a Petri dish containing Krebs Henseleit (KH) solution (115 mM NaCl; 4.6 mM KCl; 2.5 mM CaCl₂·2H₂O; 25 mM NaHCO₃; 1.2 mM KH₂PO₄; 1.2 mM MgSO₄·7H₂O; 0.01 mM EDTA; 11.1 mM glucose) at 4 °C. With the aid of a binocular lens (Leica GZ4), the blood was carefully removed from inside the vessel and the connective tissue and perivascular adipose tissue...
were separated. The artery was then cut into segments of 2 mm in length. The experiments were carried out in intact arteries with endothelium.

Each of these segments was inserted between two horizontal rigid steel wires (300 μm in diameter) according to the method described previously. Arterial segments were placed in an organ bath containing a KH solution at 37 °C continuously bubbled with a mixture of 95% O₂ and 5% CO₂, maintaining a physiological pH between 7.3 and 7.4 constantly. These were subjected to an initial tension of 1.5 g; which was periodically readjusted for 45 min until stabilization. Once the preparations were stabilized, their functional integrity was checked with KCl (75 mM). This value represented 100% of vascular contraction for each of the arterial segments. Next, concentration–response relaxation curves were performed after contraction to a single dose of NA (10⁻⁷ M), both with acetylcholine to test the integrity of endothelial function and with the different AMPK activators (AICAR 10⁻⁶ M–8 × 10⁻³ M; A-769662 10⁻⁹ M–10⁻⁴ M or IND6 10⁻⁹ M–10⁻⁴ M) to assess vasodilatation. To test the possible involvement of both AMPK and eNOS in the IND6-dependent vasodilation, arteries were preincubated for 30 min with the AMPK and eNOS inhibitors, SBI-0206965 (10⁻⁴ M) and L-NAME (10⁻⁴ M), respectively. After each curve, the preparations were washed 3 times with KH solution, and a 20-min rest period was left between each curve to ensure that the effects observed in each curve were not due to the agents used previously. The relaxation results were expressed as percentage relaxation with respect to the previous contraction obtained with NA (10⁻⁷ M). The analysis of the recordings obtained was performed with the aid of ACQ Knowledge 3.9 software (BioPac Systems INC).

Preparation of drugs. AICAR (Toronto Chemical Research) was prepared at a concentration of 1.3 × 10⁻³ M in distilled water and kept at −20 °C until use. 2-deoxyglucose (Sigma) was prepared at a concentration of 10⁻¹ M in distilled water and used immediately. SBI-0206965 (Sigma-Aldrich), A-769662 (Tocris Bioscience) and the different modulator candidates were prepared in DMSO (Sigma). NA (Sigma Aldrich) was prepared in a saline-ascorbic solution (0.9% NaCl/0.01% ascorbic acid), Ach and L-NAME (Sigma Aldrich) were prepared in 0.9% NaCl solution. A stock solution (10⁻² M) was prepared for each of them and stored at −20 °C before use (maximum 3 months).

Statistical analysis. The results obtained were expressed as the arithmetic mean ± the standard error of the arithmetic mean (S.M.E.). Comparisons of the results obtained between individual groups were made using Shapiro-Wilk analysis of variance followed by a one or two-way ANOVA and a Dunnett test (parametric) or a Kruskal–Wallis (non-parametric) to analyze differences between experimental groups with the untreated control. Significantly different groups were considered when p < 0.05. GraphPad Prism 9 software (San Diego) was used for statistical analysis. The free software ImageJ was used for image analysis.

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Author contributions
Idea, F.I.L.; C.E.; M.S.F.A. and A.C.; performed the experiments and the data analysis, M.S.G.; E.A.; M.B.; I.L.; E.V.M.; M.G.O.; J.C.; C.P.; F.I.L.; C.E.; M.S.F.A. and A.C.; writing—review and editing, M.S.G.; F.I.L.; C.E.; M.S.F.A. and A.C.; all authors have read and agreed to the published version of the manuscript.

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Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to C.E., M.S.F.A.-O. or A.C.

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