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Note

The first organometallic derivative of 11β-ethynylestradiol, a potential high-affinity marker for the estrogen receptor

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1. Introduction

When carefully selected substituents are added to the basic skeleton of hormonal steroids, there can be considerable changes in the properties of the new compound. The 11β position is particularly amenable to such changes. Mifepristone (RU 486) (Scheme 1) owes its antiprogestin properties to the $-C_6H_4$-$N(Me)_2$ group attached at position 11β [1–3], while in the estrogen series, attachment of a long chain, either *N*-butylundecanamide or a polyfluoroalkylthio group, at 11β turns the new compound into antiestrogens such as RU 39411 [4] or RU 58668 [5]. Similarly, the 11β-CH_2Cl attached to the estradiol framework (ORG 4333) causes a spectacular increase in the affinity of the modified compound for the estrogen receptor [6,7]. Although many organometallic moieties, i.e. those with a metal–carbon bond, have been attached at various positions on the estradiol skeleton [8–14], the attachment of an organometallic fragment at the strategic 11β position is very rare [3]. We present here the synthesis, several noteworthy biochemical properties and study of agonist or antagonist effect of 11β-[(ethynyl)$Co_2(CO)_6$]estradiol (**1**). Recently, antitumoral properties have been shown in vitro for some alkyne $Co_2(CO)_6$ organometallic complexes such as $Co_2(CO)_6$[2-propynyl acetyl salicylate] on melanoma and lung carcinoma cells but only at high concentrations (10–20 μM), while $Co_2(CO)_8$ itself shows no cytotoxic effect [15]. In vivo, alkylcobalt(III) chelate complexes, which are known to generate free radicals at low pH, have been shown to damage tumors such as ascite leukemia or Guerin carcinoma [16]. Assuming that recognition of the estrogen receptor is preserved in **1**, this would provide a vehicle for introduction of a potential cytotoxic substance into estrogen-responsive tumors. The lack of specificity of most antitumoral agents, and the consequent damage to healthy tissue, remains a major problem in anti-cancer chemotherapy [17].

2. Results and discussion

2.1. Synthesis of *1*

Two potential methods of arriving at 11β-ethynylestradiol (**8**), the organic precursor of **1**, are available. One, used by Teutsch and co-workers [18], via an epoxide in the 5–10 position, made it possible to introduce various substituents at the 11β position, in-

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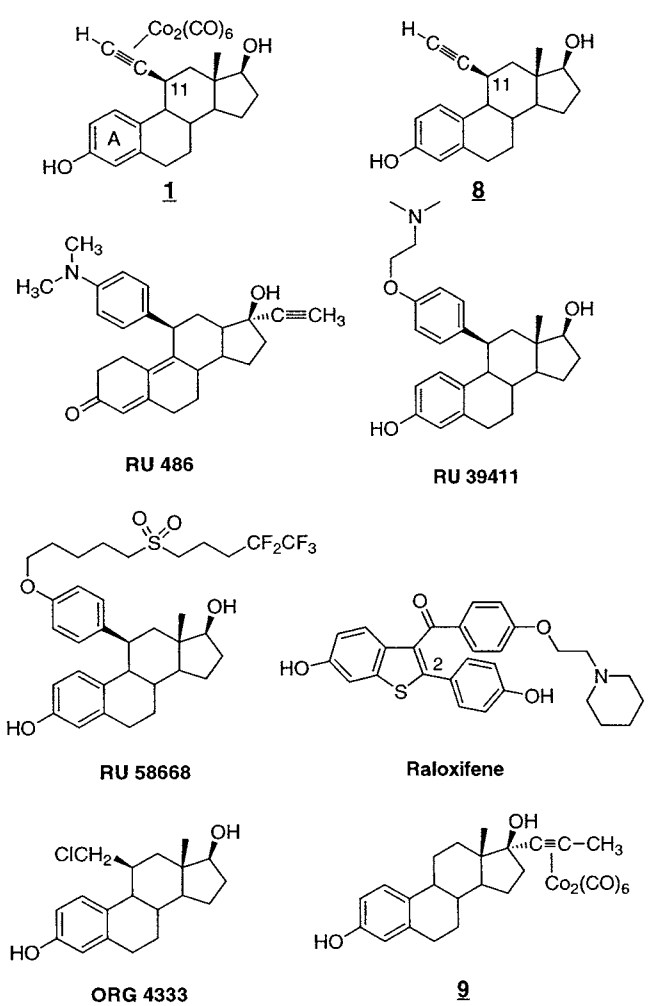
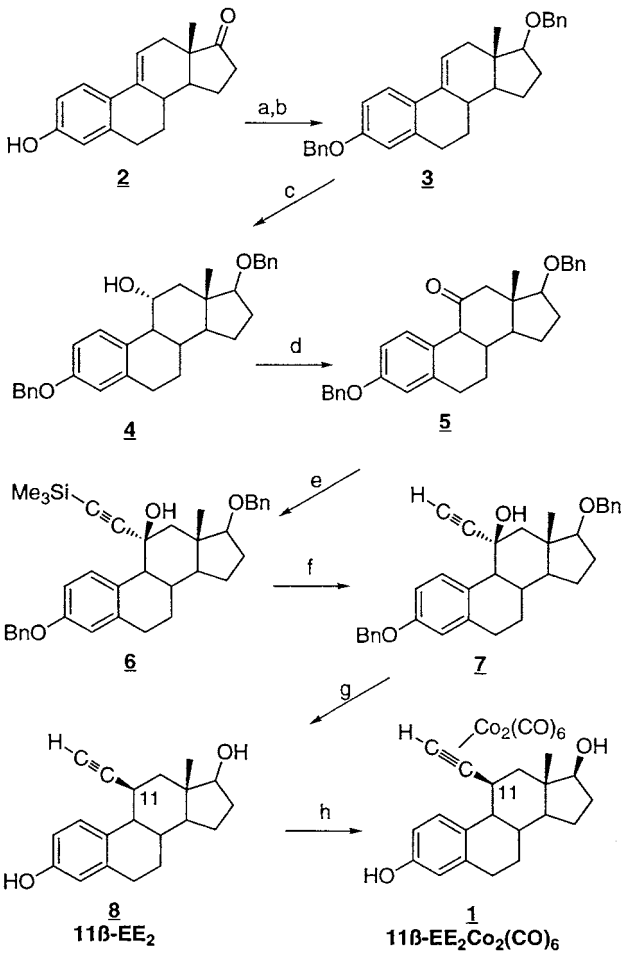
cluding arenes and alkenes [19]. However, this cumbersome approach involving the destruction and reconstruction of the aromaticity of ring A requires 11 steps. The other method, that of Napolitano and co-workers (Scheme 2) [20], is slightly more direct, starting from commercially obtained estrone which can easily be transformed into the 11-ketoestradiol derivative (**5**) that reacts with lithium (trimethylsilyl)acetylide. Then the stereoselective deoxygenation of this 11-hydroxy steroid followed by the deprotection of the OH groups gave access to 11β-ethynylestradiol (**8**). We chose to use the second method, despite steric crowding that is known to occur at position 11 of the steroid, and despite its relatively low reactivity.

Complex **1**, namely 11β-[(ethynyl)$Co_2(CO)_6$]estradiol, was prepared by reaction of 11β-ethynylestradiol with $Co_2(CO)_8$ in CH_2Cl_2 at room temperature followed by column purification (50% yield). Attempts to grow suitable crystals for X-ray diffraction have failed. However, **1** has been fully characterized by spectroscopic techniques and elemental analysis.



Scheme 1.



Scheme 2. Key: (a) $NaBH_4$, MeOH; (b) NaH, C_6H_5Br, DMF; (c) (1) catechol borane, $LiBH_4$, THF (2) NaOOH; (d) pyridinium chlorochromate, CH_2Cl_2; (e) lithium(trimethylsilyl); (f) $HSiEt_3$, $BF_3{\cdot}Et_2O$, CH_2Cl_2; (g) BCl_3, CH_2Cl_2; (h) $Co_2(CO)_8$, CH_2Cl_2.

2.2. Lipophilicity of *1*

It has been shown that there is a good correlation between the in vivo uptake rate of a molecule in fatty tissue and lipophilicity. Moreover, by altering the lipophilicity of the steroid through synthetic modification, one can change its tissue permeability by allowing more or less the ligand to enter cells in estrogen receptor (ER)-rich target tissues or non-target tissues [21]. The lipophilicity of a compound is measured by its octanol–water partition coefficient $P_{o/w}$. This measurement can be carry out by HPLC [22]. The log $P_{o/w}$ value found for **1** is 5.5 compared with 3.3 for estradiol. Thus **1** appears to be more lipophilic than estradiol itself. This seems to be a general trend for the organometallic derivatives of estradiol [7].

2.3. Biochemical tests of *1* and *8*

Some tests of the biochemical behavior of these organometallic hormones were performed. First, the

relative binding affinity (RBA) for the estrogen receptor was measured, using a previously reported method with lamb uterine cytosol as a source of estrogen receptor [23]. It is interesting to note that the relative binding affinity value of **1** was established at 18% for an incubation performed at 0°C, compared with 63% for an incubation done at 25°C. The RBA of **1** is therefore good (by definition, RBA of estradiol = 100) despite the steric crowding of the cluster. An increase in the RBA value (31.5–85.5%) was also observed for **8**, the organic precursor of **1** even if these values found after a 3 h incubation are significantly lower than those of the literature measured after 18 h incubation (51 and 447%) [24]. The increase in RBA with temperature is fairly infrequent but has been noted previously for estradiol derivatives bearing 11β electron-rich substituents [7,24,25]. It has been interpreted in terms of a slowing or possibly even prevention of the dissociation of the modified bioligand from its receptor. This interpretation suggests that possible inactivation of the estrogen receptor by this new type of organometallic hormone **1** might be expected. Results showing inactivation of the estradiol receptor obtained with **1** are listed in Table 1. Considerable non-reversibility of the exchange of the organic hormone **1** with radioactive estradiol is observed after previous incubation of the receptor in the presence of **1**. The 60% value obtained is reproducible (mean of three experiments, S.D. 0.21%). To gain information on the strength of the hormone–receptor bond, the time allowed for the exchange reaction was increased from 19.5 to 43.5 h in the presence of radioactive estradiol. The receptor inactivation rate (RI = 62%) remained comparable to that obtained after 19.5 h. This suggests fairly high binding stability, superior to that seen with the free ligand, 11β-ethynylestradiol (**8**), which under the same conditions has an inactivation rate dropping from 58 to 50%. The latter behavior can be compared to that of 11β-chloromethylestradiol reported by Katzenellenbogen and co-workers [6].

The inactivation values of the estradiol receptor found here for **1** (~ 60%) are close to those observed with hormones modified at the 17α position by osmium and ruthenium clusters, and less (80%) than those found for 17α-[(propynyl)$Co_2(CO)_6$]estradiol (**9**) [9]. In the latter case, there is an OH group adjacent to the alkyne cluster $Co_2(CO)_6$, and it is easy to imagine the in situ generation of an alkylating carbenium ion, assuming there is a Brönsted or Lewis acid close to the association site. The pK_{R+} value of **9**, in the order of −5.5, supports this hypothesis. In the present case the situation is different since there is no OH function in the α position of the organometallic moiety. The pK_{R+} value of **1** (−9.0) also makes it clear that another explanation is needed to take into account the unusual behavior of **1**. One hypothesis proposed to rationalize the surprisingly high affinity of estradiol when there is a CH_2Cl group in the 11β position is the presence of a Zn^{2+}, complexed with cysteines such as 530 and 381 of the hormone-binding domain [7]. This would enable $-CH_2Cl$ coordination. This type of association, similar to those of coordination chemistry and weaker than the covalent C–C bonds, could play a role in 11β-chloromethyl estradiol [7]. In the present case an explanation for the behavior of **1** must be sought in the nature of the $C_2Co_2(CO)_6$ cluster. It is known that these small, stable tetrahedral clusters can be rationalized according the 18-electron rule, which they obey. It has, however, also been postulated that these clusters can be seen as nido trigonal bipyramids, with 12 delocalized electrons within the framework of the cluster [26]. It follows that a vacant coordination site must exist on the surface of the cluster, and it is owing to this vacant site that specific coordination between these nido clusters and acid fragments (H^+, Zn^{2+}) is possible.

This description of the clusters in terms of delocalized electrons is based on the Wade–Mingos rules [27] and the isolobality relationships of Hoffmann [28]. It also has the advantage of providing an explanation for the unusual biological results found for compound **1**. Nevertheless, it is only by preparation of **1** in radioactive form that the existence of the phenomenon could be ascertained.

*2.4. Desorption chemical ionization mass spectrometry (DCI MS) studies of **1** and **8***

DCI MS using isobutane as the reagent gas can be used to detect the existence in a molecule of a domain

Table 1
Inactivation of the estrogen receptor (ER) by **1** [a]

Temperature of the first incubation (°C)	Length of exchange in the presence of radiaoctive estradiol (h)	Inactivation of the estradiol receptor RI (%) [a]
0	19.5	60 [b]
0	43.5	62
25	19.5	59

[a] RI (%) = the ratio of the amount of ER covalently bound in the presence of **1** versus the amount of ER found for the control (after incubation in the presence of estradiol).

[b] Mean of three experiments, S.D. = 0.21%

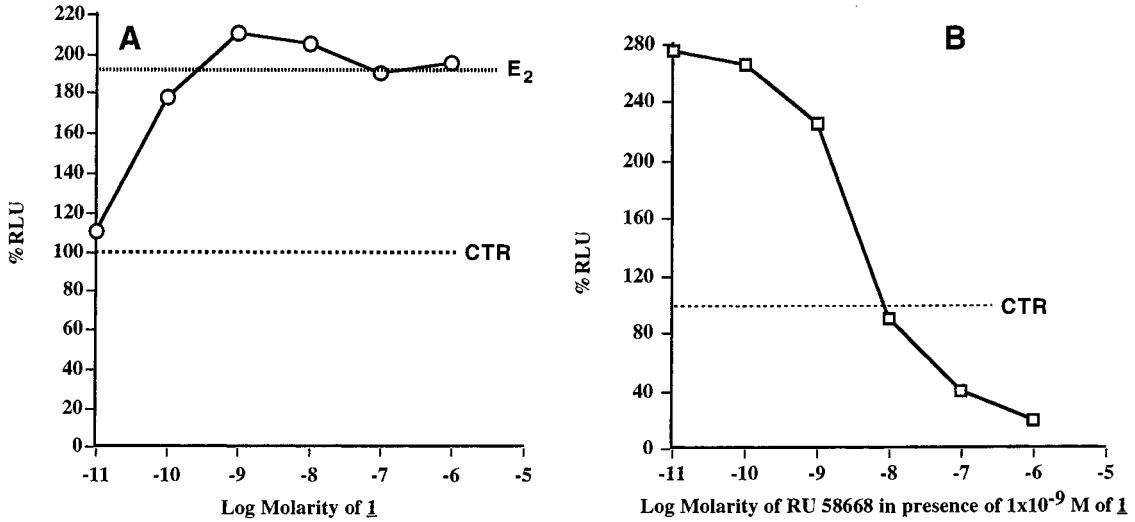


Fig. 1. Dose-dependent stimulation by **1** (A) and dose-dependent inhibition by RU 58668 in the presence of **1** (B) of the luciferase induction in MVLN cells (estrogen receptor-positive breast cancer cells).

rich in delocalized electrons [29]. Isobutane is initially ionized by conventional electron impact, then several collisions occur yielding the stable ion *tert*-butylcarbenium ion $[(CH_3)_3C]^+$. Ionization of the molecule under study (M) takes place by proton transfer generating the quasi-molecular ion $[M + H]^+$. Besides proton transfer, the formation of an adduct between $[(CH_3)_3C]^+$ and the neutral analyte (M) has been frequently observed but the abundance of such an adduct, $[M + (C_4H_9)]^+$, is generally small, not exceeding a few percentage points. In contrast the DCI mass spectra of **1** and **8** show that this adduct is the most abundant species with $[M + (C_4H_9)]^+/[M + H]^+$ ratios being equal, respectively, to 2.94 and 1.45. This unusual adduct ion formation apparently indicates that the structure of 11β-ethynylestradiol includes for both **1** and **8** a particular region, formed by the triple bound arm and the aromatic A-ring lying below, with extremely high electron density, which may easily undergo the electrophilic addition of the *tert*-butylcarbenium ion. The same domain is likely involved in the electrophilic interaction with the receptor, which determines the remarkable affinity of **1** and **8** for the estrogen receptor (vide supra). Notably the ratios $[M + (C_4H_9)]^+/[M + H]^+$ found for **9** and for 17α-ethynylestradiol, two molecules where the electron-rich domain is no longer present since the triple bond and the aromatic cloud are located far apart, are only 0.96 and 0.29.

*2.5. Test of the agonist/antagonist properties of **1** on a cell line derived from mammary tumor (MVLN)*

Due to this electron-rich domain, it became important to check the agonist/antagonist effect of **1**. Indeed, some estradiol derivatives bearing 11β substituents are among the most effective pure antiestrogens. This is the case for RU58668 [5]; however, its 11β-substituent is longer and more flexible than the $Co_2(CO)_6$ethynyl entity of **1** (Scheme 1).

In order to check this hypothesis we used a cellular test recently developed by Pons [30] to characterize (anti)estrogen molecules. In this test the expression of the luciferase activity expressed as the relative light unit (RLU) has been shown to be proportional to the estrogenicity of the compound under study. The results obtained with **1** are shown in Fig. 1. The organometallic hormore **1** appears to act as an estrogen as effective as estradiol itself (Fig. 1(A)). It seems to have no cytotoxicity for the cancer cells even at high molarity (1 μM). This estrogenic effect is progressively and totally supressed by addition of increasing concentrations of the pure antiestrogens RU 58668 (Fig. 1(B)). This estrogenic effect compares well with the one observed for 11β-chloromethyl estradiol (ORG 4333), which is also suppressed by addition of pure antagonist (data not shown). One can conclude that the antiestrogenic effect of the 11β-estradiol derivatives appears more likely to be mediated by the presence of a long, flexible chain bearing heteroatoms such as N or S, rather than by short and rigid substituents.

3. Conclusions

We have thus shown that the addition of an unsaturated, bulky and lipophilic organometallic moiety in position 11β permits strong binding with the estrogen receptor. In addition, the residence time of the modified hormone on the receptor protein appears greatly enhanced and **1** acts in vitro as a strong estrogenic hormone, devoid of pronounced cytotoxicity.

4. Experimental

4.1. General remarks

IR and NMR spectra were recorded on a Perkin–Elmer 580 B and on a JEOL-EX 400 spectrometer, respectively. DCI mass spectra were recorded on a Finnigan-MAT 95Q instrument with magnetic and electrostatic analyzers. Isobutane was used as the reagent gas at 0.5 mbar pressure. The ion source temperature was kept at 50°C, the electron emission current at 0.2 mA, and the electron energy at 200 eV. Positive ion spectra were collected. A Kontron modular HPLC instrument was employed with a Merck LiChrospher 100 RP-18 column.

4.2. Synthesis of **1**

A solution of $Co_2(CO)_8$ (3 mmol, 500 mg) and 11β-ethynylestradiol (1 mmol, 300 mg) in CH_2Cl_2 was stirred for 1 h at room temperature (r.t.) under nitrogen. The reaction mixture was filtered througth cellulose to remove any insoluble impurities, concentrated under reduced pressure, and then chromatographed on a silica gel column. An *n*-hexane–CH_2Cl_2 gradient (0–50% CH_2Cl_2) resolved several bands. The first band corresponds to unreacted $Co_2(CO)_8$, the second to adventitious $Co_4(CO)_{12}$. From the third band, **1** was obtained as a crude product (50% yield). It was re-crystallized from an *n*-hexane–acetone mixture as a pale-brown fine powder.

1: Anal. Found: C, 53.91; H, 3.98; Co, 20.33. $C_{26}H_{24}Co_2O_8$. Calc.: C, 53.63; H, 4.15; Co, 20.24%. IR (CH_2Cl_2, ν_{CO} (cm^{-1})): 2090 m, 2049 vs, 2025s, 2018s (sh); ^{1}H-NMR (400 MHz, $CDCl_3$, δ (ppm)): 7.22 [1H, d, C_1H], 6.68 [1H, dd, C_2H], 6.53 [1H, d, C_4H], 6.07 [1H, s, C_{21}H], 4.60 [1H, s, C_{17}OH], 3.76–1.13 [all the remaining resonances partially overlapped], 1.08 [3H, s, Me-18]; {^{1}H}-^{13}C-NMR (101 MHz, $CDCl_3$, δ (ppm)): 199.8 [Co–CO′s], 153.5 [C_3], 139.5 [C_5], 129.0 [C_1], 128.5 [C_{10}], 115.6 [C_4], 113.4 [C_2], 97.7 [C_{20}], 82.6 [C_{17}], 80.0 [C_{21}], 52.6–22.9 [all the remaining resonances partially overlapped], 14.9 [Me-18]. DCI MS, m/z 583 $[M+H]^+$.

4.3. Determination of the relative binding affinity (RBA) of **1** and **8** for the estrogen receptor (ER)

Aliquots (200 μl) of lamb uterine cytosol prepared as described in Ref. [23] were incubated for 3 h at 0°C with 2×10^{-9} M of [6,7-^{3}H]-estradiol (specific activity 1.96 TBq $mmol^{-1}$) in the presence of nine concentrations of the hormones to be tested. At the end of the incubation period, the free and bound fractions of the tracer were separated by protamine sulfate precipitation. The percentage reduction in binding of [^{3}H]-estradiol (Y) was calculated using the logit transformation of Y (logit Y: $\ln[y/1-Y]$ versus the log of the mass of the competing steroid). The concentration of unlabeled steroid required to displace 50% of the bound [^{3}H]-estradiol was calculated for each steroid tested, and the results expressed as RBA. The RBA value of estradiol is by definition equal to 100%.

4.4. Receptor inactivation assays of **1** and **8**

Aliquots (3×200 μl) of lamb uterine cytosol were incubated with 100 nM of **1** or **8** during 2.5 h at the temperature indicated (0 or 25°C). The unbound fraction of the hormone was removed by treatment with dextran-coated charcoal (DCC). The remaining amount of hormone reversibly bound was measured after exchange in the presence of [^{3}H]-estradiol for the length of time indicated (19.5 or 43.5 h), and separation of the free and bound fractions of the tracer by precipitation with protamine sulphate [9].

4.5. Determination of the pK_{R^+} value of **1**

1 was dissolved in acetonitrile (1×10^{-3} M solutions). A 50 μl aliquot of this solution was then added to 450 μl of various concentrations of H_2SO_4. The UV measurements were performed at 346 nm after a 10 min incubation at r.t. Final calculations were done by using the Deno acidity function [31].

4.6. Test on MVLN cells

4.6.1. Culture materials

Earle's based minimal essential medium (MEM), fetal bovine serum (FBS), L-glutamine, penicillin, gentamicin, streptomycin were obtained from Gibco (Ghent, Belgium), and plastic culture materials from Falcon (Ghent, Belgium).

4.6.2. Culture conditions

MVLN cells obtained from Pons et al. [30] are maintained in monolayer culture in Dulbecco–MEM added with 10% thermally inactivated FBS, L-glutamine (0.6 mg ml^{-1}) and a cocktail of antibiotics (gentamicin 40 μg ml^{-1}, penicillin 100 U ml^{-1}, streptomycin 100 μg ml^{-1}).

4.6.3. Luciferase assays

MVLN cells were cultured for 3–4 days in 35 mm diameter Falcon dishes (plating density 80 000/dish) in 10% depleted of endogeneous steroid (DCC treatment). Ethanolic solutions of **1** (1×10^{-11}–1×10^{-6} M) were subsequently added to the medium and the culture pursued until luciferase assay (24 h). For that purpose, the medium was removed and cells washed twice with PBS buffer. A minimal volume (250 μl) of a fivefold

diluted lysis solution (Promega E 153A) was then added to the dishes and the latter maintained under mild agitation for 20 min to extract luciferase. Lysed cells were subsequently detached with a scraper (Costar 3010) and centrifuged for 5 s at 12 000*g* to clarify the extracts. A 20μl aliquot of each extract was finally mixed at r.t. with 100 μl of luciferase reactant medium (Promega E151A/E152A) prepared according to the manufacturer's protocol. Induced light was measured with a Berthold luminometer (Lumat LB 9507). Induction of the luciferase was expressed in arbitrary units with regard to the light measured with a blank (RLU). To compare RLU data, the protein content of each extract was measured by the Coomassie method (PIERCE) and the data expressed per mg of protein. Estradiol (1×10^{-10} M) was used as positive control.

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