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Modulation of intracellular Ca^{2+} signalling in HeLa cells by the apoptotic cell death enhancer PK11195

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ABSTRACT

1-(2-Chlorophenyl-*N*-methylpropyl)-3-isoquinolinecarboxamide (PK11195) is a proven enhancer of apoptotic cell death in a variety of cellular models. This effect is independent of its established cellular target, the mitochondrial benzodiazepine receptor (mBzR), since it is able to promote cell death also in mBzR knockout cells. Thus recently it was suggested that PK11195 might exert its effect by modulating the expression and function of the oncogene Bcl-2. We have previously demonstrated that Bcl-2 modulates cellular Ca^{2+} homeostasis as its overexpression reduces the Ca^{2+} concentration in the endoplasmic reticulum (ER) ($[Ca^{2+}]_{er}$), impairing mitochondrial and cytosolic Ca^{2+} overload during cellular stress and therefore inhibiting the induction of the apoptotic cascade. Here, using ER, mitochondria and cytosolic targeted aequorin probes, we show that cellular treatment with PK11195 induces opposite changes in cellular Ca^{2+} homeostasis, increasing the $[Ca^{2+}]_{er}$ and amplifying IP_3 induced Ca^{2+} transients in mitochondria ($[Ca^{2+}]_m$) and cytosol ($[Ca^{2+}]_c$). This work provides evidence for a novel pharmacological effect of PK11195 on Ca^{2+} signalling which may be linked to its effect on Bcl-2 and account for its role in apoptotic cell death.

1. Introduction

1-(2-Chlorophenyl-*N*-methylpropyl)-3-isoquinolinecarboxamide (PK11195) [1,2] has been recently established as a chemosensitizer of tumour cells to a wide range of chemotherapeutic agents [3–7]. Although it is known as the ligand of the mitochondrial benzodiazepine receptor (mBzR) [8,9] (recently named also translocator protein, TSPO [10,11]), it has been repeatedly suggested that further targets are involved in its role of cell death enhancer. PK11195 efficiently facilitates the programmed cell death execution by a wide range of stimuli such as etoposide, doxorubicin, ceramide; besides improving the effect of the cytostatic drugs like doxorubicin and the Bcl-2 inhibitor HA14-1 [4,12,13]. Notably, PK11195 remains equally active even when the mBzR is knocked down [14,15] and thus different mechanisms for its action have been proposed [6]. It was shown to inhibit the multiple drug resistance (MDR) pumps facilitating the uptake of different therapeutics in human multidrug-resistant cells [3,16]. In parallel, also its ability to activate the p38 MAPK signaling pathway in esophageal cancer cells was demonstrated [5] as well as a specific effect on the intrinsic apoptotic pathway leading to a substantial release of cytochrome c from mitochondria was documented [17]. Still, the principal pathway targeted by the drug remains ill-defined.

Recently, it was proposed that PK11195 might reduce the expression levels and inhibit the function of the anti-apoptotic members of the Bcl-2 family. Indeed, it was shown to reverse

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the Bcl-2 mediated inhibition of apoptosis in specific cell types and to reduce the apoptosis threshold in others [12,18]. The presence of the compound facilitated the induction of apoptosis in EW36, a human B-cell lymphoma cell line that over-expresses Bcl-2 [19]. Moreover, in human cholangiocarcinoma cells, PK11195 reverted Bcl-2 mediated cytoprotection by promoting translocation of Bax to the mitochondrial outer membrane [12,13,20]. Moreover, in human hepatocellular carcinoma both Bcl-2 and Bcl-X_L are downregulated by micromolar doses of PK11195, concomitantly with an upregulation of Bax levels [4]. Members of the Bcl-2 gene family are known to exert their pro- or anti-apoptotic effect determining the state of mitochondrial permeability, by promoting or inhibiting respectively the release of proapoptotic factors like cytochrome c (cyt-c) [21] and apoptosis inducing factor (AIF) from the intermembrane space [22–24]. To this aim, a tuned regulation of Ca^{2+} fluxes is essential and a role for this family of proteins in regulating ion fluxes has been long proposed [25]. We and other groups have recently demonstrated that anti- and pro-apoptotic Bcl-2 family members regulate intracellular Ca^{2+} homeostasis in opposite ways by targeting the endoplasmic reticulum (ER) [26,27]. Bcl-2/Bcl-X_L overexpression reduces the state of filling of the ER Ca^{2+} store, impairs IP_3 induced Ca^{2+} release, and consequently protects against treatment with various proapoptotic drugs acting through mitochondrial Ca^{2+} overload following Ca^{2+} release from the ER [28–36] (see also [37] and references therein). On the contrary, Bax was shown to chronically and acutely increase ER Ca^{2+} loading, leading to uploading of mitochondrial Ca^{2+} uptake hence manifestation of apoptotic cell death [32,38,39]. This led to the recognition of a Ca^{2+} mediated mitochondrial apoptotic pathway (see above and [40]), characterized also by Ca^{2+} dependent translocation of Bax to the mitochondria [41].

We have therefore hypothesized that cellular treatment with PK11195 might be able to modulate intracellular Ca^{2+} signaling due to its regulatory role on Bcl-2 and/or Bcl-2 family interactors (e.g. Bax, Bad, Bcl-X_L). By the use of the Ca^{2+} sensitive photoprotein aequorin, we have discovered that in HeLa cells micromolar concentrations of PK11195 increase the steady-state $[Ca^{2+}]$ of the ER ($[Ca^{2+}]_{er}$) generating in turn higher IP_3 generated mitochondrial ($[Ca^{2+}]_m$) and cytosolic ($[Ca^{2+}]_c$) Ca^{2+} transients. Furthermore, this effect is plausibly not due to mBzR binding since another prototypic PBR ligand, the 7-chloro-5-(4-chlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one (Ro5-4864) [42] has no effect on cellular Ca^{2+}. Hence, we propose that the PK11195 induced alterations in Ca^{2+} signaling might be consequence of a direct blockade of Bcl-2 interaction with other Bcl-2 family members, relevant to cell death through mitochondrial membrane permeabilization.

2. Methods

2.1. Cell culture, plasmids, and transfection

For the aequorin measurements, cells were seeded onto 13 mm coverslips (BDH, Milan, Italy) and transfected with 4 μg mtAEQmut or cytAEQ using Ca^{2+}-phosphate technique; experiments were performed 36 h after transfection as previously described [43,44]. HeLa cells were grown in DMEM (Celbio) supplemented with 10% FBS, 100 units penicillin per ml and 25 μg streptomycin per ml. Cells were grown at 37 °C in a 5% CO_2 incubator.

2.2. Aequorin measurements

mtAEQmut and cytAEQ transfected cells were used 36 h after transfection. The cells were incubated with 5 μM coelenterazine for 1–2 h in Krebs Ringer Buffer (KRB) supplemented with 1 mM Ca^{2+} and then transferred to the perfusion chamber.

To test the effect of the PBR ligands, PK11195 or Ro5-4864, were added in concentration of 10 μM and 100 nM, respectively, in KRB supplemented with 1 mM $CaCl_2$ for 2 h. PK11195 was dissolved in chloroform and Ro5-4864 in dimethyl sulfoxide (DMSO), utilized at a concentration of 1/1000, v/v and 1/10,000, v/v, respectively. Solvent only containing controls have been included throughout the experiments. All aequorin measurements were carried out in KRB, supplemented with either 1 mM Ca^{2+} or the indicated $[Ca^{2+}]$. Agonists and other drugs were added to the same medium, as specified in the figure legends. The experiments were terminated by lysing cells with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. In brief, a 13 mm-round coverslip with the transfected cells was placed in a perfused, thermostatted chamber located in close proximity of a low-noise photomultiplier, with built-in amplifier-discriminator. The output of the discriminator was captured by a Thorn-EMI photon counting board and stored in an IBM-compatible computer for further analyses. The aequorin luminescence data were calibrated off-line into $[Ca^{2+}]$ values, using a computer algorithm based on the Ca^{2+} response curve of wild-type and mutant aequorins, as previously described [43–47].

For the Er-AEQ measurements, as stated also in the results, before the reconstitution, it is necessary to reduce the Ca^{2+} content of the Golgi apparatus and the ER drastically. To this end, the cells were incubated for 1 h at 4 °C, in KRB (Krebs–Ringer modified buffer: 125 mM NaCl, 5 mM KCl, 1 mM Na3PO4, 1 mM MgSO4, 5.5 mM glucose, 20 mM HEPES, pH 7.4, 37 °C) supplemented with 5 mM coelenterazine, the Ca^{2+} ionophore ionomycin (2.5 μM) and 600 μM EGTA. After this incubation, the cells were washed extensively with KRB supplemented with 2% bovine serum albumin (BSA) and 1 mM EGTA. In the experiments, additions (1 mM $SrCl_2$, 1 mM $CaCl_2$, histamine, etc.) were made to the same medium, as specified in the figure legends. As for mtAEQ and cytAEQ, the experiments were terminated by lysing the cells with 100 mM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool [48].

2.3. Statistical analysis

All statistical analyses were performed using a two tailed Student's t-test assuming normal distributions with unequal variances. Error bars presented in graphs denote standard deviation (S.D.).

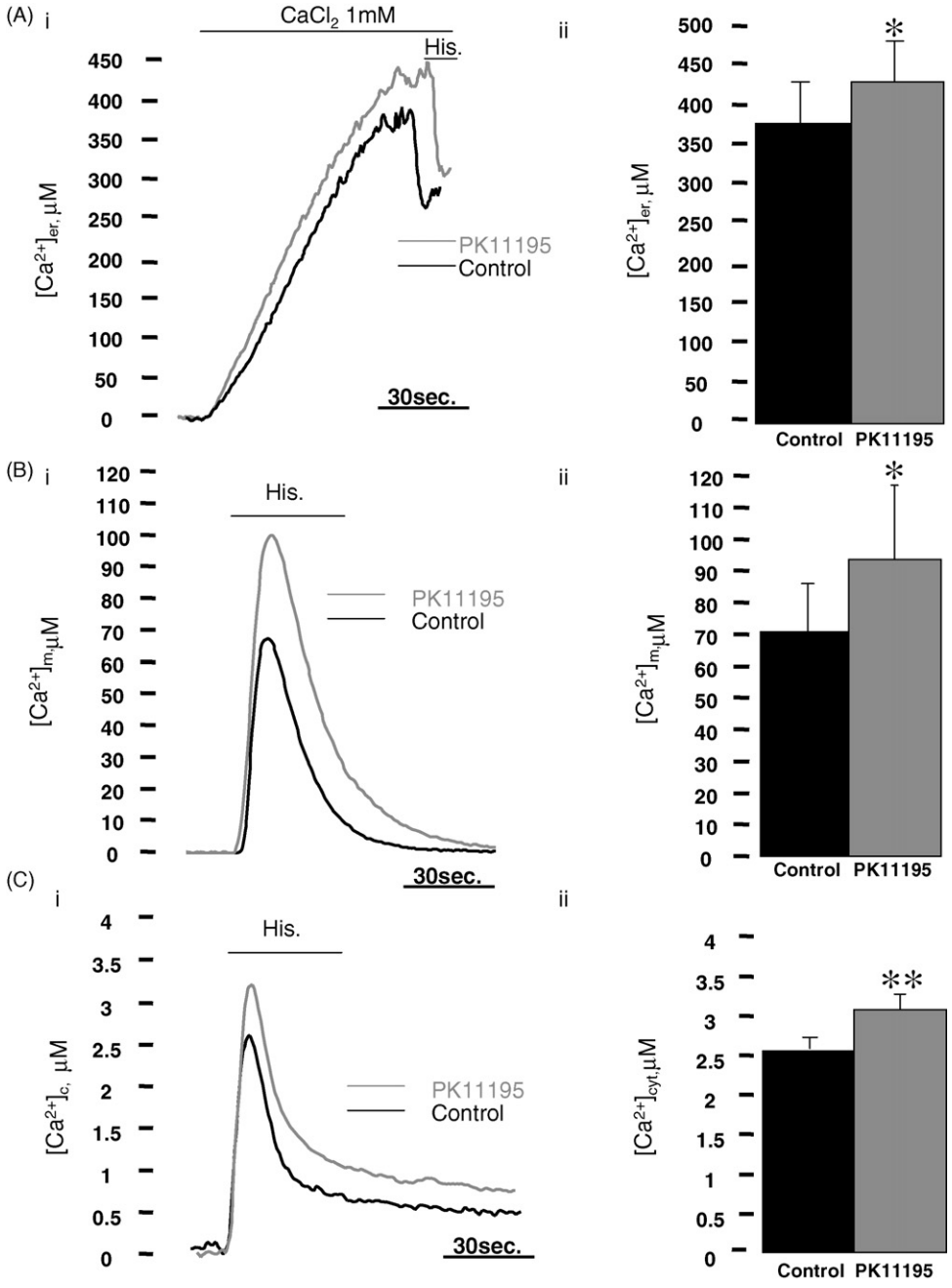

Fig. 1 – PK11195 increases the Ca^{2+} content in the endoplasmic reticulum and IP_3 generated Ca^{2+} transients in the cytosol and mitochondria of HeLa cells.

(Ai) Shows representative traces of the ER Ca^{2+} refilling in HeLa cells expressing endoplasmic reticulum targeted Aequorin (er-AEQ) treated for 2 h with 10 μM PK11195 (grey trace) or kept in PK11195 solvent as control (black trace). (Aii) Summarizes the average $[Ca^{2+}]_{er}$ values of 14 individual experiments (control: 384.93 ± 78.52 μM Ca^{2+}, PK11195: 426.55 ± 55 μM Ca^{2+}, $n = 24$ $^{*}p < 0.05$). (Bi) Shows representative records of histamine (100 μM) induced Ca^{2+} rise in mitochondria of HeLa cells expressing mitochondrially targeted mutated aequorin (mt-AEQMut). The degree of Ca^{2+} accumulation recorded in untreated (black trace) cells is statistically lower than in cells treated with 10 μM PK11195 (grey trace). Peak $[Ca^{2+}]_m$ values corresponding to 32 individual experiments are plotted on (Bii) (control: 68.17 ± 23.81 μM Ca^{2+}, PK11195: 84.82 ± 29.25 μM Ca^{2+}, $n = 33$ $^{*}p < 0.05$). (Ci) Shows histamine (100 μM) induced cytosolic Ca^{2+} transients in control (black trace) and PK11195 treated cells (grey trace) expressing cytosolic based aequorin (cyt-AEQ). As for the mitochondrial environment, PK11195 treatment promotes a statistically higher cytosolic Ca^{2+} peak also in this cellular compartment and values corresponding to 15 individual experiments are shown in (Cii) (control: 2.43 ± 0.28 μM Ca^{2+}, PK11195: 3.1 ± 0.33 μM Ca^{2+}, $n = 27$, $^{**}p < 0.01$).

2.3.1. *Reagents*

Coelenterazin was purchased from molecular probes (Eugene, OR, USA). Histamine, ATP, ionomycin, EGTA, $CaCl_2$ and all the reagents used for the Krebs Ringer Buffer were purchased from Sigma–Aldrich (ITALY). Krebs Ringer Buffer used has the following composition: 20 mM HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Ha_2HPO_4, 20 mM $NaHCO_3$, 5.5 mM glucose, 2 mM L-glutamine. PK11195 and Ro5-4864 and their respective solvents chloroform and dimethyl sulfoxide were purchased from Sigma–Aldrich (ITALY).

3. Results

3.1. *Increased ER luminal Ca^{2+} concentrations in PK11195 treated HeLa cells*

The ER is the major source of both physiological and pathological Ca^{2+} signals. To investigate the effect of PK11195 on ER Ca^{2+} handling, we used a low affinity aequorin construct, targeted to the ER [47]. HeLa cells were transfected with er-AEQ and treated with 10 μM PK11195 for 2 h or with an equivalent volume of the drug's solvent chloroform (1/1000, v/v) and the $[Ca^{2+}]_{er}$ in the two cohorts were compared. To avoid consumption of the aequorin, the luminal $[Ca^{2+}]$ of these organelles must be reduced prior to the reconstitution of the probe with prostethic group coelenterazine. This was obtained by incubation of the cells in KRB supplemented with the low affinity coelenterazine n and 2.5 μM ionomycin, a Ca^{2+} ionophore, in the absence of extracellular Ca^{2+} (see Section 2 and references therein). Aequorin luminescence signals were collected using a luminometer and calibrated into $[Ca^{2+}]$ values. Upon switching the perfusion medium to KRB buffer supplemented with 1 mM Ca^{2+}, $[Ca^{2+}]_{er}$ gradually increased, reaching plateau levels of ~390 μM Ca^{2+} in control cells whilst in PK11195 treated cells, an higher steady state level (~10%) was observed in the same compartment with values of ~430 μM Ca^{2+} (Fig. 1A). The consecutive addition of the IP_3 generating agonist histamine

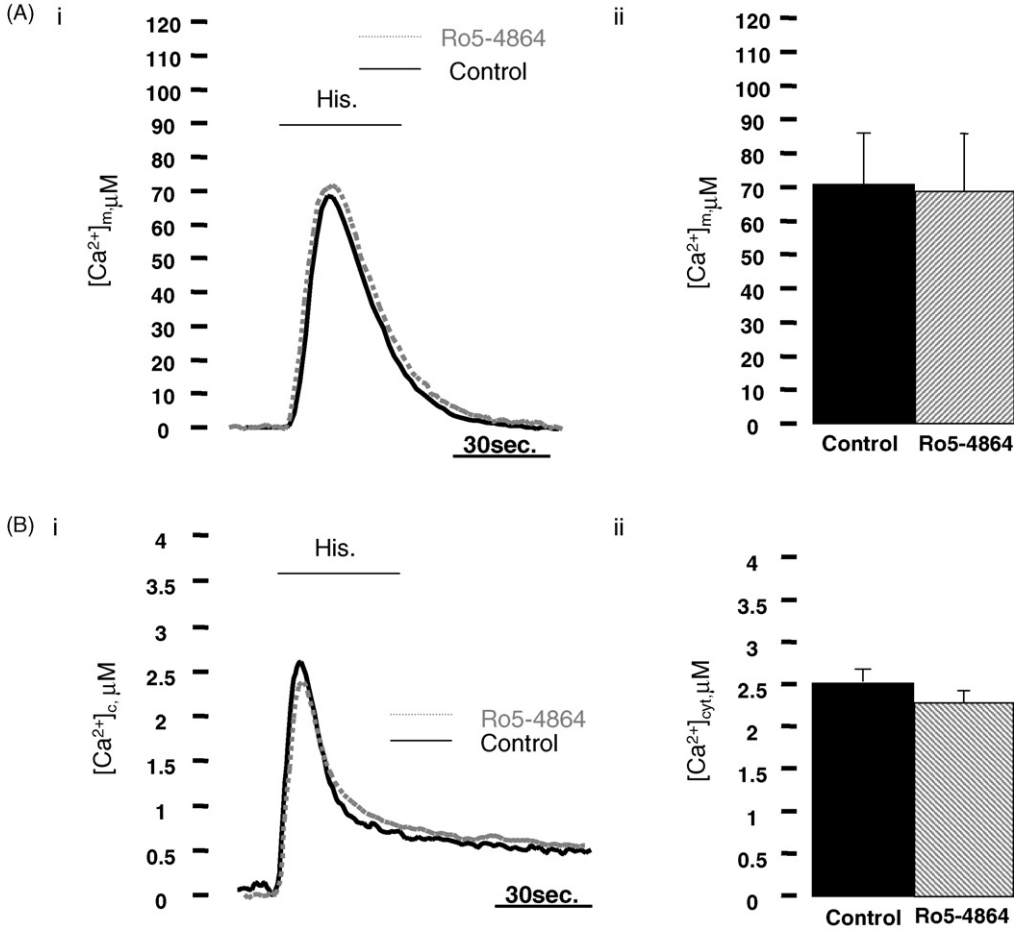

Fig. 2 – The prototypical PBR ligand Ro5-4864 does not affect the $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in HeLa cells.
(Ai) Shows Ca^{2+} traces of HeLa cells transfected with mitochondrially-targeted aequorin (mt-AEQMut) challenged with 100 μM histamine (Hist.) Ro5-4864 (dot grey trace) and control cells (black trace) present identical amplitude of the Ca^{2+} response as reported by the statistical analysis in (Aii) (control: 67.41 ± 22.68 μM Ca^{2+}, Ro5-4864: 69.41 ± 92 μM Ca^{2+}, mean ± S.D. of 28 individual experiments, $P = 0.61$). (Bi) As for the mitochondrial Ca^{2+} uptake, neither in the bulk cytosol, Ro5-4864 affects Ca^{2+} rise after histamine stimulation. Representative traces of HeLa cells expressing cyt-AEQ are pictured in the panel. Control (black trace) and Ro5-4864 (dot grey trace) cells have similar Ca^{2+} peak values as the graph plotted in (Bii) underlines (control: 2.23 ± 0.27 μM Ca^{2+}, Ro5-4864: 2.33 ± 0.35 μM Ca^{2+}, average ± S.D. of 12 individual experiments, $P = 0.79$).

resulted in a rapid decrease in $[Ca^{2+}]_{er}$ in both cohorts, indicating that the sensitivity to agonists of the Ca^{2+} stores was retained. Taken together, these measurements indicate that micromolar doses of PK11195 induce an increase of the luminal $[Ca^{2+}]$ in the ER. Solvent treated cells did not show any modification as compared to those untreated (data not shown).

3.2. Stimulus-induced increases in $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ in PK11195 treated HeLa cells

Mitochondria play an important role in intracellular Ca^{2+} homeostasis as they lie in close proximity of inositol 1,4,5-trisphosphate (IP_3)-gated channels and are capable of taking up the Ca^{2+} released by IP_3-generating agonists (e.g. histamine H1 and ATP), thereby buffering the $[Ca^{2+}]_c$ [45,49]. We hypothesized that the PK11195-induced increase in the steady state $[Ca^{2+}]_{er}$ level and the ensuing increase in the driving force for IP_3-induced Ca^{2+} release should also increase the mitochondrial Ca^{2+} uptake. To test this hypothesis, HeLa cells were transfected with a mitochondrial targeted low affinity aequorin construct (mt-AEQmut), exposed to PK11195 or only the solvent (control) and then challenged with histamine in the presence of extracellular Ca^{2+}. Fig. 1B shows that the peak mitochondrial response was markedly increased ~25% in PK11195 treated cells than solvent treated cells showing average values of ~85 μM Ca^{2+} in PK11195 and ~70 μM Ca^{2+} in control. The effect of PK11195 on mitochondrial Ca^{2+} transient was also measured with equal outcome in single cells transfected with the fluorescent Ca^{2+} probe 2 mt-YC 2.1 (data not shown) [50] confirming that PK11195-induced increment of $[Ca^{2+}]_{er}$ leads to a consequent increase in the stimulus-induced mitochondrial Ca^{2+} uptake.

Consequently, we expected to see similar result also for the $[Ca^{2+}]_c$ following agonist stimulation. To this purpose, HeLa cells were transfected with cytosolic high affinity aequorin (cyt-AEQ) [43] and treated with PK11195 or with its solvent. Also in this cellular compartment, the amplitude of the histamine induced $[Ca^{2+}]_c$ peak was increased by PK11195 treatment (Fig. 1C) resulting in a peak value higher (~25%) than controls.

3.3. Mitochondrial and cytosolic Ca^{2+} handling in Ro5-4864 treated HeLa cells

To ascertain that PK11195 mediated effect on Ca^{2+} signalling was not due to a modulation of the mBzR we tested mitochondrial and cytosolic Ca^{2+} transients in HeLa cells treated with another high affinity ligand of the mBzR, Ro5-4864. HeLa cells expressing mt-AEQmut and cyt-AEQ respectively were incubated with 100 nM Ro5-4864 or with equal volume of DMSO (1/10,000, v/v) as control for 2 h. Fig. 2 shows that Ro5-4864 treated cells did not show any statistically significant modification of the cytosolic and mitochondrial Ca^{2+} peak values. No alterations were observed either after the application of higher concentrations of Ro5-4864 (1–10 μM, data not shown). In this way, we could postulate that the PK11195 induced alterations of the intracellular Ca^{2+} signalling are most probably not mediated by the mBzR.

3.4. Capacitative Ca^{2+} influx in control and PK11195 treated cells

Previously, we have demonstrated that in Bcl-2 overexpressing cells a reduction in the steady-state of $[Ca^{2+}]_{er}$ was

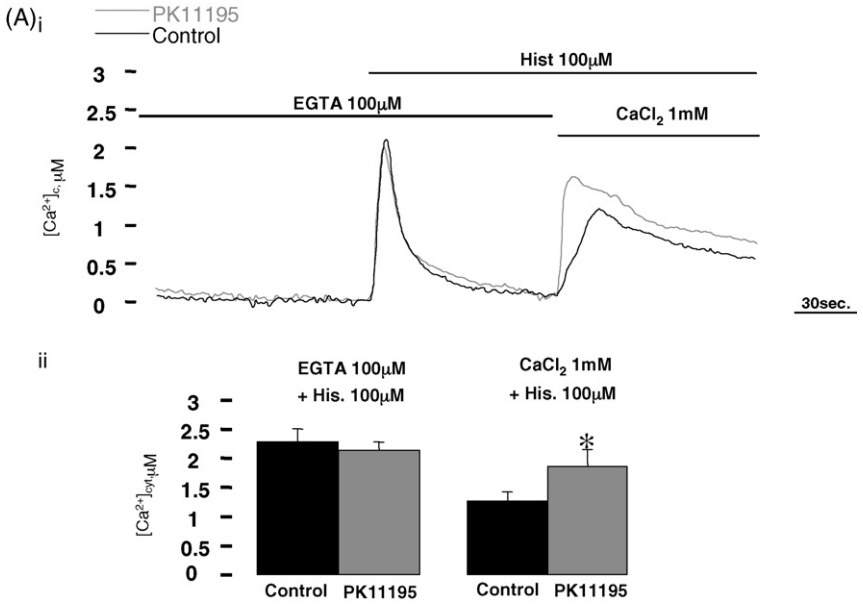

Fig. 3 – Capacitative Ca^{2+} influx in PK11195 treated HeLa cells.
(Ai) Cyt-AEQ expressing control and PK11195 treated HeLa cells were kept in Ca^{2+}-free medium containing 100 μM EGTA for about 5 min and then challenged with 100 μM histamine. Ca^{2+}-containing medium was then replaced still in presence of histamine in order to estimate the Ca^{2+} influx from extracellular medium. Panel (Aii) shows mean ± S.D. values of 12 individual experiments (control: 2.2 ± 0.14 μM Ca^{2+}, PK11195: 2.22 ± 0.21 μM Ca^{2+}, $P = 0.20$; control: 1.31 ± 0.33 μM Ca^{2+}, PK11195: 1.81 ± 0.66 μM Ca^{2+}, $^*p < 0.05$).

strikingly paralleled with a long term depression of capacitive Ca^{2+} influx [28,30]. We assumed therefore that if PK11195 modulates global Ca^{2+} signaling in the opposite way of Bcl-2, it should also increase the capacitative Ca^{2+} influx, following agonist induced emptying of the ER Ca^{2+} stores. For this purpose, PK11195 treated and control HeLa cells expressing cyt-AEQ were first challenged with histamine in Ca^{2+}-free extracellular medium containing 100 μM EGTA. This maneuver ensures that increases of the $[Ca^{2+}]_c$ will be due only to the release of Ca^{2+} from the intracellular stores. Moreover, leaving the cells in this medium leads to the partial depletion of the ER Ca^{2+} store, which we benefited to even out the steady state ER luminal $[Ca^{2+}]$ differences in control and PK11195 treated cells, and thus allowing to assess the specific effects of the drug on the Ca^{2+} influx following Ca^{2+} re-addition to the extracellular medium (Fig. 3Ai). Indeed, this procedure, observed similar Ca^{2+} release in the control and PK11195 treated groups, while PK11195 induced a markedly higher $[Ca^{2+}]_c$ increase (~28%) via the capacitative Ca^{2+} influx pathway in PK11195 treated cells (see graphs in Fig. 3Aii). This indicates a specific activation of the Ca^{2+} influx pathway in PK11195 treated cells, leading to a higher filling state of ER as observed in the presence of extracellular Ca^{2+}. This effect on the cellular Ca^{2+} homeostasis is the contrary of what have been observed in Bcl-2 overexpressing cells.

3.5. *Bcl-2 as target for PK11195 induced modulation of Ca^{2+} homeostasis: a hypothesis*

By these means, we suggest that a direct effect of PK11195 on Bcl-2 is a plausible mechanism by which steady-state levels and agonist dependent fluxes of Ca^{2+} ions occur (Fig. 4Ai). Thus PK11195 incubation affects key mechanisms of intracellular Ca^{2+} homeostasis in a Bcl-2 opposite manner: the state of refilling of the ER, the influx trough the plasma membrane (PM) and consequent mitochondrial Ca^{2+} accumulation and cytosolic Ca^{2+} transients in response to IP_3 generation (Fig. 4Aii).

4. Discussion

Using genetically encoded Ca^{2+} indicators, we have demonstrated that pharmacological treatment with PK11195 modulates intracellular Ca^{2+} signaling in HeLa cells independently of mBzR binding and consistently with a direct inhibition of Bcl-2 effect on cellular Ca^{2+} homeostasis.

In detail, 2 h treatment with 10 μM PK11195 led to: (i) a substantial increase (~10%) of the steady-state $[Ca^{2+}]$ level in the ER, (ii) higher histamine evoked mitochondrial and cytosolic Ca^{2+} transients (~25%) and (iii) increased capacitative Ca^{2+} influx (~28%).

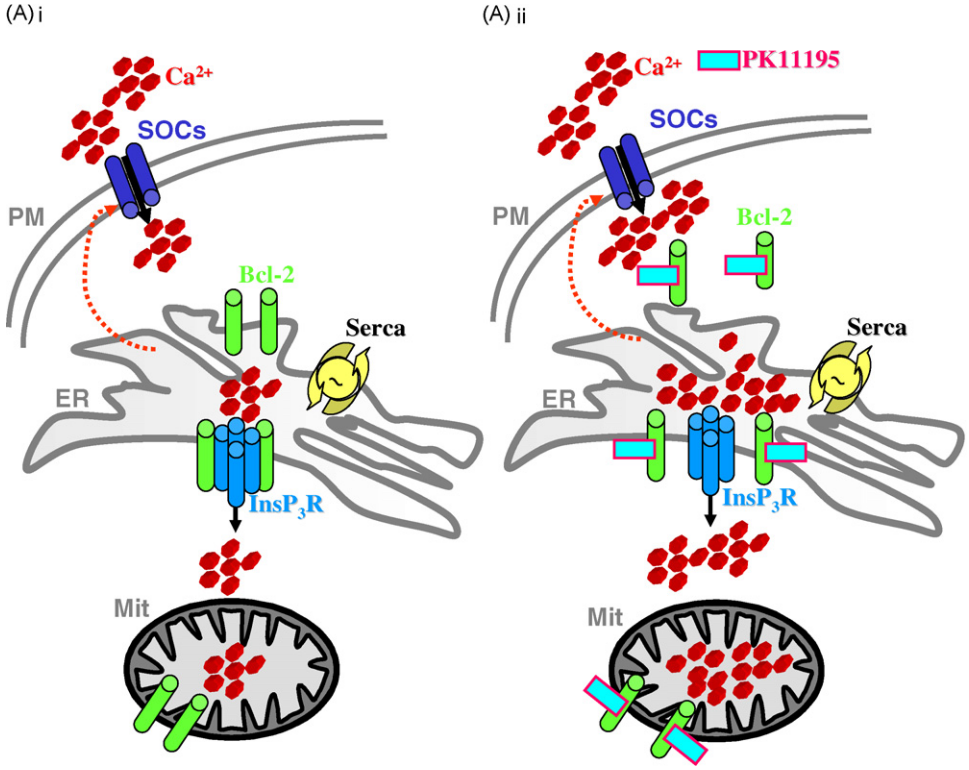

Fig. 4 – Model for the PK11195 proposed mechanism of action on intracellular Ca^{2+} signalling in HeLa cells. (Ai) shows the principal effects of the anti-apoptotic protein Bcl-2 on Ca^{2+} homeostasis. The oncogene reduces the Ca^{2+} content of the ER increasing the leak of Ca^{2+} from the lumen and downregulating the influx of the ion trough the plasma membrane (PM). This ultimately modulates the steady-state levels and the agonist dependent fluxes of intracellular Ca^{2+} in the mitochondria and cytosol. We suggest that incubation with PK11195 counteracts this Bcl-2 mediated effect reducing the leak from the ER, re-enforcing the Ca^{2+} influx and increasing the amplitude of mitochondrial and cytosolic Ca^{2+} transients following stimulation with Ca^{2+} mobilizing agonists (Aii).

Conversely, treatment with another prototypical mBzR ligand, the diazepam Ro5-4864 did not affect Ca^{2+} signalling. Interestingly, previous reports showed opposite effects on $[Ca^{2+}]_i$ for both mBzR ligands. Frigo and co-workers thus reported a Ro5-4864 mediated rise of $[Ca^{2+}]_c$ trough extracellular Ca^{2+} influx in human neutrophils and a parallel inhibitory effect for PK11195 [51]. Similarly, McLarnon and co-workers [52] proposed a PK11195 mediated inhibition of the Ca^{2+} store-operated channels (SOCs) activity in human microglia; hence in apparent contradiction with our findings. Whilst, more recently, the group of Lacapere demonstrated in human colon cancer cells HT-29 that PK11195 was able to promote a rapid and transient dose-dependent rise in intracellular $[Ca^{2+}]_c$, unaffected by extracellular Ca^{2+} [53]. However, the differences of the experimental protocol, the cell type, methods of Ca^{2+} measurements, and the way of drugs administration-tested in acute and not in incubation as in our model- do not allow a neat comparison. Nevertheless, in the cited papers, the authors aimed to characterize the mBzR contribution to intracellular Ca^{2+} signalling via its acute pharmacological modulation whilst in our case, as the role of PK11195 in apoptotic cell death was proven to be mBzR independent, we enrolled a long term pharmacological approach to define the functional results of the drug interaction with target genes in apoptotic response modulation.

Despite that, as mitochondria should amplify the differences recorded in the cytosol (Fig. 1B and C), an inhibitory role for mBzR itself on mitochondrial Ca^{2+} uptake in PK11195 treated HeLa cells cannot be excluded.

It is now widely appreciated that alteration of cellular Ca^{2+} handling is a common strategy used by different proteins in order to modulate the apoptotic response: Bcl-2 exerts some of its antiapoptotic effects by reducing $[Ca^{2+}]_{er}$ and down-regulating Ca^{2+} fluxes between the ER and mitochondria and the capacitative Ca^{2+} influx [26,28].

A similar effect was observed with the coxsackievirus 2B protein [54] and by reducing the mitochondrial Ca^{2+} uptake directly [55] (see also [56]). In the same way, experimental conditions that lowered $[Ca^{2+}]_{er}$ protect HeLa cells from ceramide-induced apoptosis [30] whilst maneuvers that increase $[Ca^{2+}]_{er}$ have the opposite effect and this is the case of the proapoptotic protein Bax [36]. This accounts for a signaling mechanism between the ER and the mitochondria in which alterations in Ca^{2+} uptake modulate the apoptotic response [57].

Key events occurring in the mitochondrial matrix are all sensitive to increases in $[Ca^{2+}]_m$ such as (i) ATP production, whose concentration is crucial for apoptosis vs. necrosis choice [58], (ii) reactive oxygen species production [59], (iii) the opening of the permeability transition pore (mPTP) followed by the release of proapoptotic proteins [60,61]. Accordingly, down-regulation of Ca^{2+} fluxes protect mitochondria from cytotoxic rises in $[Ca^{2+}]_m$.

Our findings show that PK11195 up regulates Ca^{2+} fluxes and this can be considered one of the mechanisms utilized by the compound to exert its role in promoting cell death, in line with the previously established proapoptotic role of ER-mitochondrial Ca^{2+} transfer [62,63]. In addition, we hypothesize that the combined PK11195 mechanism to increase either the IP_3-dependent release of Ca^{2+} from the ER and the capacitative Ca^{2+} entry support also a direct inhibitory effect on Bcl-2. Bcl-2 selectively modulates two hubs of Ca^{2+} signaling, the $[Ca^{2+}]_{er}$ and the capacitative Ca^{2+} entry, acting via a ''double hit'' strategy to avoid the Ca^{2+} mediated apoptosis [30]. PK11195 appears to act in an identical, but opposite manner thus it will potentiate this pathway, leading to cell death induction.

Although direct experimental evidences will be needed to confirm that this effect is mediated by a direct inhibition of the oncoprotein Bcl-2, it appears as a plausible target to justify the observed effects. An intriguing possibility might be also that PK11195 modifies the phosphorylation state of Bcl-2, as previously demonstrated for specific mitochondrial proteins [64], thus changing its interaction with other Bcl-2 family members and components of the Ca^{2+} signaling machinery on the ER surface [65,66]. Further studies will be then necessary to explore these prospects in depth thus paving the way to identify new molecular targets for modifying the apoptotic sensitivity of cancer cells.

Acknowledgments

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