2015-04-23

The PI3K/AKT/mTOR pathway is involved in direct apoptosis of CLL cells induced by ROR1 monoclonal antibodies

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Br J Haematol. 2015 May;169(3):455-8.
http://doi.org/10.1111/bjh.13228
http://hdl.handle.net/10616/44644

Letter to the Editor

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PI3K/AKT/mTOR signaling is a central pathway regulating growth of malignant cells and is constitutively activated in several types of cancer. PI3K is a key regulator of survival in cancer cells and the downstream molecules AKT and mTOR have anti-apoptotic effects (1). The class IA PI3K p110 (PI3Kδ) catalytic subunit is activated by the SRC homology 2 domain binding of p85 regulatory subunits to phosphorylated tyrosine motifs of receptor tyrosine kinases (RTKs). Mutations in the PI3K/AKT/mTOR pathway have been noted in different malignancies. In chronic lymphocytic leukemia (CLL), PI3K has been shown to be constitutively activated phosphorylating AKT and mTOR.

In an attempt to find new tumor antigens with a specific expression in CLL, we identified the RTK ROR1 (2). ROR1 is a surface receptor participating in cellular processes, as signal transduction, cell-cell interaction, proliferation, metabolism and survival. ROR1 has been suggested to be a survival factor in CLL (3). Silencing of ROR1 in CLL induced downregulation of the ROR1 gene and protein as well as apoptosis of the leukemic cells (4). Patients with progressive CLL had a higher expression of ROR1 as compared to patients with non-progressive disease (5).

We have previously shown that an anti-CRD ROR1 mAb induced specific direct apoptosis of primary CLL cells and dephosphorylated the intracytoplasmic TK domain of the ROR1 molecule (5, 6). To further

understand the effects of our anti-CRD ROR1 mAb, we analysed effects on the PI3K/AKT/mTOR pathway as well as on SRC and CREB.

Surface staining of CLL cells and PBMC of healthy donors, preparations of cell lysates and Western blot analysis have been described previously (5). The following total and phosphorylated proteins were analysed: ROR1, SRC, PI3K, AKT, mTOR and CREB. Phosphoproteins were measured before and after 2h of incubation with the mAbs. In cytotoxicity experiments, CLL cells and PBMC of healthy donors were incubated with mAbs against CRD (5), and Ig domains of ROR1 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 24-72h and measured by the MTT assay.

The percentage of ROR1 positive PBMC of CLL patients analysed by the anti-CRD ROR1 mAb was 82 ± 5% (mean ± SD) (range: 78-89%) (n=15). Normal PBMC was negative in PCR for ROR1 and < 0.5% cells were stained.

The frequency of apoptotic cells induced by different mAbs is shown in Fig 1. The anti-CRD ROR1 mAb was significantly more effective in inducing apoptosis than the anti-Ig ROR1 mAb at all time points (24h; $p<0.0381$, 48h; $p<0.002$, 72h; $p<0.002$). There was no difference between the isotype control and the anti-Ig ROR1 mAb.

The anti-CRD ROR1 mAb induced dephosphorylation of ROR1 as well as of SRC in CLL cells (Fig 2). SRC proteins have been shown to be activated in lung, breast and pancreatic carcinoma cells and involved in survival, proliferation and invasion. Phosphorylated ROR1 can physically interact with and

38 phosphorylate SRC and suggested to be a critical component for multiple signaling pathways involved in
39 tumorigenesis (7).

40 Treatment of CLL cells with the anti-CRD ROR1 mAb also decreased the level of phosphorylated AKT
41 (Fig 2). ROR1 mediated SRC phosphorylation has been shown to trigger AKT activation in lung
42 adenocarcinoma cells and ROR1 knockdown induced dephosphorylation of AKT as well as inhibited
43 growth and induced apoptosis (7).

44 Phosphorylation of PI3Kδ (Fig 2) and PI3K p85 but not the p55 isoform (data not shown) also decreased
45 in CLL cells treated with the anti-CRD ROR1 mAb. In CLL and AML cells, increased AKT activity was
46 shown to correlate with phosphorylation of PI3Kδ which was the predominant isoform. Phosphorylation
47 of PI3Kδ was mediated by SRC and p85 recruitment increased the catalytic activity of the PI3Kδ subunit.

48 Treatment of CLL cells with the anti-CRD ROR1 mAb also dephosphorylated mTOR. mTOR is
49 important for the regulation of cell growth as well as metabolism and is activated in different tumor types
50 translating proteins required for cell cycle progression from the G1 to S phase.

51 Furthermore, the anti-CRD ROR1 mAb induced dephosphorylation of the transcription factor CREB (Fig
52 2). Oncogenic transcription factors play a central role in tumorigenesis. CREB is activated through
53 phosphorylation by kinases, including AKT. CREB has been shown to be overexpressed and
54 constitutively phosphorylated in AML and NSCLC and important for the pathogenesis of these diseases.

55 Apoptosis of CLL cells induced by the anti-CRD ROR1 mAb was preceded by dephosphorylation of
56 ROR1, SRC, PI3K p85, PI3Kδ, AKT, mTOR and CREB proteins. Binding of the anti-CRD ROR1 mAb

57 to ROR1 decreased phosphorylation of SRC which might lead to dephosphorylation of the PI3K p85
58 isoform abrogating p85 recruitment and inactivation of the PI3K p110 catalytic subunit preventing signal
59 transmission downstream of PI3K. Activation of CREB might occur via the PI3K/AKT/mTOR pathway
60 which may enhance expression of genes augmenting resistance of tumor cells to apoptosis as well as
61 promoting tumor cell growth. ROR1 has been shown to utilize distinct kinase dependent and independent
62 mechanisms to sustain a favorable balance between PI3K/AKT mediated pro-survival signals and the pro-
63 apoptotic p38 pathway (7). A significant association between the expression of ROR1 and activated
64 AKT/CREB enhancing tumor cell growth in different tumor types has recently been reported (8).

65 The present study indicates that our anti-CRD ROR1 mAb inhibited the PI3K/AKT/mTOR pathway
66 which is of major importance in tumorigenesis (1). This pathway is a validated target for e.g. EGFR and
67 HER-2, two other receptor tyrosine kinases of the RTK families. ROR1 might be an interesting
68 therapeutic target using mAbs for CLL and other cancers expressing ROR1. A support of a therapeutic
69 effect of anti-ROR1 mAbs was recently reported using the anti-ROR1 D10 mAb in an animal CLL model
70 (9). The D10 ROR1 mAb is directed against a CRD close epitope and induced direct apoptosis, while
71 antibodies against the Ig-domain did not induce direct apoptosis (10), as confirmed in the present study.
72 In addition, our anti-CRD ROR1 mAb also killed leukemic cells in ADCC and CDC (5). Thus, our
73 specific anti-CRD ROR1 mAb seems to have multifunctional activities and might support a notion that
74 the ROR1 binding site is of importance for the activity of cytotoxic ROR1 antibodies.

75

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Author contributions

Contribution: AHDM performed the experiments, analysed the data and wrote the manuscript. MHF performed experiments and reviewed the manuscript. AM, ASK and EM reviewed the manuscript. AÖ provided clinical material, analysed the data and wrote the manuscript. HM designed and supervised the study, provided clinical material, analysed the data and wrote the manuscript

Conflict of interest

The authors declare no conflict of interest.

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Key words: ROR1, monoclonal antibodies, CLL, apoptosis, PI3K/AKT/mTOR pathway

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139 **Legends to figures**

140 **Figure 1.**

141 Apoptosis (%) (mean ± SD) time response curves induced by the anti-CRD ROR1 mAb in CLL cells
142 (n=7) (A) and PBMC of healthy donors (n=5) (B). For comparison a mouse mAb against the Ig domain of
143 ROR1 as well as an isotype control mAb was included. Direct apoptosis induced by anti-CRD ROR1
144 mAb was significantly higher compared to the anti-Ig ROR1 and the isotype control mAbs (anti-CRD
145 ROR1 vs anti-Ig ROR1 at 24h; $p<0.0381$, at 48h; $p<0.002$ and at 72h; $p<0.002$) (anti-CRD ROR1 vs
146 control isotype control at 24h; $p<0.002$, at 48h; $p<0.002$ and at 72h; $p<0.002$). Spontaneous apoptosis at
147 each time point was deducted. At 72h the spontaneous apoptosis for CLL cells was 27 ± 5% and for
148 PBMC of healthy donors 26 ± 6% (mean ± SD). There were no statistically significant differences in
149 apoptosis comparing the anti-CRD ROR1, the anti-Ig ROR1 and the isotype control mAbs using normal
150 PBMC as targets.

151 **Figure 2**.

152 **A)** Representative experiments of five CLL patients showing dephosphorylation of ROR1, SRC, PI3Kδ,
153 AKT, mTOR (signaling molecules) and CREB (transcription factor) within 2h of incubation of CLL cells
154 with the anti-CRD ROR1 mAb (+) and an isotype control mAb (-). Time kinetics experiments showed
155 that 2h was the optimal time point to achieve maximum dephosphorylation of the signaling proteins (data
156 not shown). The Western blot methods as well as the anti-CRD ROR1 mAb and the rabbit polyclonal
157 pROR1 antibody have been described previously (5, 6). The other antibodies were monoclonal antibodies
158 purchased from Cell Signaling Technology (Danvers, MA, USA) and Santa Cruz Biotechnology (Dallas,
159 TX, USA). **B)** Relative intensity of phosphorylated proteins to total proteins was calculated after 2h of

160 incubation without (□) and with (■) the anti-CRD ROR1 mAb. Statistically significant levels are

161 shown at the top. Intensity was measured by ImageJ software (National Institutes of Health (NIH),

162 Bethesda, MD, USA), as previously described (6).

163

Figure 1

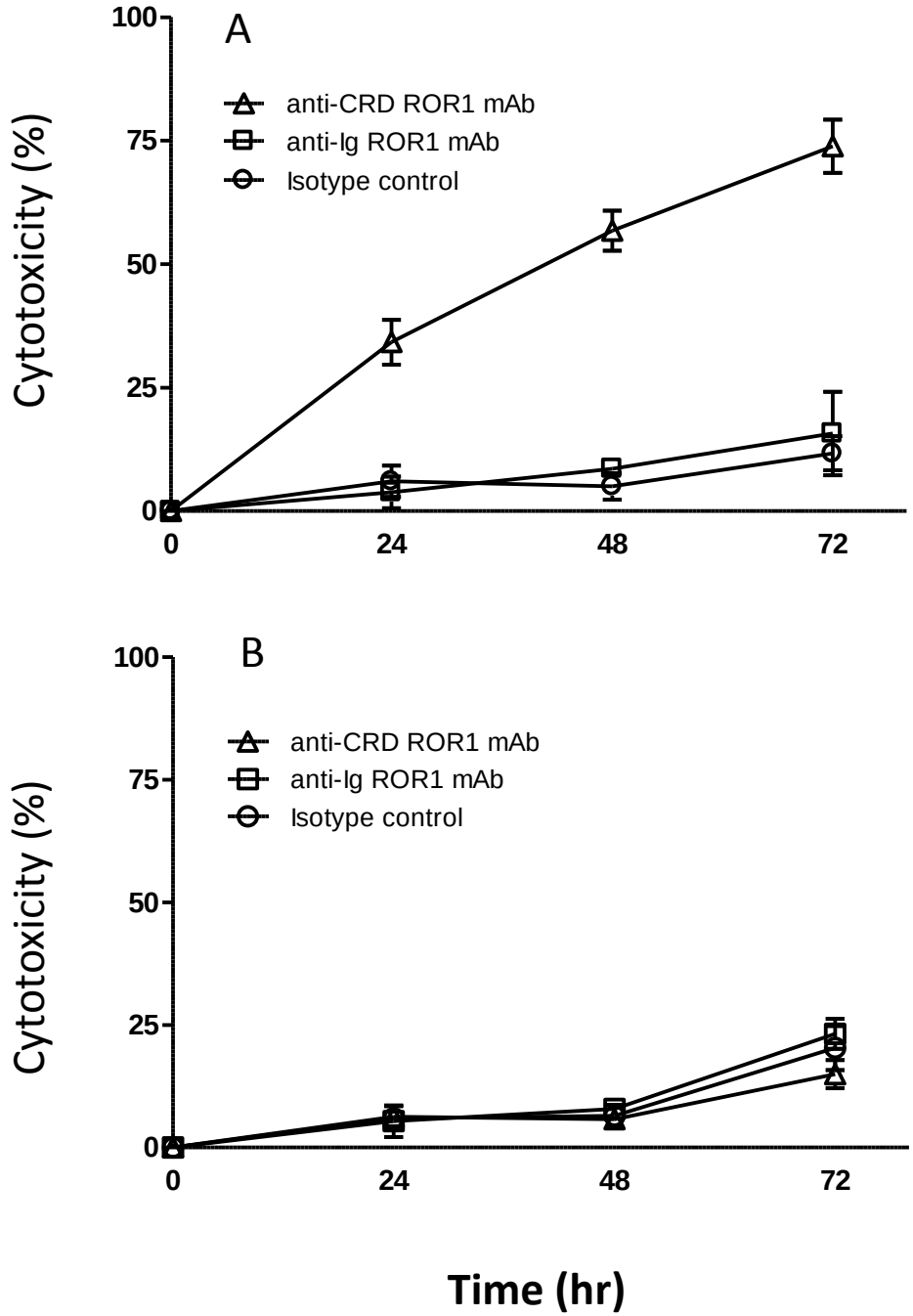
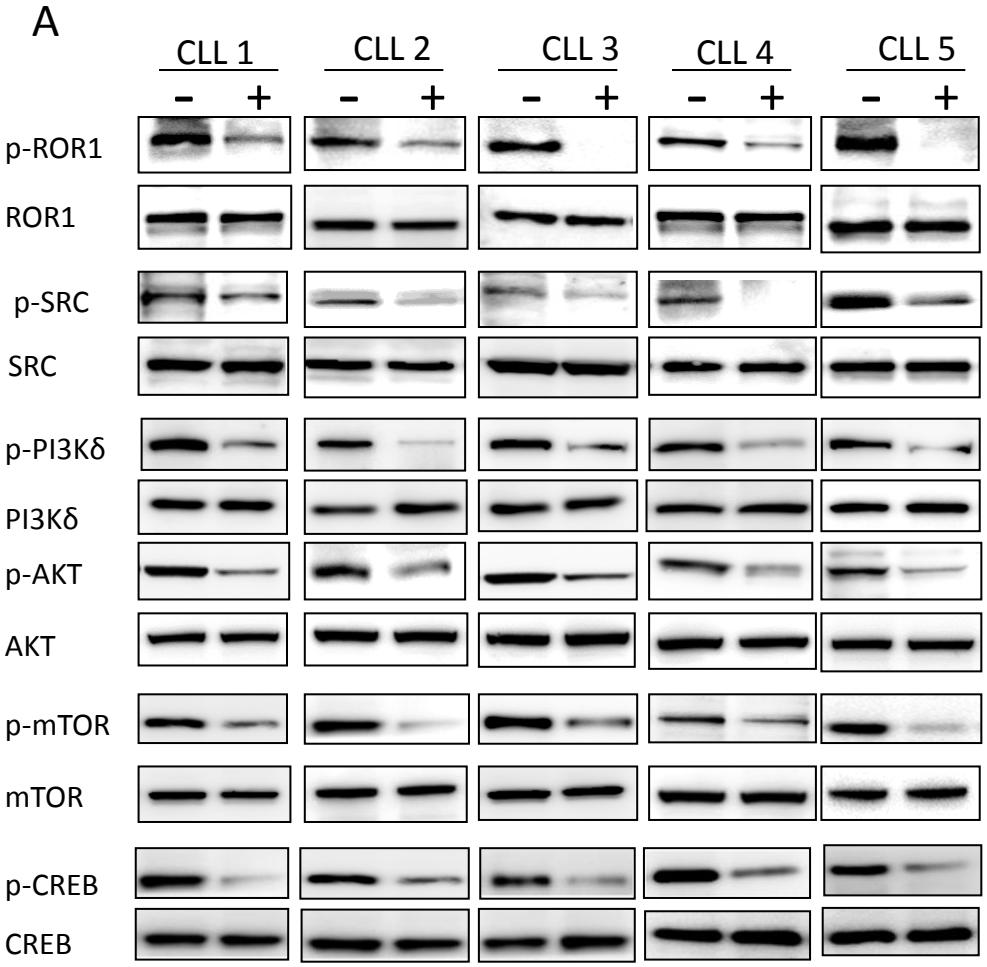
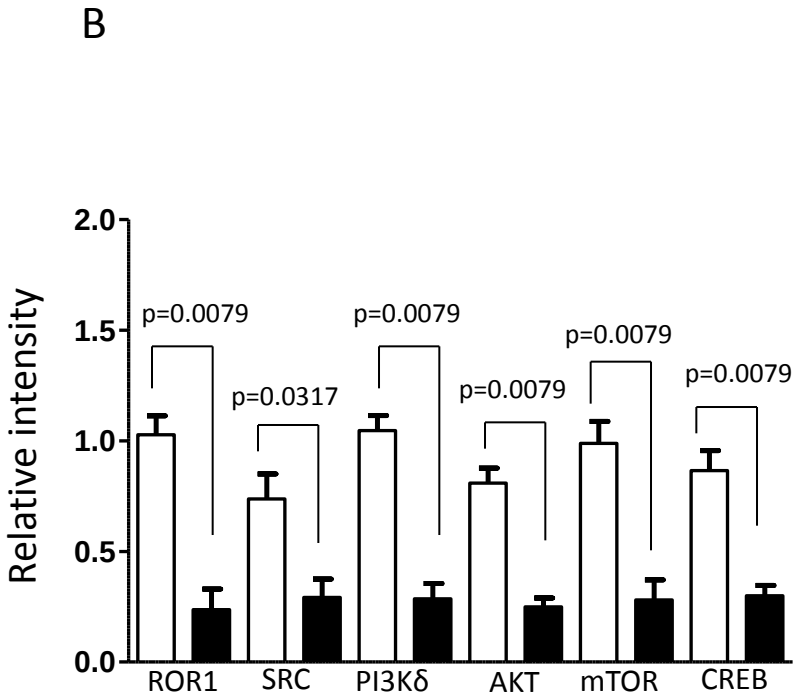

A
anti-CRD ROR1 mAb
anti-Ig ROR1 mAb
Isotype control
Cytotoxicity (%)
100
75
50
25
0
0
24
48
72
B
anti-CRD ROR1 mAb
anti-Ig ROR1 mAb
Isotype control
Cytotoxicity (%)
100
75
50
25
0
0
24
48
72
Time (hr)

Figure 2


A
CLL 1
CLL 2
CLL 3
CLL 4
CLL 5
- +
p-ROR1
ROR1
p-SRC
SRC
p-PI3Kδ
PI3Kδ
p-AKT
AKT
p-mTOR
mTOR
p-CREB
CREB


B
Relative intensity
2.0
1.5
1.0
0.5
0.0
p=0.0079
p=0.0317
p=0.0079
p=0.0079
p=0.0079
p=0.0079
ROR1
SRC
PI3Kδ
AKT
mTOR
CREB