Supplementary data

Supplementary Materials and Methods

Patients and diagnostic evaluations. A total number of eight patients with chronic myeloid leukemia (CML) (5 females, 3 males) were examined. The median age was 47.3 years (range: 26–59 years). Diagnoses and the phase of CML were established according to the classification provided by the World Health Organization (WHO) [1]. Peripheral blood (PB) and/or bone marrow (BM) cells (iliac crest or sternum) were collected at diagnosis. Karyotyping was performed following the recommendations of the European Leukemia Net (ELN) [5,6]. *BCR/ABL1* mRNA levels were determined in PB or BM cells using *ABL1* as a reference gene and the International Scale (IS) for standardized quantification [7–9]. The patients' characteristics are shown in Supplementary Table E2. All patients examined (8/8 = 100%) had chronic phase CML (CP). The patients provided written informed consent before PB or BM samples were collected. All studies including storage of cells, drug incubation experiments and the examination of leukemic stem cells (LSC) were approved by the local ethics committee of the Medical University of Vienna.

Cell sampling and storage. A total number of 8 CML samples (from 8 patients) were analyzed. Stabilizer-free heparin (Biochrom AG, Berlin, Germany) was used as anticoagulant. Freshly obtained samples (PB, BM) were subjected to flow cytometry analysis and isolation of mononuclear cells (MNC) using Ficoll gradient centrifugation. Isolated MNC were either used immediately or were frozen in liquid nitrogen until used. Frozen MNC were thawed using DNAse type I, 100 U/mL (Sigma Aldrich, St. Louis, MO, USA) to prevent cell clumping.

Lentiviral transduction of cell lines. The coding sequence of human CD26 was cloned into the lentiviral pWPTet vector [10]. pWPTet-green fluorescence protein (GFP) served as a control. VSV-G pseudotyped viral particles were produced as described [11]. KU812 cells were transduced as described and $CD26^+$ (CD26-transduced) cells and GFP^+ control cells were purified by fluorescence-activated cell sorting (FACS) and cultured in RPMI 1640 medium and 10% fetal calf serum (FCS).

Flow cytometry analyses. Phenotyping of $CD34^+/CD38^=$ stem cells (SC) and $CD34^+/CD38^+$ progenitor cells was performed in eight patients with CML. Whole BM and/or PB samples or MNC were used. Multi-color flow cytometry was performed as described [12,13] using fluorochrome-conjugated mAb shown in Supplementary Table E1. Flow cytometry was performed on a FACSCalibur (Becton Dickinson, San José, CA, USA). All staining reactions were controlled by isotype-matched control antibodies and expressed as percentage of positive cells. For staining of cytoplasmic caspase-3, KU812 cells or CML-T1 cells were incubated in control medium or in medium supplemented with various concentrations of vildagliptin at 37°C for 48 hours. After incubation, CML cell lines were fixed in 4% formaldehyde (15 minutes) and permeabilized in methanol (-20°C, 15 minutes). Thereafter, CML cells were incubated with PE-conjugated mAb C92-605 against active caspase-3 for 30 minutes. After incubation, cells are analyzed on a FACSCalibur. For analyzing apoptosis induction in primary CML LSC, MNC were incubated in control medium or in medium supplemented with various concentrations of vildagliptin and/or nilotinib at 37°C for 48 hours. After incubation, cells were stained with mAb HI30 against CD45, mAb 581 against CD34 and mAb HIT2 against CD38 (15 minutes). After incubation, cells were washed with annexin-binding buffer (ABB) (Aqua, $CaCl_2$, NaCl, Hepes, pH 7.4) and rh Annexin-V was added (15 minutes). Then, cells were resuspended in ABB containing DAPI (100 ng/ml) in order to exclude non-viable cells. Cells were analyzed on a FACSCanto (Becton Dickinson, San José, CA, USA).

Measurement of ^{3}H-thymidine uptake in CML cells. To determine the growth-inhibitory effects of various drugs, KU812 cells transfected with a control construct (GFP^+) or a CD26 construct ($CD26^+$), $CD26^+$ CML-T1 cells (1–2 x 10^4 cells/well) or primary CML MNC (1–1.5 x 10^5 cells/well) were incubated in control medium or in various concentrations of vildagliptin (10–10,000 nM), imatinib (50–250 nM), or nilotinib (2–12 nM) alone or in combination in 96-well culture plates at 37°C for 48 hours. After incubation, 0.5 µCi ^{3}H-thymidine was added (37°C, 16 hours). Cells were then harvested on filter membranes (Packard Biosciences, Meriden, CT) in a Filtermate 196 harvester (Packard Bioscience). Filters were air-dried, and the bound radioactivity was counted in a ß-counter (Top-count NXT, Packard Bioscience). All experiments were performed in triplicates.

Determination of DPPIV (CD26) enzymatic activity. As shown previously, lysates from primary, CD26 sorted CML LSC and CD26 transfected KU812 cells exhibit DPPIV activity, whereas normal stem cells lack CD26 activity [14]. In the present study, DPPIV activity was determined in lysates of KU812 $CD26^+$ cells, KU812 GFP^+ ($CD26^=$) cells, primary $CD34^+/CD38^=/CD26^+$ stem cells and $CD34^+/CD38^=/CD26^=$ (normal) stem cells from one CML patient, and the $CD26^+$ CML cell line CML-T1. Before being measured, CML-T1 cell lysates were pre-incubated with gliptins (1-1,000 nM) at 37°C for 30 minutes. Enzyme activity was measured by the DPPIV-Glo™ protease assay (Promega, Madison, WI, USA) essentially as described [14]. In brief, lysates from 20 x 10^3 cells (50 µL per well) were incubated with DPPIV-Glo reagent plus DPPIV-Glo substrate (50 µL) at room temperature for 30 minutes in white-colored 96-well plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark). After incubation, luminescence was determined in a FLx 800 Fluorescence

Microplate Reader (BioTek, Winooski, VT, USA). DPPIV enzymatic activity was expressed as relative luminescence/light units (RLU) per well.

Xenotransplantation assay. Purified $CD34^+$ CML cells were re-suspended in 0.15 ml phosphate-buffered saline (PBS) with 2% FCS and 1% penicilin/streptomycin and were injected intravenously (i.v.) into the lateral tail vein of adult NOD SCID-IL-2R$\gamma^{-/-}$ (NSG) mice (5 mice per group; 0.5 - 3 x10^6 cells per mouse). Twenty-four hours prior to injection, mice were irradiated (2.4 Gy). After i.v. injection of $CD34^+$ CML cells, one group of mice received only solvent (PBS + 10% DMSO) intraperitoneally (i.p.) for 4 weeks (control group), one group vildagliptin (3 µmol/mouse/day) via drinking water for 26 weeks; a third group of mice received imatinib (10 mg/kg/day via i.p. injection) for 4 weeks; a fourth group imatinib (10 mg/kg/day via i.p. injection) for 4 weeks plus oral vildagliptin (3 µmol/mouse/day) for 26 weeks; a fifth group nilotinib (10 mg/kg/day via i.p. injection) over 4 weeks; and a sixth group nilotinib (10 mg/kg/day via i.p. injection) over 4 weeks plus oral vildagliptin (3 µmol/mouse/day) for 26 weeks. Mice were inspected daily and were sacrificed as soon as they developed disease-related or other severe symptoms before 26 weeks. After 26 weeks (from the time of cell-injection) all (remaining) mice were sacrificed. Despite the long observation period, the dropout rate of mice (by unknown causes) was below 10%. BM cell suspensions of NSG mice were obtained by flushing femurs, tibias, and humeri. Human engrafted cells were detected by multicolor flow cytometry using mAb against CD19, CD33, and CD45. TO-PRO3 was used to exclude non-viable cells. CML-repopulation was quantified by multi-color flow cytometry and expressed as percentage $CD45^+/CD33^+$ cells of all viable BM cells in the samples. In addition, engraftment of CML cells was quantified by immunohistochemistry of BM sections obtained from pelvic samples of NSG mice (see below).

Immunohistochemistry (IHC). Immunohistochemistry was performed on formalin-fixed and paraffin-embedded BM sections obtained from NSG mice (pelvic samples) injected with $CD34^+$ CML cells and treated with imatinib, nilotinib, vildagliptin or a combination of the TKI and the CD26 blocker. Before staining, BM sections were pretreated by microwave oven. Sections were incubated with a monoclonal antibody (mAb) against human CD45 (clones: 2B11 and PD7/26, dilution 1:50; Dako, Denmark). Antibodies were diluted in 0.05 M Tris-buffered saline (pH 7.5) and 1% bovine serum albumin (Sigma). After washing, slides were incubated with biotinylated anti-mouse IgG supplemented with normal goat serum (both from Vector, Burlingame, CA, USA) for 30 minutes, washed, and exposed to Vectastain ABC KIT (Vector) for 30 min. 3-amino-9-ethyl-carbazole (AEC) was used as the chromogen.

Quantitative PCR (qPCR). To confirm CML engraftment in NSG mice after 26 weeks, BCR/ABL1 mRNA levels were quantified in human cells as described previously [14]. In brief, total RNA was extracted from enriched $CD34^+$ human cells before injection and from engrafting human cells in NSG mice, using the RNeasy Micro Kit (Oiagen, Hilden, Germany). Pretreated with DNAse (Promega, Medison, WI), cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA), random primers, dNTPs (2 mM) (Invitrogen), and RNasin as described [14]. To quantify BCR/ABL1 mRNA levels, qPCR was performed using primers specific for human BCR/ABL1 (huBCR/ABL1-fwd, TCCGCTGACCATCAATAAGGA; huBCR/ABL1-rev, CACTCAGACCCTGAGGCTCAA) and human ABL1 (huABL1-fwd, TGTATGATTTTGTGGCCAGTGGAG; huABL1-rev, GCCTAAGACCCGGAGCTTTTCA). qPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystem, Foster City, CA) using iTAq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA). Human ABL1 served as reference gene.

References

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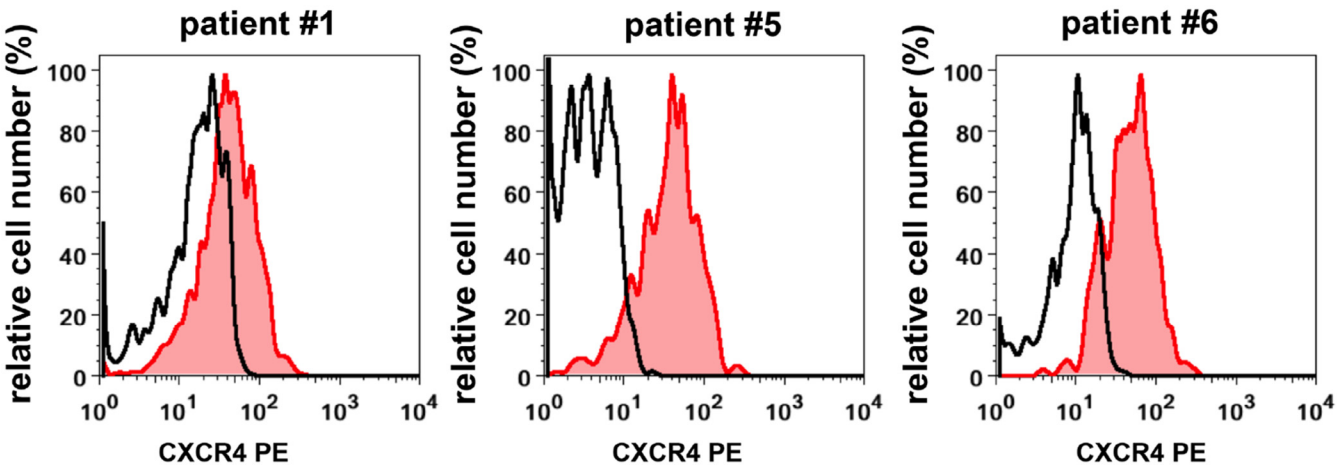
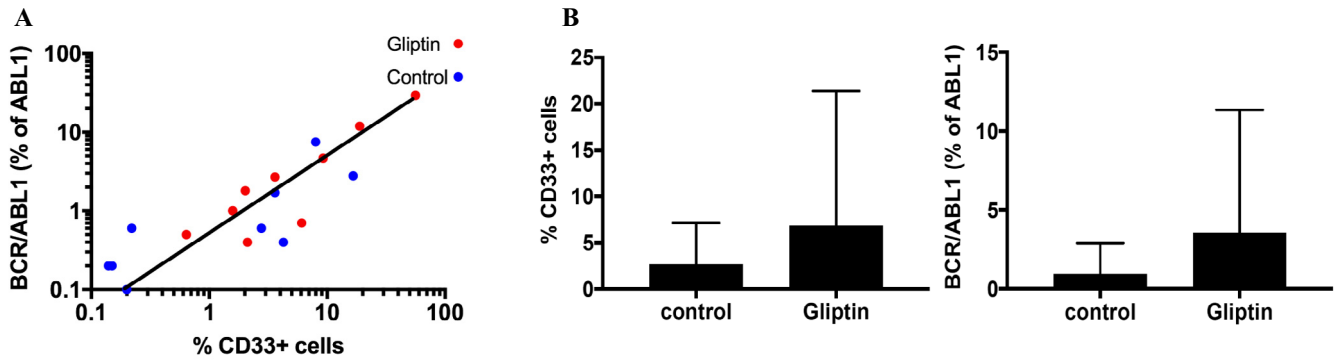
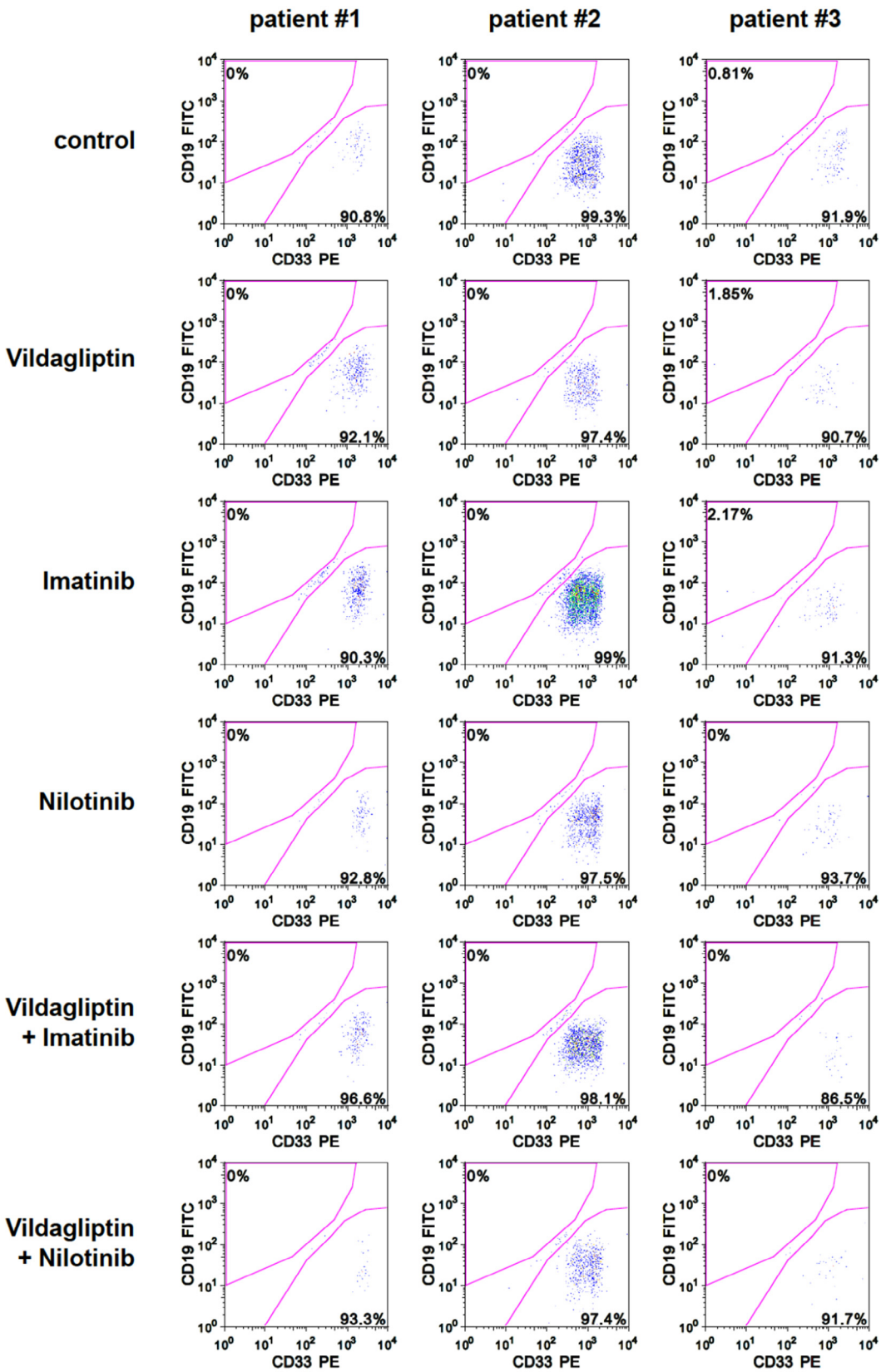


Supplementary Figure E1. Expression of SDF-1 receptor CXCR4 (CD184) on CML LSC. In three patients with CML (#1, #5, and #6 in Supplementary Table E2), leukemic cells were stained with a monoclonal antibody (mAb) against CXCR4; and the reactivity of $CD34^+/CD38^=/CD26^+$ CML stem cells (LSC) with this mAb (red histogram) was determined by multi-color flow cytometry. Reactivity with the isotype-matched control antibody is also shown (open histogram).



Supplementary Figure E2. Engraftment of $CD33^+$ CML cells in NSG mice receiving vildagliptin. Purified $CD34^+$ CML cells (chronic phase CML, n = 3) were injected intravenously into NSG mice. Mice were treated with vildagliptin per os (3 µmol/mouse/day) or control drinking water for 16 weeks. Then, mice were sacrificed and bone marrow (BM) cells were examined for the presence of human $CD33^+$ cells, $CD19^+$ cells and human BCR/ABL1 (as percent of ABL1) by qPCR as described in the text of this supplement. In all three experiments, only $CD33^+$ cells engrafted. "A": To confirm CML engraftment, BCR/ABL1 mRNA levels were quantified and correlated with the percentage of $CD33^+$ cells. As visible, a good correlation between BCR/ABL1 mRNA levels and the percentage of $CD33^+$ cells was demonstrable. In "B" a summary of engraftment levels (percent human $CD33^+$ cells and BCR/ABL1 levels in BM cells) in all mice treated with solvent control or vildagliptin are shown.



Supplementary Figure E3. Expression of CD33 and CD19 on the surface of engrafted cells. Engrafted human cells detected in the bone marrow of NSG mice in the three experiments (#1, #2 and #3 in Supplementary Table E2) in which mice received solvent control, imatinib, nilotinib, vildagliptin or a combination of either imatinib and vildagliptin or nilotinib and vildagliptin. The figure shows typical staining results obtained in three mice from each group (solvent control, imatinib, nilotinib, vildagliptin or drug combinations). Cells were stained with a mAb against human CD19 and a mAb against human CD33. The reactivity with these mAb was determined by multi-color flow cytometry. As visible, engrafted cells expressed almost exclusively CD33 (86.5–99.3%), whereas no $CD19^{+}$ populations of human cells (B cells) could be detected.

Supplementary Table E1. Specification of monoclonal antibodies (mAb) used in this study

CD	antigen	clone	isotype	conjugate	reactivity	company*
n.c.	Isotype control	MOPC-21	mouse, IgG1	PE	human	BD Biosciences
n.c.	Isotype control	20102	mouse IgG2a	PE	human	R&D systems
CD19	B4	4G7	mouse, IgG1	FITC	human	BD Biosciences
CD26	DPPIV	M-A261	mouse, IgG1	PE	human	BD Biosciences
CD33	Siglec-3	WM53	mouse, IgG1	PE	human	BD Biosciences
CD34	HPCA-1	581	mouse, IgG1	FITC	human	BD Biosciences
CD34	HPCA-1	581	mouse, IgG1	PE	human	BD Biosciences
CD38	T10	HIT2	mouse, IgG1	APC	human	BD Biosciences
CD45	LCA	2D1	mouse, IgG1	PerCP	human	BD Biosciences
CD45	LCA	HI30	mouse, IgG1	APC-H7	human	BD Biosciences
CD184	CXCR4	12G5	mouse, IgG2a	PE	human	BD Biosciences
n.c	Caspase-3	C92-605	mouse, IgG1	PE	human	BD Biosciences

CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate; PerCP, peridinin chlorophyll protein; APC, allophycocyanin; n.c., not (yet) clustered; n.a.; not available; Ig, immunoglobulin; DPPIV, dipeptidyl-peptidase IV; HPCA-1, human precursor cell antigen-1; LCA, leukocyte common antigen.

*Company Location: BD Bioscience, San José, CA, USA; Dako, Glostrup, Denmark; eBioscience, San Diego, CA, USA; R&D Systems, Minneapolis, MN, USA.

Supplementary Table E2. Patients' characteristics—chronic myeloid leukemia (CML)

No	diagnosis	karyotype	age (yrs)	gender (m/f)	WBC (G/L)	Hb (g/dL)	PLT (G/L)	basophils PB (%)	blasts PB (%)	blasts BM (%)	BCR/ABL1* (%)	CD26 expression on $CD34^+/CD38^-$ (%)
1	CML CP	46,XX,t(9;22)	57	f	365.6	8.8	516	5	8	<5	n.t.	64.1
2	CML CP	46,XX,t(9;22)	26	f	506.92	7.9	197	3	9	n.t.	47.22	96.1
3	CML CP	46,XX,t(9;22)	56	f	398.94	8.6	165	7	1	1	31.63	62.4
4	CML CP	46,XY,t(9;22)	50	m	162.20	16.4	164	4	2	<5	43.45	73.9
5	CML CP	46,XY,t(9;22)	59	m	83.73	13.5	297	4	2	3–4	29.99	54.8
6	CML CP	46,XY,t(9;22)	49	m	232.75	9.3	1062	6	3	1	39.27	95.8
7	CML CP	46,XX,t(9;22)	46	f	570.5	7.5	342	3	1	2	57.54	85.1
8	CML CP	46,XX,t(9;22)	35	f	396.93	7.2	425	6	2	1	43.84	59.3

No, number; yrs, years; m, male; f, female; WBC, white blood count; Hb, hemoglobin; PLT, platelet count; PB, peripheral blood; BM, bone marrow; G/L, 10^9 cells per liter; g/dL, gram per deciliter; CP, chronic phase; n.t., not tested. *BCR/ABL1 mRNA levels were determined in PB cells using ABL1 as a reference gene and the International Scale (IS) for standardized quantification.