(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 March 2013 (14.03.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/034685 A1

(51) International Patent Classification:
A61K 31/00 (2006.01) *A61K 31/415* (2006.01)
A61K 31/352 (2006.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/EP2012/067504

(22) International Filing Date:
7 September 2012 (07.09.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/531,861 7 September 2011 (07.09.2011) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2013/034685 A1

(54) Title: MEANS AND METHODS FOR TREATING AND/OR PREVENTING NATURAL AHR LIGAND-DEPENDENT CANCER

(57) Abstract: The present invention relates to the field of cancer therapeutics and treatment of cancer. In particular, it relates to a method for treating and/or preventing a natural AHR ligand-dependent cancer comprising administering to a subject suffering from said cancer a therapeutically effective amount of an AHR inhibitor. Moreover, contemplated is a AHR inhibitor for use in treating and/or preventing a natural AHR ligand-dependent cancer.

Means and methods for treating and/or preventing natural AHR ligand-dependent cancer

The present invention relates to the field of cancer therapeutics and treatment of cancer. In particular, it relates to a method for treating and/or preventing a natural AHR ligand-dependent cancer comprising administering to a subject suffering from said cancer a therapeutically effective amount of an AHR inhibitor. Moreover, contemplated is a AHR inhibitor for use in treating and/or preventing a natural AHR ligand-dependent cancer.

Tumor micro-environment represents a particular challenge in an effective tumor therapy since it has multiple influences on the malignancy of a tumor (Tennant 2010, Nat Rev Cancer 10, 267).

Tryptophan (Trp) metabolism is an example for the importance of the tumor micro-environment. Its functional relevance as a pivotal endogenous mechanism for limiting the immune response has been demonstrated in animal models already (Munn 2007, J Clin Invest 117, 1147).

In particular, the activation of the Trp metabolism correlates with diseases and disorders of the immune system such as tumor immunity, autoimmunity, infectious diseases and maintenance of the immune privilege (Opitz 2007, Cell Mol Life Sci 64, 2452). Degradation of Trp by indoleamine-2,3-dioxygenases 1 and 2 (IDO1/2) in tumors and tumor draining lymph nodes inhibits antitumor immune responses and is associated with a poor prognosis in various malignancies (Lob 2009, Nat Rev Cancer 9 (6): 445). Inhibition of IDO1/2 suppresses tumor formation in animal models and is currently tested in phase I/II clinical trials in cancer patients (Muller 2005, Nat Med 11(3): 312, Uyttenhove 2003, Nat Med 9(10), 1269; DiPuccio 2010, Expert Opin Ther Pat 20, 229; Ball 2007, Gene 396(1), 203; Metz 2007, Cancer Res 67(15), 7082).

Another enzyme known to be involved in the Trp metabolism in neurons and hepatocytes is the tryptophan 2,3- dioxygenase (TDO) which synthesizes the first step of the Trp degradation as well (Thackray 2008, Biochem Soc Trans 36, 1120). TDO has also been reported as a

potential target for tumor drugs (WO2010/008427) The relevance of Trp catabolism for human tumor formation and progression, however, remains elusive.

Kynurenin (Kyn) is a Trp metabolite having immunosupressive functions. However, its molecular targets and the mechanism how this effect is elicited is not yet understood. Exogenous Kyn has been reported to, inter alia, activate the Aryl- Hydrocarbon Receptor (AHR) transcription factor in dendritic cells and T-cells (Mezrich 2010, J Immunol 185, 3190; Nguyen 2010, Proc Natl. Acad Sci, USA, 107, 19961).

The AHR is a transcription factor of the basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) family, which is activated by xenobiotics such as benzoapyrene and 2,3,7,8-tetrachlordibenzodioxin (TCDD). In the nucleus the AHR forms a heterodimer with the AHR nuclear translocator (ARNT) that interacts with the core binding motif of the dioxin-responsive elements (DRE) located in regulatory regions of AHR target genes (Reyes 1992, Science 256, 5060; Abel 2010, Biol Chem 391, 1235).

AHR is known to be involved into chemical carcinogenesis elicited by, e.g., halogenated aromatic hydrocarbons. Further, it has been reported that green tea extracts can act as antagonists of the AHR and, thereby, can prevent the harmful effects of such halogenated aromatic hydrocarbons (Palermo 2003, Chem Res Toxicol 16, 865). Moreover, constitutive expression of the AHR gene is known to be involved in cellular survival in glioblastoma cells (Gramatzki 2009, Oncogene 28, 2593).

In light of the above, the provision of means and methods for effectively treating tumors the malignancy of which are dependent on metabolic processes such as the Trp catabolism are not yet available but would be nevertheless highly desirable.

SUMMARY OF THE INVENTION

The present invention relates to a method for treating and/or preventing a natural AHR ligand-dependent cancer comprising administering to a subject suffering from said cancer a therapeutically effective amount of an AHR inhibitor.

In a preferred embodiment of the method of the invention, said cancer is selected from the group consisting of: brain tumors, preferably, glioma, melanoma, colorectal adenocarcinoma, colon carcinoma, renal cell carcinoma, NSCLC, breast cancer, hepatocellular carcinoma, ovarian carcinoma, head and neck carcinoma, bladder cancer, pancreatic adenocarcinoma, mesothelioma, and SCLC.

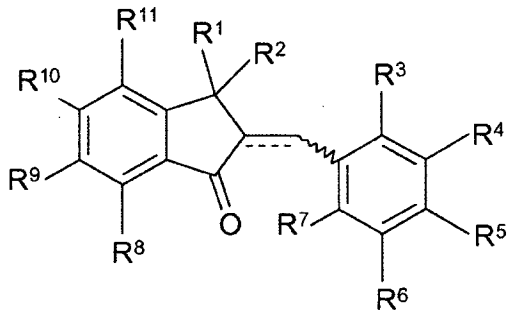
In a preferred embodiment of the method of the invention, said AHR inhibitor is a small molecule compound.

In a more preferred embodiment of the method of the invention, said small molecule compound is a plant compound or derivative thereof.

In a more preferred embodiment of the method of the invention, said plant compound or derivative thereof is a flavone or derivative thereof. Most preferably, said flavone or derivative thereof is 3,4-dimethoxyflavone, 3′-methoxy-4′-nitroflavone, 4′,5,7-Trihydroxyflavone (apigenin), or 1-Methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phenyl-1H-pyrazole-5-carboxamide (CH223191; CAS number 301326-22-7).

In another more preferred embodiment of the method of the invention, said plant compound or derivative thereof is reveratrol or a derivative thereof, epigallocatechin or epigallocatechingallate.

In another preferred embodiment of the method of the invention, said small molecule compound is a compound characterized by the following general formula (I):



wherein

(i) R^1 and R^2 independently of each other are hydrogen or a C_1 to C_{12} alkyl,

(ii) R^3 to R^{11} independently from each other are hydrogen, a C_1 to C_{12} alkyl, hydroxyl or a C_1 to C_{12} alkoxy, and

(iii) the broken line represents either a double bond or two hydrogens.

In another preferred embodiment of the method of the invention, said AHR inhibitor is an antibody which specifically binds to and inhibits the AHR protein.

In another preferred embodiment of the method of the invention, said AHR inhibitor is the AHR repressor protein or an inactive AHR nuclear translocator (ARNT).

In another preferred embodiment of the method of the invention, said AHR inhibitor is a nucleic acid inhibitor.

In another more preferred embodiment of the method of the invention, said nucleic acid repressor specifically binds to an AHR encoding polynucleotide and is selected from the group consisting of: a ribozyme, an antisense molecule, an inhibitors oligonucleotide, a micro RNA, and an siRNA.

Moreover, contemplated by the invention is an AHR inhibitor for use in treating and/or preventing a natural AHR ligand-dependent cancer.

FIGURES

Figure 1 shows that TDO degrades Trp to Kyn in human brain tumors. **a,** Trp (left) and Kyn (right) content in the supernatants of human astrocytes (hAs), glioma cell lines and GIC (T323) cultured for 72 h and measured by HPLC (n=4). **b,** Correlation between *TDO* mRNA and Kyn release of human glioma cells measured by quantitative RT-PCR and HPLC (n=4). **c,** Kyn concentrations in the supernatants of U87 glioma cells cultured for 48 h in the presence of the TDO inhibitor 680C91 (black bars) or its solvent (white bars; n=4, P= 0.005, 0.002 and 0.0009 for 1, 5 and 10 μM TDOI, respectively). **d,** Kyn release of glioma cells after knockdown of *TDO* (black bars, P = 0.000007, 0.0007 and 0.00006, respectively), *IDO1* (dark gray bars) or *IDO2* (light gray bars) by siRNA (n=3). **e,** Weak neuronal TDO expression in healthy brain tissue (upper panel). TDO expression in glioblastoma (WHO grade IV, lower panel); red: TDO staining; * necrosis; arrowheads: border to infiltrated brain tissue. Inset: single tumor cells (arrows) infiltrating the adjacent brain tissue. Magnification: 40x, insets 400x (upper panel), 100x (lower panel). **f,** Plot of TDO expression [H-score] in brain tumors of increasing malignancy (WHO grade II- IV; grade II, n=18, grade III, n=15, grade IV, n=35). **g,** Correlation of the Ki-67 proliferative index with the TDO H-score in gliomas of different WHO grades (n = 42). **h,** Trp (left) and Kyn (right) concentrations in the sera of 24 glioblastoma patients and 24 age- and sex-matched healthy controls, measured by HPLC. **i,** Quantification of quinolinic acid staining in healthy human brain tissue (white bar, n=5) and glioblastoma tissue (black bar, n=5). The data distribution in **(f)** and **(g)** is presented as box plots, showing the 25th and 75th percentile together with the median, whiskers represent the 10th and 90th percentile, respectively.

Figure 2 shows paracrine effects of TDO-mediated Kyn release by glioma cells on immune cells. **a,** Correlation of the allogeneic proliferation of PBMC cocultured with different glioma cell lines with the Kyn release of the glioma cells (n=3). **b,** Allogeneic proliferation of PBMC cocultured with *TDO*-expressing control U87 glioma cells (sh-c) in comparison to U87 glioma cells with a stable short hairpin RNA-mediated knockdown of *TDO* (sh-*TDO*), with or without 100 μM Kyn (black bars), in comparison to PBMC alone with or without 100 μM Kyn (white bars, n=3). **c,** Quantification of LCA+ cells (left graph) and CD8+ cells (right graph) stained in human glioma sections with low TDO expression (H-score < 150, white bar, n=12 for LCA, n=10 for CD8) and in human glioma sections with high TDO expression (Hscore ≥ 150, black bar, n=17 for LCA and n=10 for CD8). **d,** Growth of *Tdo*-deficient GL261 murine glioma cells stably transfected with *Tdo* (solid circles) or empty vector (open circles) injected s.c. into the flank of C57BL/6N mice was monitored using metric callipers (n=6). Tumor weight was calculated using the equation: tumor weight (g) = (length (cm) × width (cm)2) × 0.5. **e,** IFN-γ-release of T cells of mice bearing subcutaneous *Tdo*-expressing tumors (black bar) in comparison to T cells of mice bearing *Tdo*-deficient tumors (white bar) after restimulation with glioma lysates measured by ELISpot (n=3). **f,** Lysis of GL261 murine glioma cells by spleen cells of mice with *Tdo*-expressing GL261 tumors in comparison to those with a subcutaneous *Tdo*-deficient GL261 tumors measured by chromium release (n=4). **g,** Quantification of the migrated distances of sh-c (open squares) and sh-*TDO* (solid circles) cells into a collagen matrix (n=3, P=0.004, 0.0005 and 0.01 for 24, 48 and 72 h, respectively). **h,** Clonogenic survival of sh-c (white bar) and sh-*TDO* (black bar) U87 cells (n=3). **i,** Matrigel boyden chamber assay of U87 glioma cells in the absence or presence of 70 μM Trp without or with 30 μM or 60 μM Kyn (n=3). **j,** Clonogenic survival of LN-18 glioma cells in the absence or presence of 70 μM Trp without or with 30 μM or 60 μM Kyn (n=3). **k,** Representative cranial MRIs, H&E and nestin stainings of *CD1 nu/nu* mice implanted with sh-c (upper panel) or sh-*TDO* (lower panel) U87 glioma cells. The images are representative of two independent experiments (n=6). **l,** Tumor weight of sh-c (white bars) and sh-*TDO* (black bars) U87 glioma cells injected s.c. in the flank of *CD1 nu/nu* mice, that were treated either with anti-asialo GM1 antibody (ASIALO) for NK cell depletion or control IgG (IgG) (n=8).

Figure 3 shows that Kyn activates the AHR. **a,** Connection of the 25 genes that were most strongly induced by Kyn treatment in U87 cells after 8 h to AHR signaling (red: upregulation, green: downregulation). **b,** Translocation of GFP-tagged AHR into the nucleus of mouse hepatoma cells, which do not degrade Trp, after 3 h treatment with 50 μM Kyn, 50 μM Trp or 1 nM TCDD (neg. control: medium). **c,** Ratios of nuclear to cytoplasmic fluorescent intensity in cells with GFP-tagged AHR after 3 h of indicated treatment (neg. control: medium, pos. control: 1 nM TCDD, 50 μM Kyn). The data distribution is represented by box plots, showing the 25th and 75th percentile together with the median, whiskers represent the 10th and 90th

percentile, respectively (P<0.001, one way ANOVA on ranks, followed by Dunns' method). **d,** AHR Western blots of two different nuclear and cytoplasmic fractions each of control (1,2), Kyn-treated (3,4) and TCDD-treated (5,6) human LN-229 glioma cells. **e,** Dioxinresponsive element (DRE) chemical activated luciferase gene expression in U87 glioma cells treated with indicated Kyn concentrations (n=2). **f,** Radioligand binding assay with indicated concentrations of L-3H-Kyn using mouse liver cytosol from *Ahr*-proficient and *Ahr*-deficient mice. Specific binding was calculated by subtracting the radioactivity measured in *Ahr*deficient cytosol from that of *Ahr*-proficient cytosol (n=4) **g,** *CYP1A1* mRNA expression in sh-*AHR* LN-308 glioma cells (black bars) in comparison to controls (sh-c, white bars) treated with 100 μM Kyn, 1 nM TCDD or controls (n=4). **h,** mRNA expression of AHR target genes in sh-*TDO* (black bars) in comparison to sh-c U87 glioma cells (white bars, n=4).

Figure 4 shows that the autocrine and paracrine effects of TDO-derived Kyn are mediated via the AHR **a,** Immunofluorescence stainings of LCA and TIPARP in human glioma sections with low or high TDO expression. Magnification: 400x. **b,** Quantification of LCA+ cells (left) and CD8+ cells (right) stained in human glioma sections with low AHR expression (Histoscore < 150, white bar, n=10 for LCA and n=8 for CD8) and in human glioma sections with high AHR expression (Histoscore ≥ 150, black bar, n=12 for LCA and n=12 for CD8. **c,** Tumor weight measured 15 days after s.c. injection of murine GL261 glioma cells with and without *Tdo* expression in the flanks of *Ahr*-proficient (white bars) or *Ahr*-deficient mice (black bars, n=6). **d,** Quantification of LCA+ immune cells stained in the subcutaneous *Tdo*-proficient and *Tdo*deficient GL261 tumors in *Ahr*-proficient and *Ahr*-deficient mice presented as box plots, showing the 25th and 75th percentile and the median (n=4). **e,** Migration of sh-c LN-308 glioma cells (white bars) and LN-308 glioma cells with knockdown of the *AHR* by two different shRNAs (sh-*AHR*1, gray bars and sh-*AHR*2 black bars) in the presence or absence of 100 μM Kyn (n=4). **f,** Clonogenicity of sh-c (white bars) and sh-*AHR* (black bars) LN-308 glioma cells with or without 100 μM Kyn (n=3). **g,** Growth of *AHR*-proficient (solid circles) and *AHR*-deficient (open circles) human LN-308 glioma cells injected s.c. into the flank of *CD1nu/nu* mice was monitored using metric callipers (n=7). Tumor weight was calculated using the equation: tumor weight (g) = (length (cm) × width (cm)2) × 0.5.

Figure 5 shows that TDO-derived Kyn activates the AHR in diverse human cancers and AHR activation predicts survival in glioma patients **a,** Correlation of TDO expression (red) and AHR expression (brown) in consecutive sections of human glioblastoma tissue. Arrows indicate vessels for orientation. Magnification 40x, insets 200x. **b,** Correlation between TDO and AHR expression in human glioma tissue based on H-scores of TDO and AHR, calculated using Spearman rank correlation (n=26). **c,** Correlation between *TDO* and *CYP1B1* expression in microarray data of human glioblastoma (n=396) analysed by Spearman rank correlation. **d,** Correlation between *TDO* and *CYP1B1* expression in microarray data of human bladder

cancer (left, n=58), human lung cancer (middle, n=122) and human ovarian carcinoma (right, n=91) analysed by Spearman rank correlation. **e,** Survival probabilities of glioma patients (WHO grade II-IV) with high expression (red) of *TDO* or the *AHR* compared to patients with intermediate (blue) or low (green) expression of these genes derived from Rembrandt. For statistical analysis see Supplementary note 21. **f,** Survival probabilities of glioblastoma patients with high expression (red) of the AHR target gene *CYP1B1* compared to patients with low (green) expression of *CYP1B1* derived from the glioblastoma data set of The Cancer Genome Atlas (TCGA) network (n=362). **g,** Synoptical figure highlighting the autocrine and paracrine effects of TDO-derived Kyn on cancer cells and immune cells via the AHR.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for treating and/or preventing natural AHR ligand-dependent cancer comprising administering to a subject suffering from said cancer a therapeutically effective amount of an AHR inhibitor.

The term "treating" as used herein refers to any improvement of the cancer that occurs in a treated subject compared to an untreated subject. Such an improvement can be a prevention of a worsening or progression of the cancer. Moreover, such an improvement may also be an amelioration or cure of the cancer or its accompanying symptoms. It will be understood that a treatment may not be successful for 100% of the subjects to be treated. The term, however, requires that the treatment is successful for a statistically significant portion of the subjects (e.g. a cohort in a cohort study). Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools, e.g., determination of confidence intervals, p-value determination, Student's t-test, Mann-Whitney test etc.. Details are found in Dowdy and Wearden, Statistics for Research, John Wiley & Sons, New York 1983. Preferred confidence intervals are at least 90%, at least 95%, at least 97%, at least 98% or at least 99 %. The p-values are, preferably, 0.05, 0.01, 0.005, or 0.0001.

The term "preventing" as used herein refers to avoiding the onset of cancer as used herein or its accompanying syndromes. It will be understood that prevention refers to avoiding the onset of cancer within a certain time window in the future. Said time window shall, preferably, start upon administration of a compound in the sense of the invention and lasts for at least 1 month, at least 6 months, at least 9 months, at least 1 year, at least 2 years, at least 5 years, at least 10 years or even for the remaining physiological life span of a subject. It will be understood that a prevention may not be successful for 100% of the subjects to be treated. The term, however, requires that the prevention is successful for a statistically significant portion

of the subjects (e.g. a cohort in a cohort study). Whether a portion is statistically significant can be determined without further ado by the person skilled in the art using various well known statistic evaluation tools discussed also elsewhere herein in detail.

The term "natural AHR ligand-dependent cancer" as used herein refers refers to any malignant neoplasm which is dependent on the constitutive activation of AHR elicited by a natural AHR ligand. Preferably, said natural AHR ligand is kynurenin (Kyn). Kynurenin is, preferably, produced by tryptophan degradation as a consequence of increased expression of tryptophan degrading enzymes. More preferably, the cancer according to the invention is, thus, cancer associated with increased tryptophan-2,3-dioxygenase (TDO) activity. TDO activity as referred to herein can be, preferably, assessed by measuring the kynurenin and/or tryptophan concentrations present in a cancer tissue or cancer cells. Moreover, increased TDO activity can also be assessed by determining the amount of TDO enzyme or transcripts encoding said TDO enzyme in a cancer tissue or cancer cells. The amount of TDO enzyme can be determined by antibody-based techniques, such as ELISA, while the amount of transcripts can be determined by nucleic acid hybridization techniques, such as Northern blots, or by nucleic acid amplification techniques, such as RT-PCR. Particular preferred techniques for determining whether there is increased TDO associated with a cancer are described in the accompanying Examples, below, or are disclosed in WO2010/008427, the respective disclosure content of which is herewith incorporated by reference. Preferably, said aforementioned cancer is selected from the group consisting of: brain tumors, preferably, glioma, melanoma, colorectal adenocarcinoma, colon carcinoma, renal cell carcinoma, non-small cell lung cancer (NSCLC), breast cancer, hepatocellular carcinoma, ovarian carcinoma, head and neck carcinoma, bladder cancer, pancreatic adenocarcinoma, mesothelioma, and and small cell lung cancer (SCLC). Alternatively, and also more preferably, the cancer according to the invention is, thus, cancer associated with increased indoleamine-2,3-dioxygenase 1 or 2 (IDO1 or 2) activity. Preferred cancers envisaged in this context are well known in the art; see, e.g., Lob 2009, Nat Rev Cancer 9(6), 445, the respective disclosure content of which is herewith incorporated by reference.

An "AHR inhibitor" in the sense of the invention is a compound capable of inhibiting either directly or indirectly the activity of the Aryl-Hydrocarbon Receptor (AHR) Polypeptide. The AHR polypeptide as referred to in accordance with the present invention is a member of the family of basic-helix-loop-helix transcription factors. It is a cytosolic transcription factor that is normally inactive and present in a complex with several chaperones. Several ligands which can activate or inhibit AHR have been described already, among them artificial or naturally occurring ones. The first ligands to be discovered were synthetic and members of the halogenated aromatic hydrocarbons (polychlorinated dibenzodioxins, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), dibenzofurans and biphenyls) and polycyclic aromatic

hydrocarbons (3-methylcholanthrene, benzo(a)pyrene, benzanthracenes and benzoflavones). Naturally occurring compounds that have been identified as ligands of AHR include derivatives of tryptophan such as kynurenin, indigo and indirubin, tetrapyroles such as bilirubin, arachidonic acid metabolites such as lipoxin A4 and prostaglandin G, modified low-density lipoprotein, several dietary carotinoids, and 7-ketocholesterol. Upon ligand binding, the chaperones dissociate resulting in AHR translocating into the nucleus and dimerizing with ARNT (AHR nuclear translocator). The complex of AHR and ARNT influences gene transcription.

The AHR polypeptide contains several domains critical for function and is classified as a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family of transcription factors. Its bHLH motif is located in the N-terminal of the protein. The members of the bHLH superfamily have two functionally distinctive and highly conserved domains. The first is the basic-region which is involved in the binding of the transcription factor to DNA. The second is the helix-loop-helix (HLH) region that facilitates protein-protein interactions. AHR further comprises two PAS domains, PAS-A and PAS-B, which are stretches of 200-350 amino acids that exhibit a high sequence homology to the protein domains which were found in the Drosophila genes period (Per) and single-minded (Sim). Moreover, similar domains are present in ARNT. The PAS domains support specific secondary interactions with other PAS domain containing proteins, as is the case with and ARNT, so that heterozygous and homozygous protein complexes can form. The ligand binding site of AHR is contained within the PAS-B domain and contains several conserved residues critical for ligand binding. In particular, the amino acids Tyr310, Phe324, His326 and/or Arg352 appear to be involved in ligand binding. Finally, a Q-rich domain is located in the C-terminal region of the protein and is involved in co-activator recruitment and transactivation.

Preferably, the AHR polypeptide is human AHR and, more preferably, human AHR encoded by a polynucleotide as shown under Genbank accession number: NM_001621.4 (GI: 229577137) or has an amino acid sequence as shown under this accession number. Moreover, in accordance with the present invention, variants of the AHR polypeptide referred to before are envisaged. Variants of the aforementioned polynucleotides comprising one or more nucleotide substitutions, deletions and/or additions and, preferably, result in an encoded amino acid having one or more amino acid substitutions, deletions and/or additions, i.e. a polypeptide variant according to the invention. A variant polynucleotide shall, preferably, comprise a nucleic acid sequence being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the specific nucleic acid sequences referred to above. Moreover, a variant polynucleotide may have, preferably, a nucleic acid sequence which encodes an amino acid sequence being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least

80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the amino acid sequences referred to above. The term "identical" as used herein refers to sequence identity characterized by determining the number of identical amino acids between two nucleic acid sequences or amino acid sequences wherein the sequences are aligned so that the highest order match is obtained. It can be calculated using published techniques or methods codified in computer programs such as, for example, BLASTP, BLASTN or FASTA (Altschul 1990, J Mol Biol 215, 403). The percent identity values are, in one aspect, calculated over the entire amino acid sequence or over at least 50% of the nucleotides of the longer sequence. A series of programs based on a variety of algorithms is available to the skilled worker for comparing different sequences. In this context, the algorithms of Needleman and Wunsch or Smith and Waterman give particularly reliable results. To carry out the sequence alignments, the program PileUp (Higgins 1989, CABIOS 5, 151) or the programs Gap and BestFit (Needleman 1970, J Mol Biol 48; 443; Smith 1981, Adv Appl Math 2, 482), which are part of the GCG software packet (Genetics Computer Group 1991, 575 Science Drive, Madison, Wisconsin, USA 53711), may be used. The sequence identity values recited above in percent (%) are to be determined, in another aspect of the invention, using the program GAP over the entire sequence region with the following settings: Gap Weight: 50, Length Weight: 3, Average Match: 10.000 and Average Mismatch: 0.000, which, unless otherwise specified, shall always be used as standard settings for sequence alignments. It will be understood that the aforementioned variants shall still exhibit essentially the same biological activities specified for AHR above.

A compound which directly inhibits the AHR activity is, preferably, a compound which is capable of interacting physically with the AHR polypeptide and, thereby, inhibiting its activity. Such an inhibition may occur if the compound binds to an interaction domain of the AHR or its ligand binding domain and thereby inhibits the biological function of the AHR as specified elsewhere herein. Preferably, the inhibitor blocks the ligand binding domain for kynurenin, i.e. interacts with the ligand binding domain of the PAS-B domain or the ligand binding domain formed by Tyr310, Phe324, His326 and Arg352 (amino acid positions corresponding to human AHR). Alternatively, the compound may elicit an allosteric effect on the AHR polypeptide resulting in an inhibition of the biological function as well. An indirect inhibition can be elicited by a compound which reduces or prevents the transcription and/or translation of AHR polypetides and, thus, the amount of available AHR polypeptides in a cell. The AHR inhibitor shall at least reduce the AHR activity to a statistically significant extent. Of course, preferably, the inhibitor will reduce the AHR activity below the detectable limits. Qualitative and/or quantitative inhibition of AHR activity can be measured by assays well known in the art and, preferably, by those disclosed in the accompanying Examples, below. The activity of AHR can be detected by determining induction of the gene expression of its endogenous target gene CYP1A1 by an ethoxyresorufin-O-deethylase (EROD) assay.

Alternatively, activity of AHR can be detected by using a reporter gene assay wherein the expression of the reporter gene is controlled by a dioxin-responsive element (DRE) dependent promoter. Particular preferred assays for determining AHR activity are disclosed in the accompanying Examples in detail.

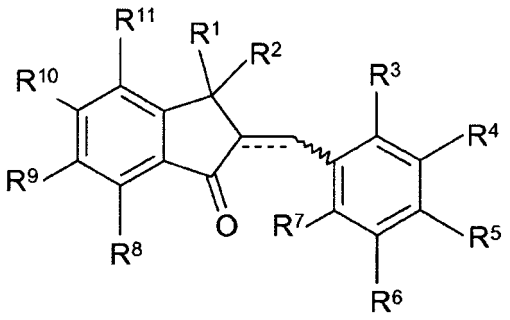
Preferably, an AHR inhibitor is a small molecule compound.

A "small molecule compound" in the sense of the invention is an organic molecule having a molecular weight of less than 10 kDa, less than 5 kDa, less than 2 kDa, less than 1 kDa or less than 500 Da. Preferably, the small molecule is not a polymer. Preferably, a small molecule as referred to in accordance with the present invention is cell-permeable and can diffuse into the cytoplasm in order to bind to the AHR polypeptide. Small molecules as referred to herein can be artificially synthesized and can be comprised in chemical libraries to be screened for potential AHR inhibitors. Alternatively, the small molecules can be obtained from natural sources such as tissues, cells or whole organisms by way of extraction. Suitable sources are, in particular, plants, plant tissue or microorganisms. However, other sources for small molecule inhibitors for AHR can be envisaged as well. For example, 7-ketocholesterol is apparently a competitive inhibitor of AHR in humans (Savouret 2001, J. Biol. Chem. 276 (5): 3054–9).

In a preferred embodiment of the method of the invention, said small molecule compound is a plant compound or derivative thereof.

A "plant compound or derivative thereof" as used herein is a small molecule obtainable by way of extraction from a plant, plant tissue or plant cell. Usually, small molecule plant compounds are metabolites such as primary or, particularly preferred, secondary plant metabolites. In a more preferred embodiment of the method of the invention, said plant compound or derivative thereof is a flavone or a derivative thereof. Most preferably, said flavone or derivative hereof is 3,4-dimethoxyflavone, 3′-methoxy-4′-nitroflavone, 4′,5,7-Trihydroxyflavone (apigenin) or 1-Methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phenyl-1H-pyrazole-5-carboxamide. In another more preferred embodiment of the method of the invention, said plant compound or derivative thereof is resveratrol (trans-3,5,4'-Trihydroxystilbene) or a derivative thereof, epigallocatechin or epigallocatechingallate.

In another preferred embodiment of the method of the invention, wherein said small molecule compound is a compound characterized by the following general formula (I):



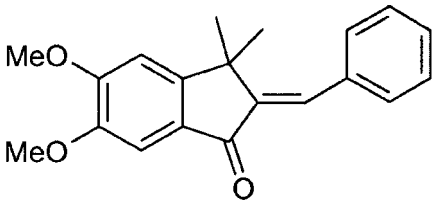
wherein

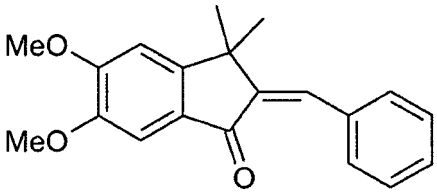
(i) R^1 and R^2 independently of each other are hydrogen or a C_1 to C_{12} alkyl,

(ii) R^3 to R^{11} independently from each other are hydrogen, a C_1 to C_{12} alkyl, hydroxyl or a C_1 to C_{12} alkoxy, and

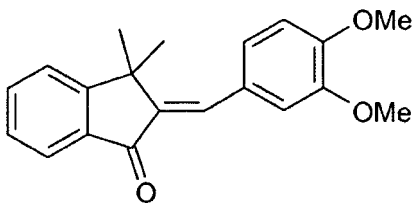
(iii) the broken line represents either a double bond or two hydrogens.

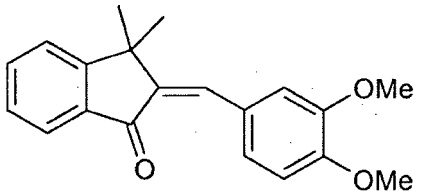
In particular, more preferred is a compound having any of the following formulas (II) to (V):



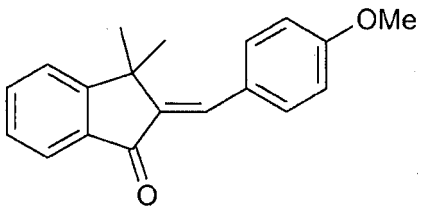


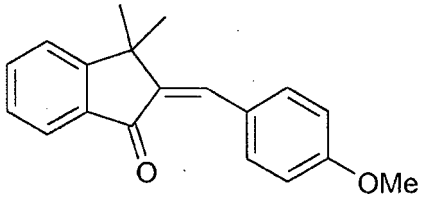
(II)



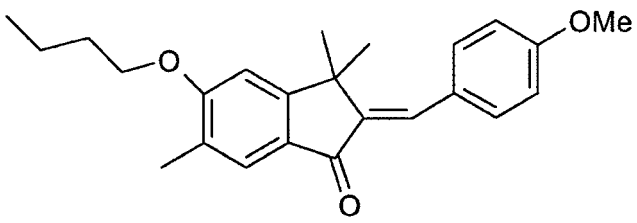


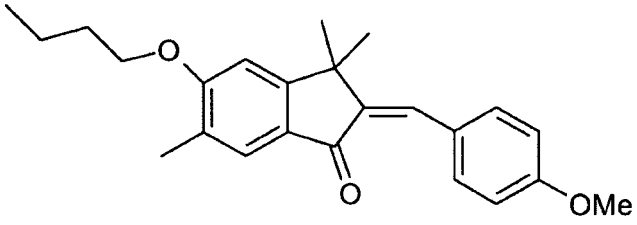
(III)





(IV)





(V)

Further more preferred compounds and methods for the manufacture thereof are disclosed in WO2007/128723, the respective disclosure content of which is herewith incorporated by reference.

In another preferred embodiment of the method of the invention, said AHR inhibitor is an antibody which specifically binds to and inhibits the AHR protein.

The term "antibody" as used in this context refers to all kinds of antibodies which specifically bind to the AHR polypeptide and which inhibit the AHR activity as specified elsewhere herein. Preferably, such an inhibitory antibody of the invention shall specifically bind to an epitope within the AHR polypeptide which is located in the ligand binding domain. Alternatively, an epitope binding of which by the antibody shall inhibit AHR activity may be located in the DNA binding domain of AHR or in domains responsible for interaction with the ARNT polypeptide. Suitable domains are discussed elsewhere herein in detail. Preferably, the antibody of the present invention is a monoclonal antibody, a polyclonal antibody, a single chain antibody, a chimeric antibody or any fragment or derivative of such antibodies. Such fragments and derivatives comprised by the term antibody as used herein encompass a bispecific antibody, a synthetic antibody, an Fab, $F(ab)_2$ Fv or scFv fragment, or a chemically modified derivative of any of these antibodies. Specific binding as used in the context of the antibody of the present invention means that the antibody does not cross react with other polypeptides. Specific binding can be tested by various well known techniques.

Antibodies or fragments thereof, in general, can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. Monoclonal antibodies can be prepared by the techniques which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals and, preferably, immunized mice (Köhler 1975, Nature 256, 495, and Galfré 1981, Meth. Enzymol. 73, 3). Preferably, an immunogenic peptide having the epitope referred to above is applied to a mammal. Depending on the host species, various adjuvants can be used to increase the immunological response. Such adjuvants encompass, preferably, Freund's adjuvant, mineral gels, e.g., aluminum hydroxide, and surface active substances, e.g., lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol.

In another preferred embodiment of the method of the invention, said AHR inhibitor is the AHR repressor protein or an inactive AHR nuclear translocator (ARNT).

The term "AHR repressor (AHRR)" as used herein refers to a putative tumor supressor gene that negatively regulates the activity of AHR and the AHR/ARNT complex. Preferably, a polynucleotide encoding the AHRR polypeptide as well as an amino acid sequence for the AHRR polypeptide itself as referred to herein are shown in Genbank accession number: BC151852 (GI: 156229770). Moreover, an AHRR polypeptide in accordance with the present invention may be a variant of the aforementioned specific polynucleotides or polypeptides. Variants of the aforementioned polynucleotides comprising one or more nucleotide substitutions, deletions and/or additions and, preferably, result in an encoded amino acid having one or more amino acid substitutions, deletions and/or additions, i.e. a polypeptide variant according to the invention. A variant polynucleotide shall, preferably, comprise a nucleic acid sequence being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the specific nucleic acid sequences referred to above. Moreover, a variant polynucleotide may have, preferably, a nucleic acid sequence which encodes an amino acid sequence being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the amino acid sequences referred to above. How the sequence identity between two given sequences can be calculated is disclosed elsewhere herein in detail.

As discussed before, inactive versions of the ARNT polypeptide can be designed by the skilled person without further ado based on the aforementioned specific polynucleotide or amino acid sequences or the variants thereof. Moreover, these inactive ARNT polypeptides or polynucleotides encoding them can be introduced into the cancer cells to be treated by

methods well known in the art. In particular, gene transfer via viral expression systems is envisaged in accordance with the present invention as a delivery system for polynucleotides encoding inactive ARNT. Suitable techniques are well known in the art (see above).

The term "AHR Nuclear Translocator (ARNT)" as used herein refers to a binding protein for the AHR transcription factor. Details are found elsewhere in this specification already. The ARNT polypeptide referred to herein as an inhibitor of AHR is a polypeptide which is still capable of interacting with AHR but which prevents nuclear translocation or which directs the AHR/ARNT complex to the protein degradation machinery of the cell. How such modified inhibitory ARNT polypeptides can be designed is well known to the skilled person. Preferably, a polynucleotide encoding the (unmodified) ARNT polypeptide as referred to herein is shown in Genbank accession number: NM_001197325.1 (GI: 309747070). Preferably, said polynucleotide encodes a polypeptide having an amino acid sequence as shown in Genbank accession number: (protein) NP_001184254.1 (GI: 309747071). Moreover, an ARNT polypeptide in accordance with the present invention may be a variant of the aforementioned specific ARNT polynucleotides or polypeptides. Variants of the aforementioned polynucleotides comprising one or more nucleotide substitutions, deletions and/or additions and, preferably, result in an encoded amino acid having one or more amino acid substitutions, deletions and/or additions, i.e. a polypeptide variant according to the invention. A variant polynucleotide shall, preferably, comprise a nucleic acid sequence being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the specific nucleic acid sequences referred to above. Moreover, a variant polynucleotide may have, preferably, a nucleic acid sequence which encodes an amino acid sequence being at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the amino acid sequences referred to above. How the sequence identity between two given sequences can be calculated is disclosed elsewhere herein in detail.

AHRR polypeptides or polynucleotides encoding them can be introduced into the cancer cells to be treated by methods well known in the art. In particular, gene transfer via viral expression systems is envisaged in accordance with the present invention as a delivery system for polynucleotides encoding inactive ARNT. Suitable techniques are well known in the art and described, e.g. in Gardlík 2005, Med Sci Monit. 11 (4): RA110–21; Salmons 1993, Hum Gene Ther. 4 (2): 129–41.

In another preferred embodiment of the method of the invention, wherein said AHR inhibitor is a nucleic acid inhibitor.

A "nucleic acid inhibitor" as referred to herein is a nucleic acid molecule such as an aptamer which inhibits either the activity of the AHR polypeptide by binding to the polypeptide in a similar manner as described for the antibodies above or to a nucleic acid molecule which due to being complementary to the polynucleotide encoding the AHR polypeptide binds to the said polynucleotide and inhibits transcription or translation thereof. For example, an inhibitory nucleic acid may act as a triple-helix forming oligonucleotide by interfering with proper transcription of the AHR gene. Moreover, an inhibitory nucleic acid may be a ribozyme which specifically binds and degrades the AHR transcripts. Alternatively, it may be an antisense, siRNA or micro RNA capable of binding to the transcript and degrading it or at least inhibiting efficient translation thereof. The latter type of inhibitory nucleic acids is characterized in that they usually comprise a nucleic sequence which is complementary to a sequence in the AHR transcripts. Such a complementary sequence shall be of sufficient length and shall comprise a sufficient number of matching nucleotides as to allow for specific hybridization with the transcript in the cell. Such nucleic acid inhibitors can be expressed in a cancer cell upon delivery by a gene transfer system as referred to elsewhere herein. The inhibitory nucleic acids can be expressed, preferably, under an expression control sequence. Thus, the mediation of RNAi to inhibit expression of the target gene can be modulated byan expression control sequence which can be regulated by a exogenous stimulus, such as the tet operator whose activity can be regulated by tetracycline or heat inducible promoters or under the control of a tumor-specific or tissue-specific promoter. However, the nucleic acid inhibitors can also be delivered by liposome-based delivery systems.

Thus, in another more preferred embodiment of the method of the invention, said nucleic acid inhibitor is selected from the group consisting of: a ribozyme, an antisense molecule, an inhibitory oligonucleotide, an aptamer, a micro RNA, and an siRNA.

A "ribozyme" in accordance with the present invention is a RNA molecule comprising a sequence complementary to the AHR transcript. Moreover, the ribozyme comprises a nucleic acid sequence which is capable of eliciting the hydrolysis of the phosphodiester bonds within the AHR transcript. Ribozymes as referred to in accordance with the preesnt invention can be so-called hammerhead ribozymes, hairpin ribozymes or VS ribozymes. The ribozyme technology is well known in the art and a suitable ribozyme can be designed and applied by the skilled artisan without further ado; see, e.g., Khan 2006, Clin. Chim. Acta 367 (1–2): 20–27; Kalota 2004, Cancer Biology & Therapy 3:1 4-12.

An "antisense molecule" as used herein refers to a therapeutic antisense RNA being complememtary to the AHR transcript or to a morpholino oligonucleotide capable of binding the AHR transcript. The antisense tecnology including the application of morpholino

oligonucleotides is well known in the art; see, e.g., Kalota 2004, Cancer Biology & Therapy 3:1 4-12; Morcos 2007, Biochem Biophys Res Commun 358 (2): 521–7.

Inhibitory oligonucleotides as used herein, preferably, relate to small double stranded DNA molecules which are either capable of binding to specific regions of a target genomic DNA whereby gene silencing is achieved (so-called triple helix forming oligonucleotides) or to oligonucleotides which act as decoys to sequester transcription factor specifically required for the transcription of a target gene These techniques have also been successfully used in vivo and also to some extend resulted already in therapeutics. (see also Kalota 2004, Cancer Biology & Therapy 3:1 4-12.).

The term "aptamer" as used herein refers to nucleic acid aptamers that specifically bind to the AHR polypeptide. A pool of aptamers can be generated by using, e.g., the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technology. The selection step can be made for those aptamers which apecifically bind to the AHR polypeptide. Among the specifically binding aptamers, those which either block ligand binding or those which block interaction domains and are, thus, suitable inhibitors in the sense of the present invention can be identified. The technology for generating aptamers is well established in the art; see, e.g., Tuerk 1990, Science. Aug 3;249(4968):505–510; or Ellington 1990, Nature. Aug 30;346(6287):818-822.

A "microRNA" in the sense of the invention refers to a single stranded RNA molecule which is at least partially complementary to a nucleic acid sequence comprised by the AHR transcript. MicroRNAs have usually a length of about 19 to 26 nucleotides. MicroRNAs are synthesized as a precursor, the so-called pri-microRNA, which comprises a harpin structure and two complementary self-complementary regions forming the stem of the hairpin. One of the self-complementary nucleic acid sequences is the microRNA. The pri-microRNA has a length of about 70 nucleotides and is processed within the target cell into the mature microRNA. The mature microRNA is capable of down-regulating gene expression by either affecting translation or stability of the mRNA to be transcribed upon hybridization thereto. How to design a microRNA and its pri-microRNA precursor is well known to the skilled person. In particular, the self-complementary regions of an endogenous pri-microRNA molecule are replaced by a pair of self-complementary regions comprising one self-complementary region being at least partially complementary to the AHR transcript. The microRNA technology is descried in, e.g., Bartel 2009, Cell 136 (2): 215–33, Trang 2008, Oncogene 27 Suppl 2: S52–7 or Li 2009, The AAPS journal 11 (4): 747–57.

A "short hairpin RNA (shRNA)" as referred to in accordance with the present invention has a similar structure as described for pri-microRNAs above. However, the shRNA is, usually,

shorter in length. More preferably, an shRNA as referred to in accordance with the present invention as an AHR inhibitor is a nucleic acid molecule comprising or essentially consisting of the nucleic acid sequence as shown in any one of SEQ ID NOs: 1 to 4. The design and application of shRNAs is well known in the art and described, e.g., in McIntyre 2006, BMC Biotechnol. 6: 1 or Cao 2005, J Appl Genet. 46 (2): 217–25.

The term "small interfering RNA (siRNA)" refers to a nucleic acid molecule which is a double stranded RNA agent that is complementary and able to base-pair with a portion of an AHR transcript. siRNA acts to specifically guide enzymes in the host cell to cleave the target RNA. By virtue of the specificity of the siRNA sequence and its homology to the RNA target, siRNA is able to cause cleavage of the target RNA strand, thereby inactivating the target RNA molecule. Preferably, the siRNA which is sufficient to mediate RNAi comprises a nucleic acid sequence comprising an inverted repeat fragment of the target gene and the coding region of the gene of interest (or portion thereof). The complementary regions of the siRNA allow sufficient hybridization of the siRNA to the target RNA and thus mediate RNAi. In mammalian cells, siRNAs are approximately 19-25 nucleotides in length. The siRNA sequence needs to be of sufficient length to bring the siRNA and target RNA together through complementary base-pairing interactions. The length of the siRNA is preferably greater than or equal to ten nucleotides and of sufficient length to stably interact with the target RNA; specifically 15-30 nucleotides; more specifically any integer between 15 and 30 nucleotides, most preferably 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, and 30. By sufficient length it is meant an oligonucleotide of greater than or equal to 15 nucleotides that is of a length great enough to provide the intended function under the expected condition. By stable interaction it is meant interaction of the small interfering RNA with target nucleic acid (e.g., by forming hydrogen bonds with complementary nucleotides in the target under physiological conditions). Generally, such a degree of complementary is 100% between the siRNA and the RNA target, but can be less if desired, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%. For example, 19 bases out of 21 bases may be base-paired. In some instances, where selection between various allelic variants is desired, 100% complementary to the target gene is required in order to effectively discern the target sequence from the other allelic sequence. Methods relating to the use of RNAi to silence genes in organisms, including C. elegans, Drosophila, plants, and mammals, are known in the art (see, e.g., Fire 1998, Nature 391:806-811; Fire 1999, Trends Genet. 15, 358-363; WO2001/29058; WO2009/932619).

Finally, contemplated, in general, by the invention is a AHR inhibitor, preferably as defined herein above, for use in treating and/or preventing cancer associated with increased tryptophan-2,3-dioxygenase (TDO) activity.

Cancer-associated immunosuppression by Trp degradation has to date been attributed pivotally to the enzymatic activity of IDO in cancer cells and tumor-draining lymph nodes. Thus, IDO inhibition is currently being evaluated as a therapeutic strategy to treat cancer in clinical trials despite some off-target effects on human cancer cells. In the studies underlying this invention it was shown that TDO is strongly expressed in cancer and equally capable of producing immunosuppressive Kyn. In IDOnegative glioma cells, TDO appears to be the sole determinant of constitutive Trp degradation, indicating that TDO represents a novel therapeutic target in glioma therapy. In fact, an orally available TDO inhibitor has recently been developed. Inhibition of TDO may not only restore antitumor immune responses but also act on the tumor cell intrinsic malignant phenotype as we delineated the importance of constitutive Trp degradation to sustain the malignant phenotype of cancer by acting on the tumor cells themselves. Emerging evidence points towards a tumor-promoting role of the AHR. AHR activation promotes clonogenicity and invasiveness of cancer cells. Transgenic mice with a constitutively active AHR spontaneously develop tumors and the repressor of the AHR (AHRR) is a tumor suppressor gene in multiple human cancers. The aberrant phenotype of *Ahr*-deficient mice points to the existence of endogenous AHR ligands. While different endogenously produced metabolites such as arachidonic acid metabolites, bilirubin, cAMP, tryptamine and 6-formylindolo[3,2-b]carbazole (FICZ) have been shown to be agonists of the AHR, their functionality has not been convincingly demonstrated in a pathophysiological context such as cancer or immune activation. The search for endogenous ligands of the AHR therefore is ongoing.

In accordance with the present invention, these two important pathways contributing to cancer progression by showing that Trp catabolism leads to AHR activation and provide evidence of a pathophysiological human condition that is associated with the production of sufficient amounts of a functionally relevant endogenous AHR ligand. The results of the studies underlying the present invention reveal a differential response of primary immune cells and transformed cancer cells to AHR-mediated signals, which is in line with various toxicological studies using the classical exogenous AHR ligands, TCDD and 3-MC. Exposure to these xenobiotics leads to profound suppression of cellular and humoral immune responses, while also promoting carcinogenesis and inducing tumor growth. These cell-specific differences in AHR effects are likely to depend on the expression of factors differentially regulating AHR signal transduction such as the AHRR as well as cell-specific transcription factor crosstalk shaping the response to AHR activation. It is likely that Kyn-mediated activation of the AHR is not only relevant in the setting of cancer. For instance, activation of the mouse and human AHR by agonistic ligands induces regulatory T cells. Interestingly, *Ahr*-deficient mice suffer from exacerbated CNS autoimmunity in the absence of an exogenous ligand, while Trp catabolites suppress CNS autoimmunity suggesting that activation of Trp catabolism

represents an endogenous feedback loop to restrict inflammation via the AHR. In fact, exogenous Kyn is involved in the regulation of immune cells in mice via the AHR. Kyn concentrations sufficient to activate the AHR are also generated by IDO in response to inflammatory stimuli. In a broader context, a significant number of malignancies arise from areas of mostly chronic infection and inflammation, where Trp catabolism in the tumor microenvironment is activated and sustains local immune suppression. Activation of the AHR by Kyn generated in response to inflammatory stimuli may thus constitute a previously unrecognized pathway connecting inflammation and carcinogenesis.

All references cited in this specification are herewith incorporated by reference with respect to their entire disclosure content and the disclosure content specifically mentioned in this specification.

EXAMPLES

The invention will now be described by the following examples which, however, must not be construed as limiting the scope of the invention.

Example 1: Materials and general methods

Cells and reagents

The origin and culture conditions of cell lines are detailed in the Supplementary Methods. All glioma initiating cells (GIC) were established from freshly resected tumors and used during the first passages. All cells were routinely tested for bacterial contamination. Trp-free RPMI 1640 (Promocell) and dialysed FBS (Invitrogen) were used to cultivate cells under Trp-free conditions. L-Trp and L-Kyn were from Sigma-Aldrich. Interferon-γ (IFN-γ) was from Immunotools (Friesoythe, Germany). TCDD and 3-methylcholanthrene (3-MC) were from Sigma-Aldrich and 3,4-dimethoxylflavone (3,4-DMF) was from Alfa Aesar (Karlsruhe, Germany). The TDO inhibitor ((E)-6-fluoro-3-[2-(3-pyridyl)vinyl]-1H-indole) 680C91 was synthesised by condensation of 6-Fluoroindole-3-carboxaldehyde with pyridine-3-acetic acid in the presence of piperidine.

Mice

C57BL/6N and CD-1 nu/nu mice were purchased from Charles River (Sulzfeld, Germany). *Ahr*-deficient mice (B6.129-*AHRtm1Bra*/J) were kindly provided by Charlotte Esser (Düsseldorf, Germany). C57BL/6N that were age-matched with the *Ahr*-deficient mice were from Harlan Laboratories (Rossdorf, Germany).

TDO expression analysis

TDO expression was analysed by immunohistochemistry in human tumors. Its relevance for Trp degradation was determined using genetic knockdown or overexpression of TDO. Trp and Kyn were measured in cell culture supernatants, human sera and xenograft tissue by HPLC. Mixed leukocyte reactions, chromium release, ELISpot and staining of immune cells in tumor tissues were used to assess the immune effects of TDO activity. Cell cycle analysis, matrigel and spheroid invasion assays, scratch assays, sphere formation assays and clonogenicity assays were employed to analyse the autocrine effects of TDO activity. All animal procedures followed the institutional laboratory animal research guidelines and were approved by the governmental authorities. Orthotopic implantation of human glioma cells with and without stable knockdown of *TDO* into *CD1nu/nu* mice, s.c. injection of these cells into NK-depleted or wildtype *CD1nu/nu* mice and s.c. injection of murine *Tdo*-proficient and

Tdo-deficient GL261 cells into syngeneic C57BL/6N mice were performed to analyse the autocrine and paracrine effects of TDO activity *in vivo*. Microarray analysis of Kyn-treated human glioma cells was performed to identify signalling pathways activated by Kyn. Analysis of AHR translocation, DRE-luciferase assays and radioligand binding assays confirmed activation of the AHR by Kyn. Pharmacological inhibition and stable knockdown of the AHR (*in vitro* and in *vivo*) proved that the effects of Kyn are AHR-dependent. Injection of *do*proficient and *Tdo*-deficient tumor cells into *Ahr*+/+ and *Ahr*-/- mice was used to address the contribution of host effects to TDO-mediated cancer promotion. Finally, stainings, mircoarray data and clinical data of human tumor tissues were used to analyse whether TDO activates the AHR in human cancers and how this affects survival.

Analysis of Trp and Kyn concentrations by high performance liquid chromatography (HPLC)
HPLC analysis was performed using a Beckman HPLC with photodiode array (PDA) detection and Lichrosorb RP-18 column (250 mm x 4 mm ID, 5 µm, Merck, Darmstadt, Germany). Kyn and Trp concentrations were measured in the medium of 3 x 105 cells. Human serum was obtained from 24 glioblastoma patients (10 females, 14 males, median age 54.5 years) and 24 age- and sex-matched healthy controls (10 females, 14 males, median age 53.5 years) after informed consent and analysed for Trp and Kyn concentrations. For measurement of Kyn concentrations in U87 xenografts, the U87 tumors were excised, weighed, immediately frozen in liquid nitrogen and processed.

Quantitative (q)RT-PCR
Total RNA was isolated with the Qiagen RNeasy kit and cDNA was synthesised with the Applied Biosystems reverse transcription kit (Foster City, CA, USA). QRT-PCR was preformed in an ABI 7000 thermal cycler with SYBR Green PCR Mastermix (both Applied Biosystems). All primers were separated by at least one intron on the genomic DNA to exclude amplification of genomic DNA. PCR reactions were checked by including no-RT controls, by omission of templates and by both melting curve and gel analysis. Standard curves were generated for each gene. Relative quantification of gene expression was determined by comparison of threshold values. All results were normalised to GAPDH.

siRNA experiments
To knockdown IDO1 (INDO), IDO2 and TDO (TDO2) SMART-pool siRNA by Dharmacon RNA Technologies (Lafayette, CO, USA) was used. The target sequences were as follows:

Human INDO (Genbank accession number NM_002164):
5'-UCACCAAAUCCACGAUCAUUU-3' (SEQ ID NO: 5);
5'-UUUCAGUGUUCUUCGCAUAUU-3' (SEQ ID NO: 6);
5'-GUAUGAAGGGUUCU GGGAAUU -3' (SEQ ID NO: 7);

5'-GAACGGGACACUUUGCUAAUU-3' (SEQ ID NO: 8)

Human IDO2 (Genbank accession number NM_194294):
5'-CAAACUUCCUCAAUUGAUU-3' (SEQ ID NO: 9);
5'-UUGGAAAGCUAUCACAUAU-3' (SEQ ID NO: 10);
5'-GAGUAUGGCUUUCUUCUUC-3' (SEQ ID NO: 11);
5'-GCACCCAGUUGAAGUUUAA-3' (SEQ ID NO: 12)

Human TDO2 (Genbank accession numberNM_005651):
5'-UCAUAAGGAUUCAGGCUAA-3' (SEQ ID NO: 13);
5'-AGUGAUAGGUACAAGGUAU-3' (SEQ ID NO: 14);
5'-GGAUUUAACUUCUGGGGAA-3' (SEQ ID NO: 15);
5'-GCGAAGAAGACAAAUCACA-3' (SEQ ID NO: 16)

TDOA shRNA sense:
5'-GGAAAGAACTCCAGGTTTATTCAAGAGATAAACCTGGAGTTCTTTCC-3' (SEQ ID NO: 17)

TDOA shRNA antisense:
5'-CCTTTCTTGAGGTCCAAATAAGTTCTCTATTTGGACCTCAAGAAAGG-3' (SEQ ID NO: 18)

TDOB shRNA sense:
5'-TCATAAGGATTCAGGCTAATTCAAGAGATTAGCCTGAATCCTTATGA-3' (SEQ ID NO: 19)

TDOB shRNA antisense:
5'-AGTATTCCTAAGTCCGATTAAGTTCTCTAATCGGACTTAGGAATACT-3' (SEQ ID NO: 20)

ON-TARGETplus siCONTROL Non-targeting Pool (D-001810-10- 05, Dharmacon) and a transfection without siRNA were used as negative controls. Cells were transfected using the lipofectamine RNAiMAX from Invitrogen. Knockdown efficiency was analysed by qRT-PCR.

Stable knockdown cells
U87 human glioma cells were transfected with pSUPER.puro plasmid (OligoEngine, Seattle, WA, USA) expressing sh-*TDO* or scrambled control using FUGENE HD transfection reagent (Roche, Mannheim, Germany). 72 h after transfection medium was exchanged to DMEM

containing 5 µg/ml puromycin (AppliChem GmbH). If not indicated otherwise sh-*TDO*A was used. For knockdown of the AHR in LN308 glioma cells the pSingle-tTS-shRNA vector was purchased from Clontech (CA, USA). Annealed ds oligonucleotides encoding the desired shRNA sequences with XhoI/HindIII overhangs were cloned into the vector using the XhoI/HindIII cloning sites. Short hairpin sequences for control/AHR or TDO shRNA silencing including the XhoI/HindIII overhangs were as follows:

scrambled shRNA antisense oligo:
5'-AGCTTGGATCCAAAAAAGTACTTCCACCTCAGTTGGCTCTCTTGAAGCCAACTG AGGTGGAAGTACC-3' (SEQ ID No: 21),

scrambled shRNA sense oligo:
5'-TCGAGGTACTTCCACCTCAGTTGGCTTCAAGAGAGCCAACTGAGGTGGAAGTA CTTTTTTGGATCCA-3' (SEQ ID NO: 22),

AHR shRNA antisense oligo:
5'-AGCTTGGATCCAAAAAAGCGTTTACCTTCAAACTTTATCTCTTGAATAAAGTTT GAAGGTAAACGCC-3' (SEQ ID NO: 1),

AHR shRNA sense oligo:
5'-TCGAGGCGTTTACCTTCAAACTTTATTCAAGAGATAAAGTTTGAAGGTAAACG CTTTTTTGGATCCA-3' (SEQ ID NO: 2),

AHR shRNA antisense oligo (Dharmacon siRNA #6 of Smart pool of AHRAHR siRNA):
5'-AGCTTGGATCCAAAAAAGGAACTCAAGCTGTATGGTATCTCTTGAATACCATA CAGCTTGAGTTCCC-3' (SEQ ID NO: 3),

AHR shRNA sense oligo (Dharmacon):
5'-TCGAGGGAACTCAAGCTGTATGGTATTCAAGAGATACCATACAGCTTGAGTTC CTTTTTTGGATCCA-3' (SEQ ID NO: 4).

The recombinant vector was transfected into LN-308 and LN-18 glioma cells and clonal transformants were selected with 1 mg/ml neomycin (Sigma-Aldrich). The knockdown was induced using 2 µg/ml doxicyclin (Sigma-Aldrich), cells were analysed 72 h after induction. If not indicated otherwise sh-*AHR*1 was used.

Stable overexpression

GL261 cells were transfected with either pcDNA3.1 (-) (Invitrogen), expressing Tdo cDNA (NM_ 019911) or empty vector using FUGENE HD reagent (Roche). Clonal transformants were selected using 1 mg/ml neomycin (Sigma-Aldrich).

Tissue specimens and immunohistochemistry

Sections cut to 3 μm were incubated and processed with the respective antibody using a Ventana BenchMark XT® immunostainer (Ventana). For quantitative analysis of TDO staining, the percentage of stained tumor cells and intensity of staining were evaluated in representative magnification fields (200x) on tissue sections using optical microscopy (Olympus BX51).

Immunofluorescent stainings

For immunofluorescence, sections of gliomas with low TDO expression and gliomas with high TDO expression were incubated with rabbit anti-TIPARP (1:50) and mouse anti-LCA (1:50) antibodies overnight at 4°C after 30 min of heat-mediated antigen retrieval in Ventana cell conditioner 1. Then, donkey anti-rabbit AlexaFluor 568 (1:500, Invitrogen) and donkey anti-mouse DyLight 488 (1:100, Jackson ImmunoResearch, West Grove, PA, USA) secondary antibodies were applied for 5 h. Micrographs were taken at a Olympus BX-50 microscope (Olympus GmbH, Hamburg, Germany) using the Zeiss Axiocam MRm (Zeiss, Jena, Germany).

Mixed leukocyte reaction (MLR)

Glioma cells were seeded in 96-well plates in RPMI 1640 containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. 24 h after seeding 2 x 105 irradiated (30 Gy) PBMC as stimulators and 2 x 105 PBMC from unrelated donors as responders were added. Six-day MLR were performed and cultures were pulsed with [3H]-methylthymidine (PerkinElmer, Waltham, MA, USA) for the last 18 h. Cells were harvested, and radionuclide uptake was measured by scintillation counting. Experiments were repeated with six unrelated PBMC donors.

Reporter assay

Dual luciferase/renilla assays were performed as detailed in the Supplementary Methods. For analysis of DRE-driven reporter gene activity in response to various Trp metabolites, the pGL3-promotor expression plasmid and the control plasmid pRL-SV40, expressing renilla luciferase were used (Promega, Heidelberg, Germany).

Invasion assays

In matrigel assays cell invasion was evaluated by counting the number of cells that had migrated across the membrane in 5 independent microscopic high-power fields and expressed

as percentage of invasiveness relative to control using a microgrid. In spheroid assays microscopic photographs of the area covered by each spheroid were taken at 0, 24, 48 and 72 h after implantation. For quantification, the mean area which was covered by invaded glioma cells at an indicated time point was measured in intervals of 24 h and compared with the area at 0 h with ImageJ.

Chromium release assay

Inhibition of immune cell cytotoxicity was assessed using the standardised 51chromium release assay (Supplementary Methods). Specific lysis in percent was calculated as follows: [Experimental 51Cr Release - Minimum Release] / [Maximum Release- Minimum Release] x 100. This experiment was performed with at least 4 different PBMC donors.

Enzyme Linked Immuno Spot Technique assay (ELISpot)

Dendritic cells (DC) were isolated from the bone marrow of healthy C57BL/6N mice and cultured in RPMI 1640 containing 20 ng/ml GM-CSF (Immunotools) for 5 days. Spleens from tumor-bearing mice were removed and mashed through a 40-µm cell strainer. Erythrocytes were lysed and T cells were isolated by MACS using the pan T cell isolation kit II (Miltenyi GmbH). 2 x 105 DC were seeded in an ELISpot plate (Millipore) that had been coated with anti-IFNγ antibody (Mabtech AB, Nacka Strand, Sweden) and pulsed with 10 µg GL261 lysate - generated in PBS by repeated freeze thaw cycles - for 4 h before addition of 1 x 105 T cells. After 36 h, IFNγ-producing T cells were detected with biotinylated anti-IFNγ antibody, streptavidin-ALP and BCIP/NBTPLUS (Mabtech) and quantified using an ImmunoSpot Analyzer (Cellular Technology Limited, Shaker Heights, OH, USA).

Detection of AHR translocation

For detection of AHR translocation 7000 Tao BpRc1c cells with a GFP-tagged AHR per well were exposed to 50 µM Kyn or 50 µM Trp, fixed in 3.7% formaldehyde in PBS, permeabilised in 0.1% Triton X100, incubated with 1 µg/ml Hoechst 33342 (Invitrogen) and imaged on a BD Pathway™ Imager 855 in a non-confocal mode using a 20x U-Apo 340 objective (Olympus, NA 0.75). Further analysis of fluorescence intensity was performed using the Attovision software (BD Biosciences). In addition, the AHR protein content in the nuclear and the cytoplasmic fractions of LN-229 glioma cells was compared by immunoblotting.

Radioligand binding assay with 3H-labelled Kyn

L-3H-Kyn with a specific activity of 11 Ci/mmol was obtained from Quotient Bioresearch (Radiochemicals) Ltd. (Cardiff, UK). The binding assays with L-3H-Kyn using mouse liver cytosol from *Ahr*-proficient and *Ahr*-deficient mice were performed. Specific binding was defined as the difference of radioactivity between *Ahr*-proficient and *Ahr*-deficient cytosol.

Animal experiments

All animal procedures followed the institutional laboratory animal research guidelines and were approved by the governmental authorities. Human glioma cells were either injected s.c. or stereotactically implanted into the right striatum of six 6-12-week-old athymic mice (*CD1nu/nu*) and monitored. NK cell depletion was performed by biweekly i.p. injection of rabbit anti-asialo GM1 antibody (Wako Chemicals, Duesseldorf, Germany) starting 2 days before tumor cell injection. Controls were injected with rabbit IgG (Calbiochem, Darmstadt, Germany). For induction of AHR knockdown in vivo doxycycline was administered to the mice at a concentration of 2 mg/ml in ucrosecontaining drinking water. Murine glioma cells were injected s.c. into the right flank of 6-12- week-old wildtype C57BL6/N mice or AHR-/- C57/Bl6 mice.

Magnetic resonance imaging (MRI)

MRI scans shown in Fig.3g were performed using a custom-developed transmit/receive small animal coil in a conventional whole-body 1.5 T MRI scanner (Symphony, Siemens, Erlangen, Germany).

Microarray

U87 glioma cells were treated with 100 μM Kyn for 8 h or 24 h, after which the cells were harvested and RNA isolated using the RNAeasy-Kit (Qiagen). The RNA was subjected to microarray analyses as detailed in the Supplementary Methods. For each of the four treatments (8 h, 24 h, Kyn-treated, untreated) two arrays were hybridised and the mean log2 ratios of gene expression in Kyn-treated vs. untreated samples were calculated. Further analyses of the data are detailed in the Supplementary Methods. For clinical samples microarray and clinical data were acquired from the REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT) (Supplementary Methods). Survival analysis within the data set of untreated primary glioblastoma (n=362) of The Cancer Genome Atlas (TCGA) network was performed using the Kaplan-Meier-analysis module of the R2 microarray analysis and visualisation platform (http://r2.amc.nl).

Statistical analysis

Data are expressed as mean ± s.e.m. Analysis of significance was performed using the Student's t-test (SigmaPlot). P values < 0.05 were considered significant. Correlation of Ki67 and TDO was analysed by Spearman rank correlation (SPSS, IBM, Somers, NY, USA). Correlations between TDO and AHR (Fig. 6c) as well as *TDO* and *CYP1B1* (Fig. 6d) were analysed by Spearman rank correlation (Sigmaplot). Nuclear fluorescence intensity was analysed using One-Way ANOVA on ranks ($p=<0.001$) followed by Dunn's Method ($p<0.05$).

Example 2: Autocrine and paracrine effects of Kyn generated by TDO

In accordance with the studies underlying the present invention, a screen of human cancer cell lines revealed constitutive degradation of Trp and release of high micromolar amounts of Kyn in brain tumor cells, namely glioma cell lines and gliomainitiating cells (GIC), but not human astrocytes (Fig. 1a). Surprisingly, IDO1 and IDO2 did not account for the constitutive Trp catabolism in brain tumors. Instead, tryptophan-2,3-dioxygenase (TDO), which is predominantly expressed in the liver and believed to regulate systemic Trp concentrations, was strongly expressed in human glioma cells and correlated with Kyn release (Fig. 1b). Pharmacological inhibition or knockdown of *TDO* blocked Kyn release by glioma cells, while knockdown of *IDO1* and *IDO2* had no effect (Fig. 1c,d), thus confirming that TDO is the central Trp-degrading enzyme in human glioma cells. In human brain tumor specimens TDO protein levels increased with malignancy and correlated with the proliferation index (Fig. 1e-g). As described previously (Miller 2004, Neurobiol Dis 15(3), 618, healthy human brain showed only weak TDO staining in neurons (Fig. 1e). TDO expression was not confined to gliomas but was also detected in other types of cancers referred to elsewhere herein including hepatocellular carcinoma, colon carcinoma, breast cancer, NSCLC, ovarian carcinoma, malignant melanoma (brain metastatses), and renal cell carcinoma.

Reduced Trp concentrations were measured in the sera of glioma patients (Fig. 1h). These may not have translated into increased Kyn levels (Fig. 1h), because Kyn is taken up by other cells and metabolized to quinolinic acid. Indeed, accumulation of quinolinic acid was detected in TDO-expressing glioblastoma tissue (Fig. 1i). Kyn suppresses allogeneic T cell proliferation9. Allogeneic T cell proliferation inversely correlated with Kyn formation by glioma-derived TDO (Fig. 2a). Knockdown of *TDO* in glioma cells (Supplementary Fig. 4c,d; Supplementary Note 9) restored allogeneic T cell proliferation, while addition of Kyn to the *TDO* knockdown cells prevented the restoration of T cell proliferation (Fig. 2b). Kyn concentration-dependently inhibited the proliferation of T cell receptor-stimulated CD4+ and CD8+ T cells (Supplementary Fig. 4e). In addition, knockdown of *TDO* resulted in enhanced lysis of glioma cells by alloreactive PBMC (Supplementary Fig. 4f). Finally, decreased infiltration with leukocyte common antigen (LCA) positive and CD8+ immune cells was observed in sections of human glioma with high TDO expression in comparison to those with low TDO expression (Fig. 2c), indicating that Kyn formation by TDO may suppress antitumor immune responses. *In vivo* experiments in immunocompetent mice demonstrated that tumors expressing TDO grew faster and displayed a higher proliferation index than TDO-deficient control tumors (Fig 2d). TDO activity in tumors suppressed antitumor immune responses *in vivo* as evidenced by reduced interferon-γ (IFN-γ) release by tumor-specific T cells and tumor

cell lysis by spleen cells of mice bearing TDO-expressing tumors in comparison with mice bearing TDO-deficient tumors (Fig. 2e,f).

Next the autocrine effects of Kyn on glioma cells were assessed. While no differences in cell cycle progression were detected between controls and glioma cells with *TDO* knockdown, knockdown of *TDO* reduced motility and clonogenic survival (Fig. 2g,h). This was mediated by Kyn as exogenous addition of Kyn restored motility and clonogenic survival in the absence of Trp (Fig. 2i,j), suggesting that Kyn increases the motility of malignant glioma cells. In GIC sphere formation was enhanced in response to Kyn. Finally, tumor formation was impaired when *TDO* knockdown tumors were orthotopically implanted in the brains of nude mice, which are devoid of functional T cells (Fig. 2k).

Example 3: TDO mediated inhibition of N cells

To analyse whether TDO-mediated inhibition of antitumor NK cell responses, which are functional in nude mice, may account for impaired formation of *TDO* knockdown tumors, subcutaneous tumor growth was compared in the presence or absence of NK cells. NK cell depletion enhanced the growth of both control and *TDO* knockout tumors but did not restore the growth of *TDO* knockout tumors to that of controls (Fig. 2l), suggesting that Kyn generated by constitutive TDO activity enhances the malignant phenotype of human gliomas in an autocrine manner in the absence of functional antitumor T cell and NK cell responses.

Example 4: Molecular mechanism of Kyn activity via AHR

To better understand the molecular mechanisms underlying the autocrine effects of Kyn on glioma cells, microarray analyses of Kyn-treated glioma cells was performed revealing broad induction of AHR response genes by Kyn (Fig. 3a). Pathway analyses showed that the 25 genes that were most strongly induced by Kyn treatment in U87 cells at 8 h and at 24 h were all directly or indirectly regulated by the AHR (Fig. 3a).

Malignant glioma cell lines as well as GIC express the AHR constitutively and upregulation of AHR target genes by Kyn was confirmed in two different glioma cell lines. Kyn led to translocation of the AHR into the nucleus after 1 h, thus showing an immediate effect of Kyn on the AHR (Fig. 3b,c). In accordance, Western blot analyses of Kyn-activated tumor cells showed reduced cytoplasmic localisation paralleled by increased nuclear accumulation of the AHR comparable to that induced by TCDD (Fig. 3d).

Kyn concentration-dependently induced DRE-luciferase activity in glioma cells with an EC50 of 36.6 μM (Fig. 3e). AHR activation was unique to Kyn in a panel of Trp catabolites. An ethoxyresorufin-O-deethylase (EROD) assay confirmed the induction of the functional AHR target gene cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) with an EC50 of 12.3 μM for Kyn. Radioligand binding assays using mouse liver cytosol from *Ahr*-proficient and *Ahr*deficient mice demonstrated that Kyn binds to the AHR with a KD (app) of ≈ 4 μM (Fig. 3f). Activation of the AHR and upregulation of AHR-regulated gene expression in response to Kyn were inhibited by the AHR antagonist 3,4-DMF or knockdown of the *AHR* (Fig. 3g), indicating that Kyn is a specific agonist of the AHR. The involvement of the same or similar AHR residues in the binding to Kyn, TCDD and 3-methylcholanthrene (3-MC) was confirmed by the fact that 3,4-DMF inhibited the activation of the AHR by all three ligands. Importantly, the endogenous Kyn production of glioma cells was sufficient to activate the AHR, as knockdown of *TDO* decreased the expression of AHR regulated genes (Fig. 3h). As mean Kyn concentrations of 37.01 +/- 13.4 μM were measured in U87 xenografts (n=6), sufficient Kyn concentrations to activate the AHR were also reached *in vivo*. In accordance with an activation of the AHR by TDO-derived Kyn, expression of the AHR target gene TIPARP in LCA+ immune cells was observed only in human glioma sections expressing TDO (Fig. 4a).

To determine whether TDO influences antitumor immune responses *via* the AHR the infiltration of immune cells in human glioma sections in relation to their AHR expression was analysed. Indeed, infiltration by LCA+ and CD8+ immune cells was decreased in sections of human gliomas with high AHR expression compared to those with low AHR expression (Fig. 4b). To analyse the contribution of host AHR expression to tumor growth, the growth of murine tumors with and without *Tdo* expression in *Ahr*-deficient and *Ahr*-proficient mice was compared. The growth of *Tdo*-expressing tumors was attenuated in *Ahr*-deficient mice when compared with *Ahr*-proficient mice (Fig. 4c) indicating that AHRmediated host effects enhance tumor growth. Staining of LCA+ immune cells in the tumors revealed that expression of TDO reduced the infiltration with LCA+ immune cells in *Ahr*proficient mice, but not in *Ahr*-deficient mice (Fig. 4d), suggesting that TDO-mediated suppression of anti-tumor immune responses *via* the AHR contributes to the host effects enhancing the growth of *Tdo*-expressing tumors. In addition, while in *Ahr*proficient mice *Tdo* expression strongly enhanced tumor growth in comparison to tumors not expressing *Tdo*, the same effect was observed in *Ahr*-deficient mice, albeit to a much lower extent (Fig. 4c). As murine glioma cells express functional AHR, these results suggest that the increase in tumor growth mediated by TDO in *Ahr*-deficient mice is due to autocrine effects of TDO on the tumor cells themselves. This notion is supported by the fact that Kyn failed to induce motility of human glioma cells after *AHR* knockdown (Fig. 4e). Also, the increase in clonogenic survival in response to Kyn was abolished in glioma cells with a knockdown of the *AHR* (Fig. 4f). Finally, *in vivo* experiments

demonstrated that induced knockdown of the *AHR* in human glioma cells inhibited tumor growth in immunocompromised mice (Fig. 4g), underscoring the importance of AHR signaling for the autocrine effects of Trp degradation.

Next it was investigated whether TDO-derived Kyn activates the AHR in human brain tumor tissue. Indeed, TDO expression correlated with the expression of the AHR and AHR target genes in human glioma tissue (Fig. 5a,b,c), indicating that constitutive TDO expression in glioma cells produced sufficient Kyn levels to activate the AHR. To address whether the TDO-Kyn-AHR signalling pathway is also activated in cancers other than glioma, we analysed microarray data of diverse human tumor entities. Interestingly, *TDO* expression correlated with the expression of the AHR target gene *CYP1B1* not only in glioma (Fig. 5c), but also in B -cell lymphoma, Ewing sarcoma, bladder carcinoma, cervix carcinoma, colorectal carcinoma, lung carcinoma and ovarian carcinoma (Fig. 5d). This finding indicates that the TDO-Kyn-AHR pathway is not confined to brain tumors but appears to be a common trait of cancers. Analysis of the Rembrandt database revealed that the overall survival of glioma patients (WHO grade II-IV) with high expression of *TDO*, the *AHR* or the AHR target gene *CYP1B1* was reduced compared to patients with intermediate or low expression of these genes (Fig. 5e). Finally, in patients with glioblastoma (WHO grade IV)14, the expression of the AHR targets *CYP1B1*, *IL1B*, *IL6* and *IL8*, which are regulated by TDO-derived Kyn in glioma cells (Fig. 3h), were found to predict survival even independent of WHO grade (Fig. 5f), thus further underscoring the importance of AHR activation for the malignant phenotype of gliomas. In summary these data suggest that endogenous tumor-derived Kyn activates the AHR in an autocrine/paracrine fashion to promote tumor progression (Fig. 5g).

Claims

1. A method for treating and/or preventing a natural AHR ligand-dependent cancer comprising administering to a subject suffering from said cancer a therapeutically effective amount of an AHR inhibitor.

2. The method of claim 1, wherein said cancer is selected from the group consisting of: brain tumors, preferably, glioma, melanoma, colorectal adenocarcinoma, colon carcinoma, renal cell carcinoma, non-small cell lung cancer (NSCLC), breast cancer, hepatocellular carcinoma, ovarian carcinoma, head and neck carcinoma, bladder cancer, pancreatic adenocarcinoma, mesothelioma, and and small cell lung cancer (SCLC).

3. The method of claim 1, wherein said AHR inhibitor is a small molecule compound.

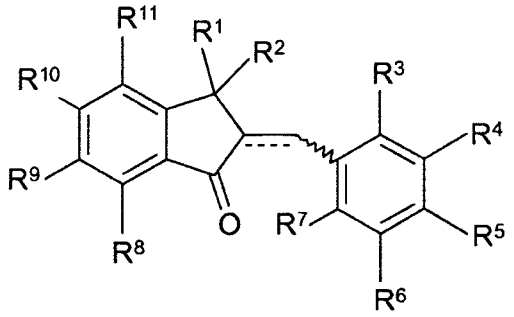
4. The method of claim 3, wherein said small molecule compound is a plant compound or derivative thereof.

5. The method of claim 4, wherein said plant compound or derivative thereof is a flavone or derivative thereof.

6. The method of claim 4, wherein said flavone or derivative thereof is 3,4-dimethoxyflavone, 3′-methoxy-4′-nitroflavone, 4′,5,7-Trihydroxyflavone (apigenin) or 1-Methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazenyl]phen yl-1H-pyrazole-5-carboxamide.

7. The method of claim 4, wherein said plant compound or derivative thereof is reveratrol or a derivative thereof, epigallocatechin or epigallocatechingallate.

8. The method of claim 3, wherein said small molecule compound is a compound characterized by the following general formula (I):



wherein

(i) R^1 and R^2 independently of each other are hydrogen or a C_1 to C_{12} alkyl,

(ii) R^3 to R^{11} independently from each other are hydrogen, a C_1 to C_{12} alkyl, hydroxyl or a C_1 to C_{12} alkoxy, and

(iii) the broken line represents either a double bond or two hydrogens.

9. The method of claim 1, wherein said AHR inhibitor is an antibody which specifically binds to and inhibits the AHR protein.

10. The method of claim 1, wherein said AHR inhibitor is the AHR repressor protein or an inactive AHR nuclear translocator (ARNT).

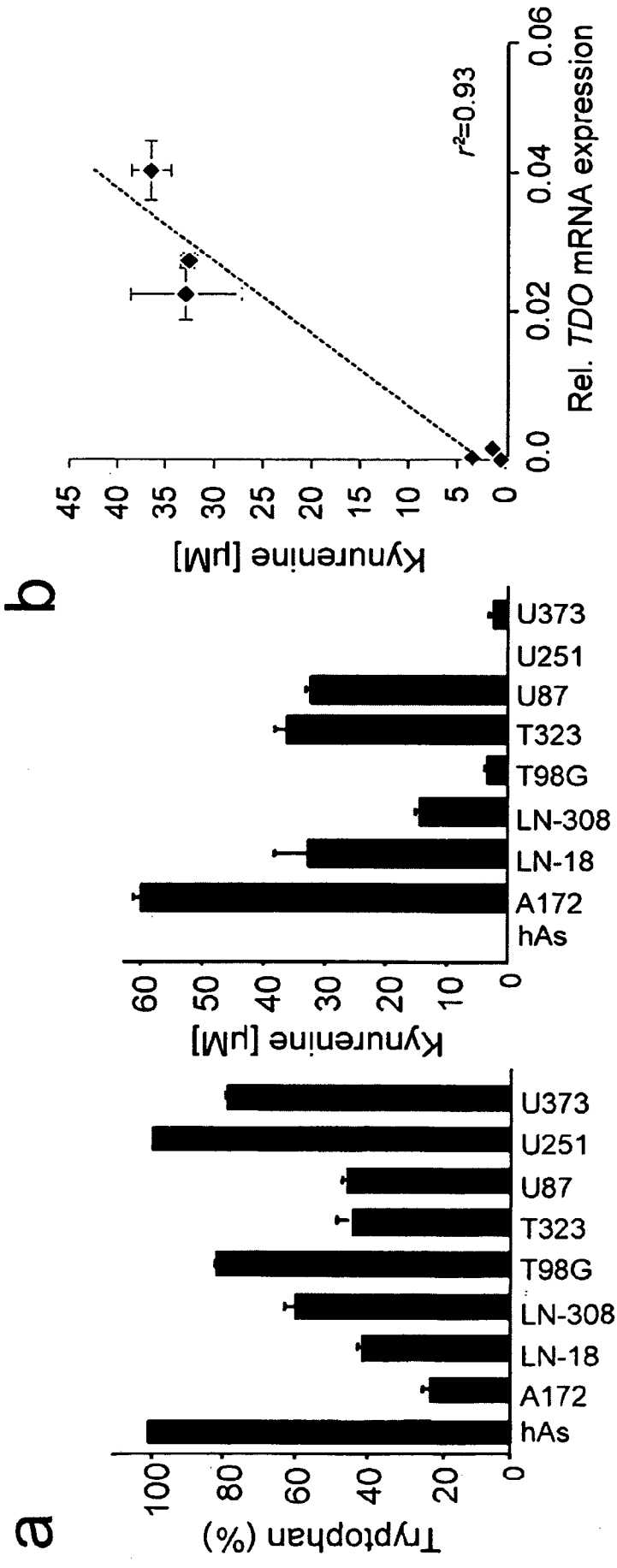
11. The method of claim 1, wherein said AHR inhibitor is a nucleic acid inhibitor.

12. The method of claim 11, wherein said nucleic acid repressor specifically binds to an AHR encoding polynucleotide and is selected from the group consisting of: a ribozyme, an antisense molecule, an inhibitors oligonucleotide, an aptamer, a micro RNA, and an siRNA.

13. An AHR inhibitor for use in treating and/or preventing a natural AHR ligand-dependent cancer.

14. The AHR inhibitor of claim 13, wherein said AHR inhibitor is an AHR inhibitor as defined in any one of claims 3 to 12.

15. The AHR inhibitor of claim 13, wherein said cancer is a cancer as defined in claim 2.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Kynurenine [µM]
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5
10
15
20
25
30
35
40
45
Rel. TDO mRNA expression
0.0
0.02
0.04
0.06
r²=0.93
a
Tryptophan (%)
0
20
40
60
80
100
Kynurenine [µM]
0
10
20
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40
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60
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U87
T323
T98G
LN-308
LN-18
A172
hAs

Fig. 1

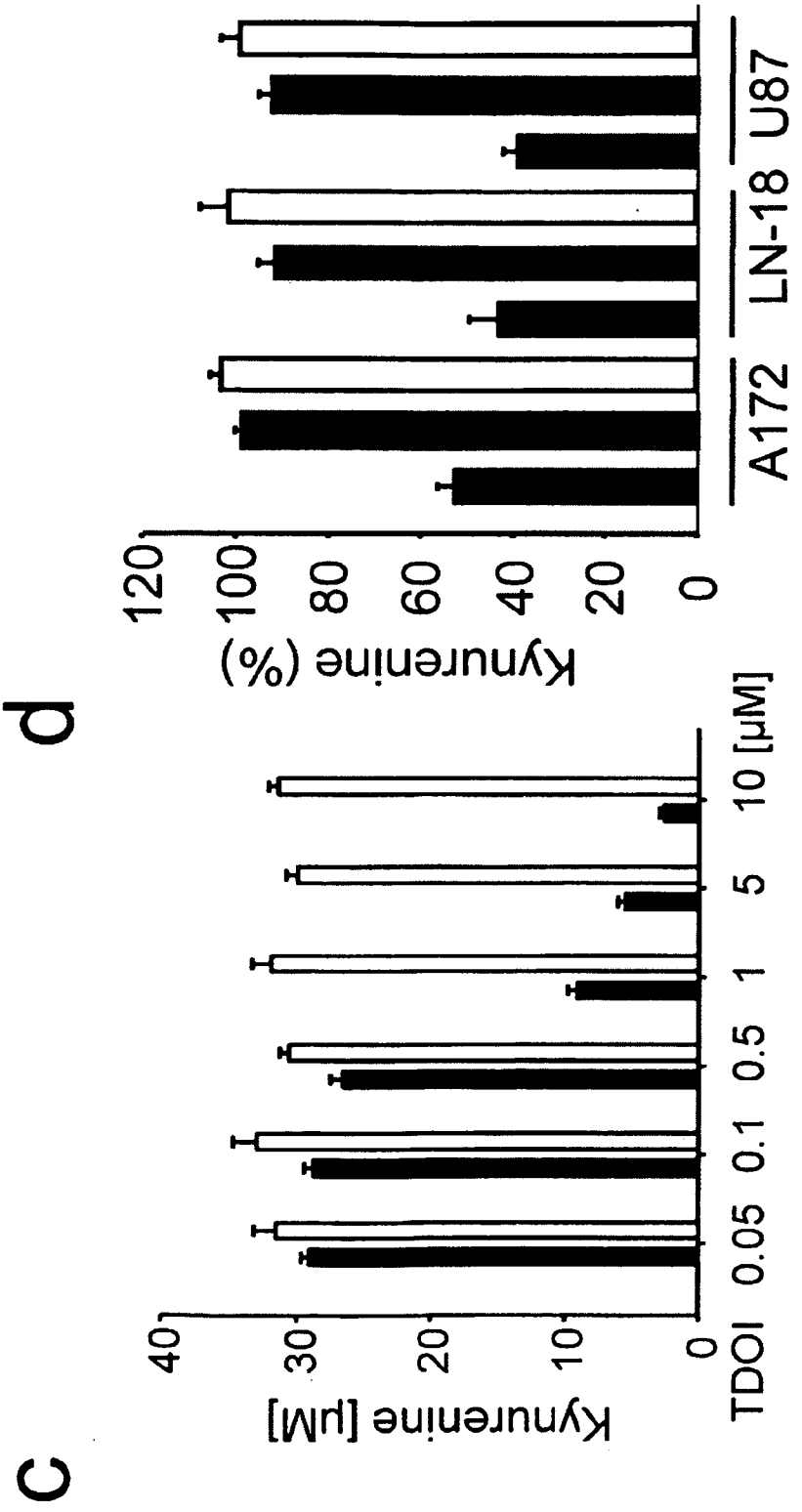
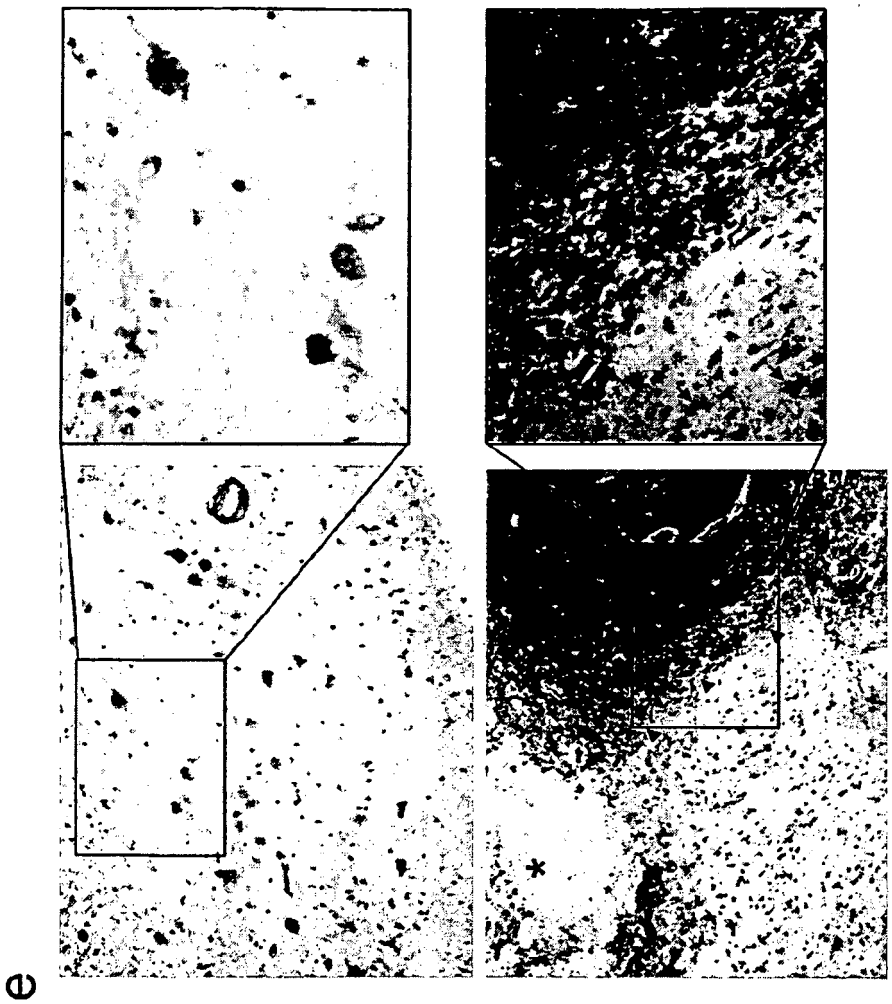


Fig. 1 (continued)



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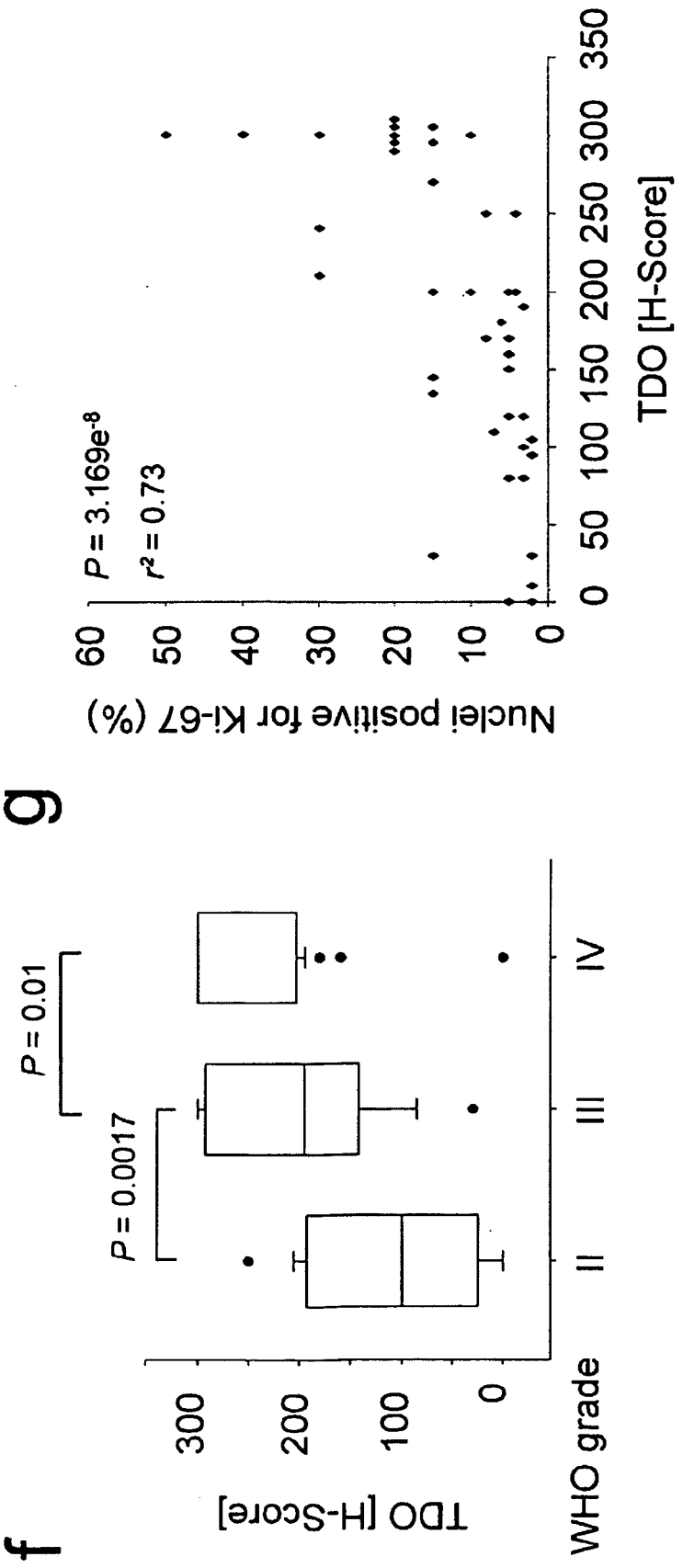

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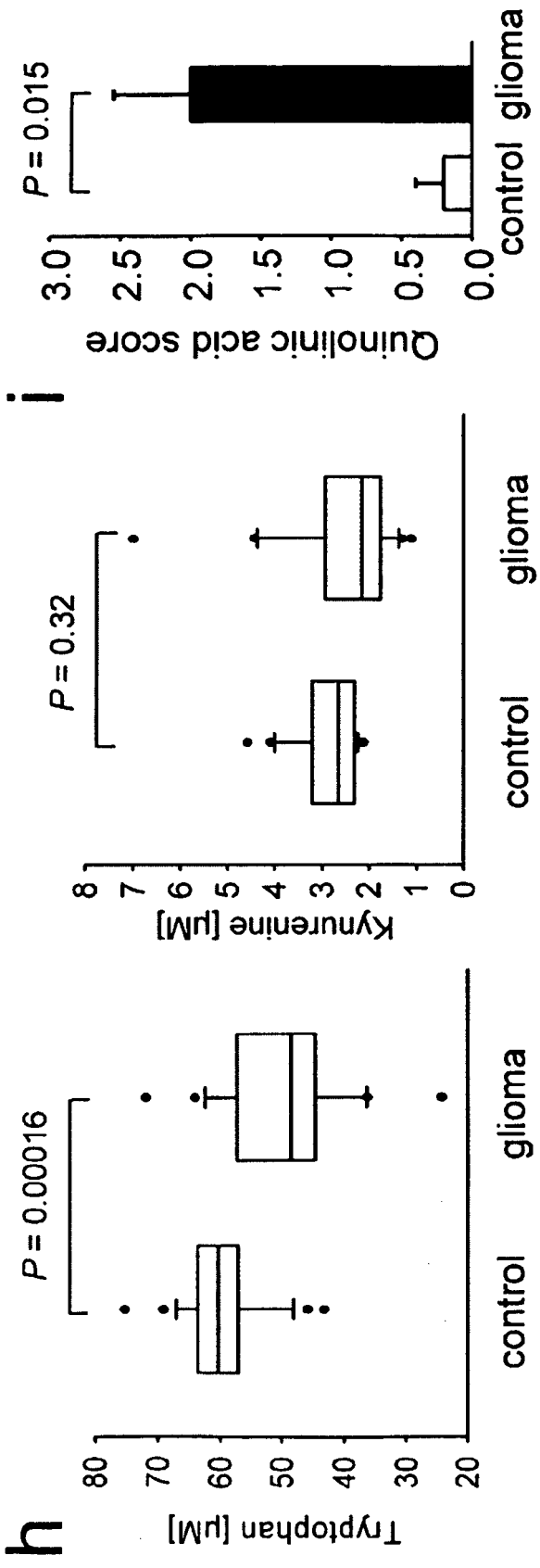

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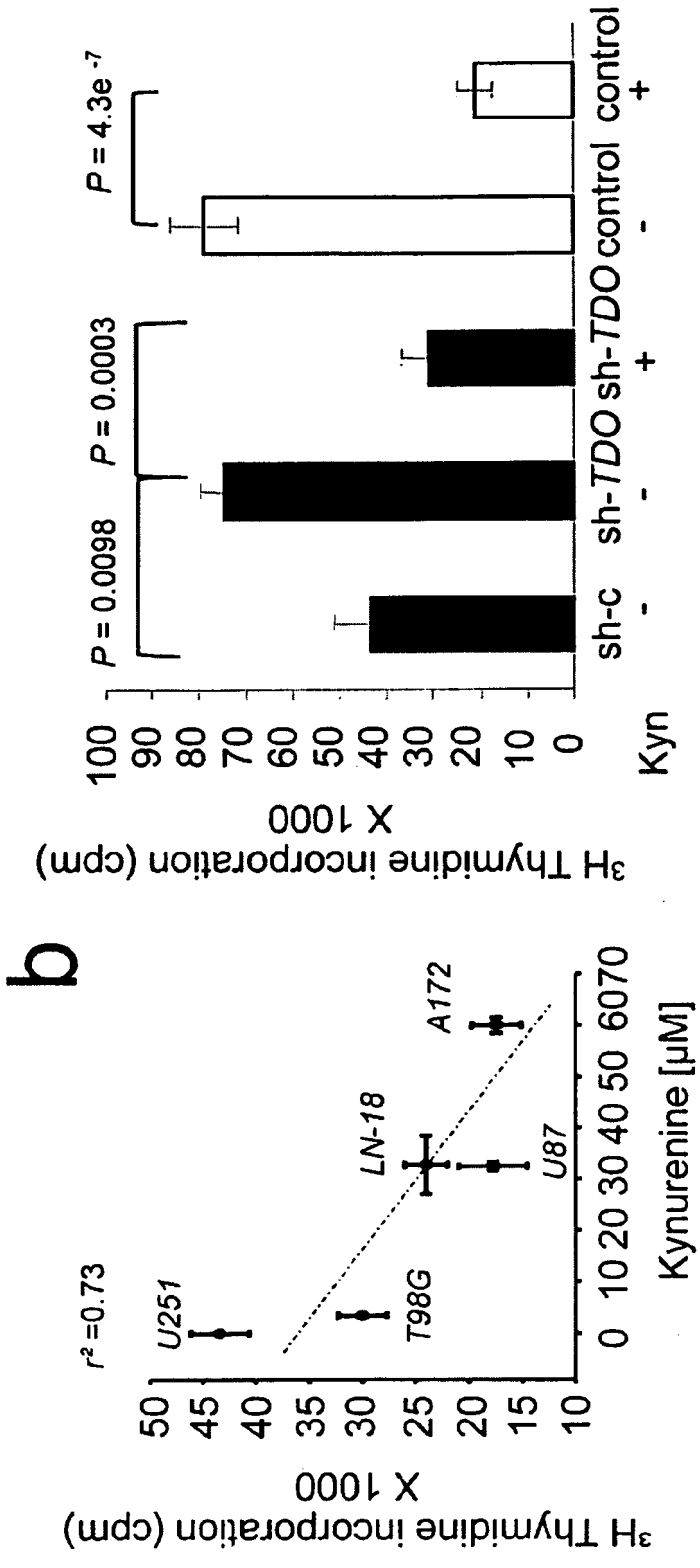
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3H Thymidine incorporation (cpm)
X 1000
100
90
80
70
60
50
40
30
20
10
0
P = 0.0098
P = 0.0003
P = 4.3e -7
sh-c
sh-TDO
sh-TDO
control
control
-
-
+
-
+
Kyn
a
3H Thymidine incorporation (cpm)
X 1000
50
45
40
35
30
25
20
15
10
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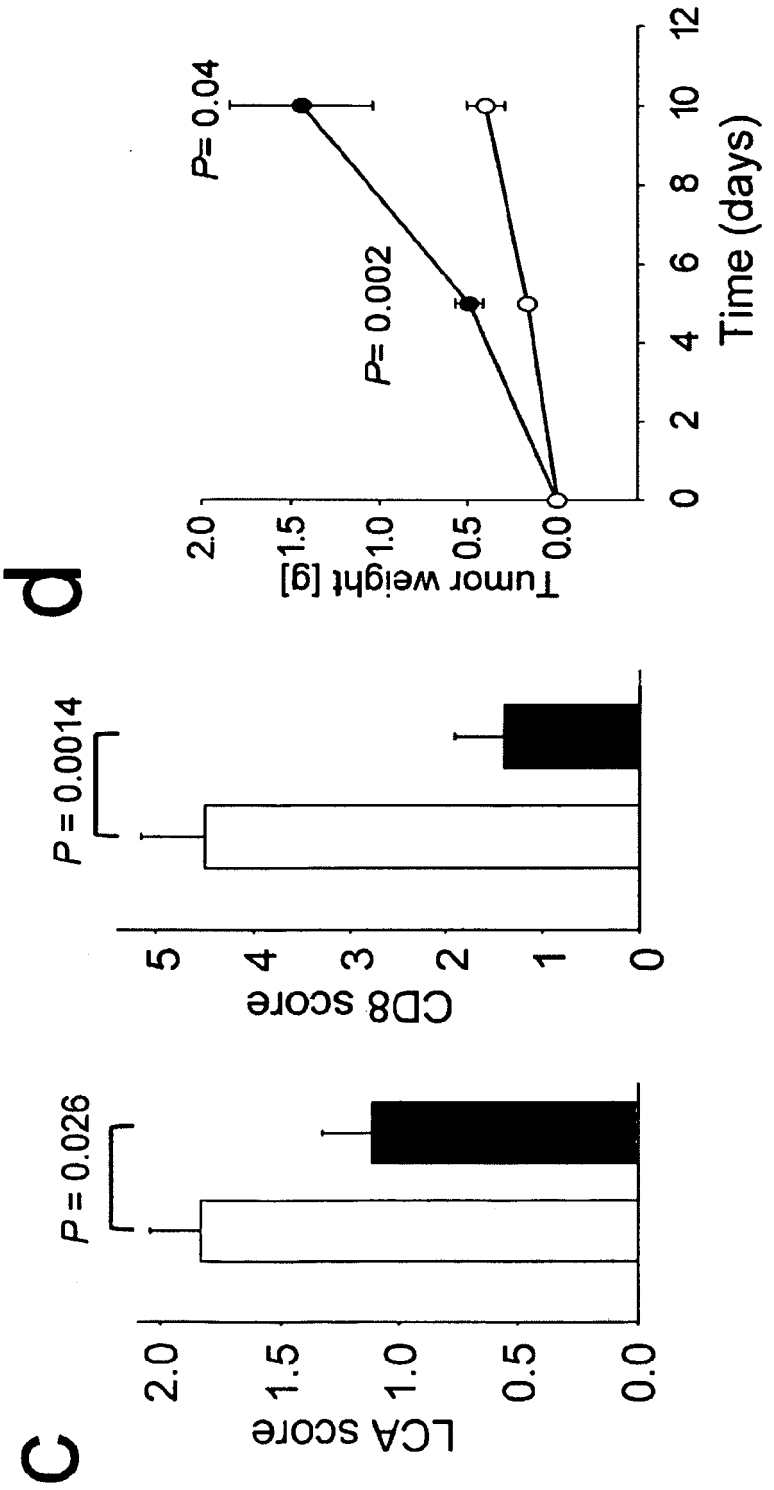
U251
T98G
LN-18
U87
A172
0 10 20 30 40 50 60 70
Kynurenine [μM]

Fig. 2



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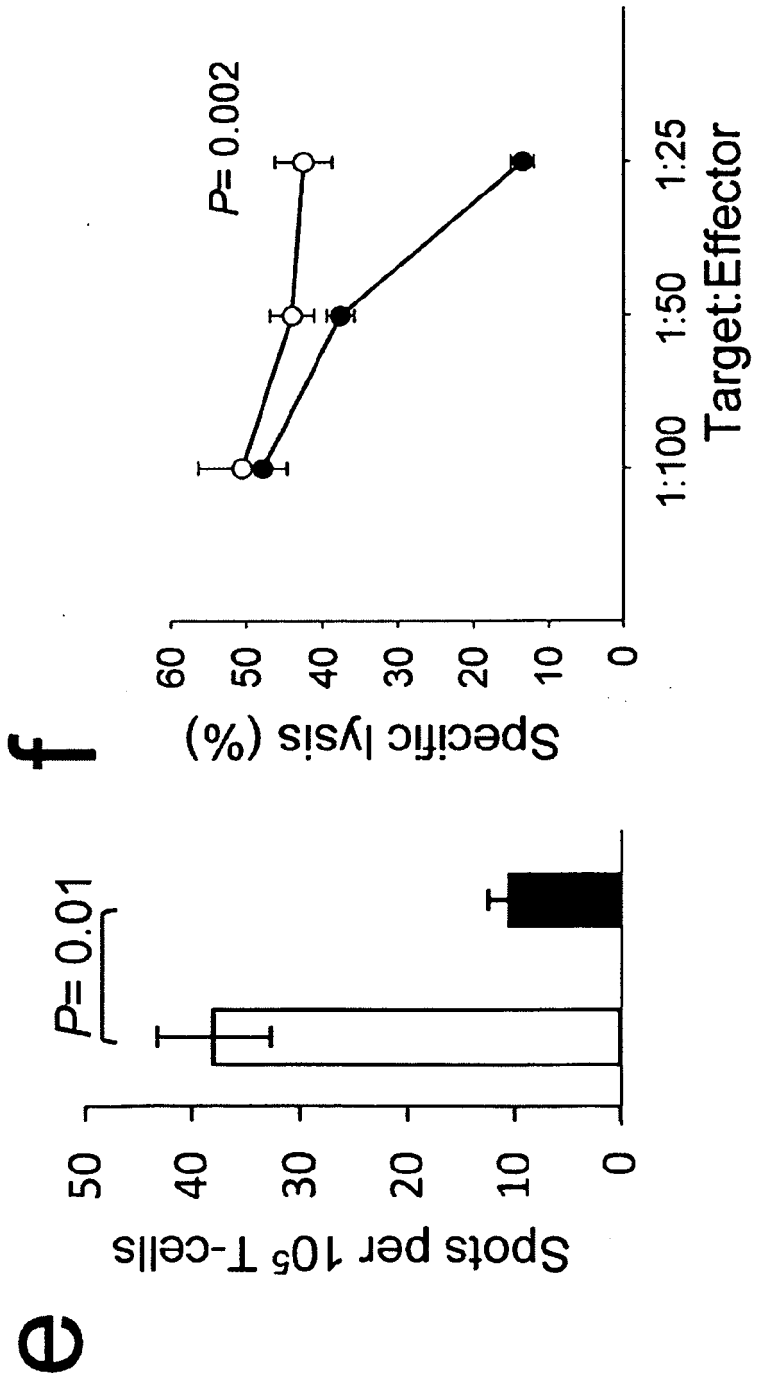

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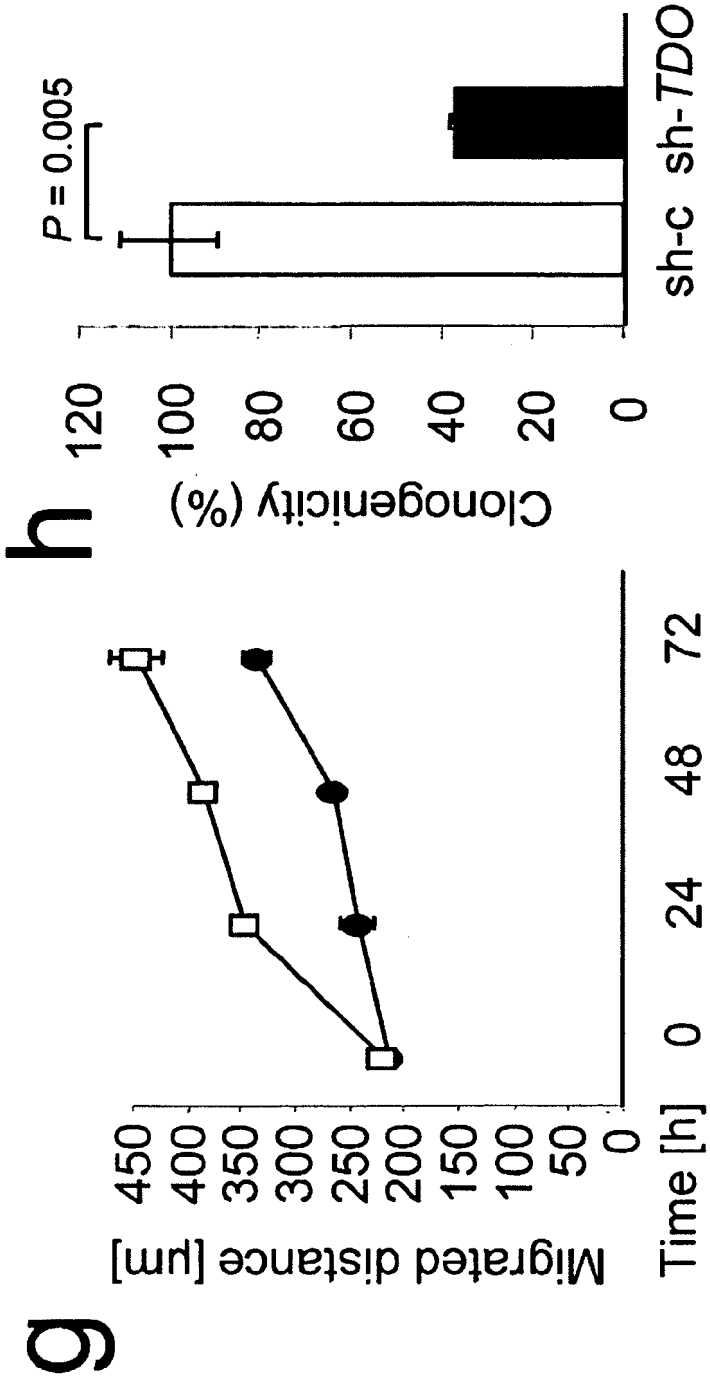

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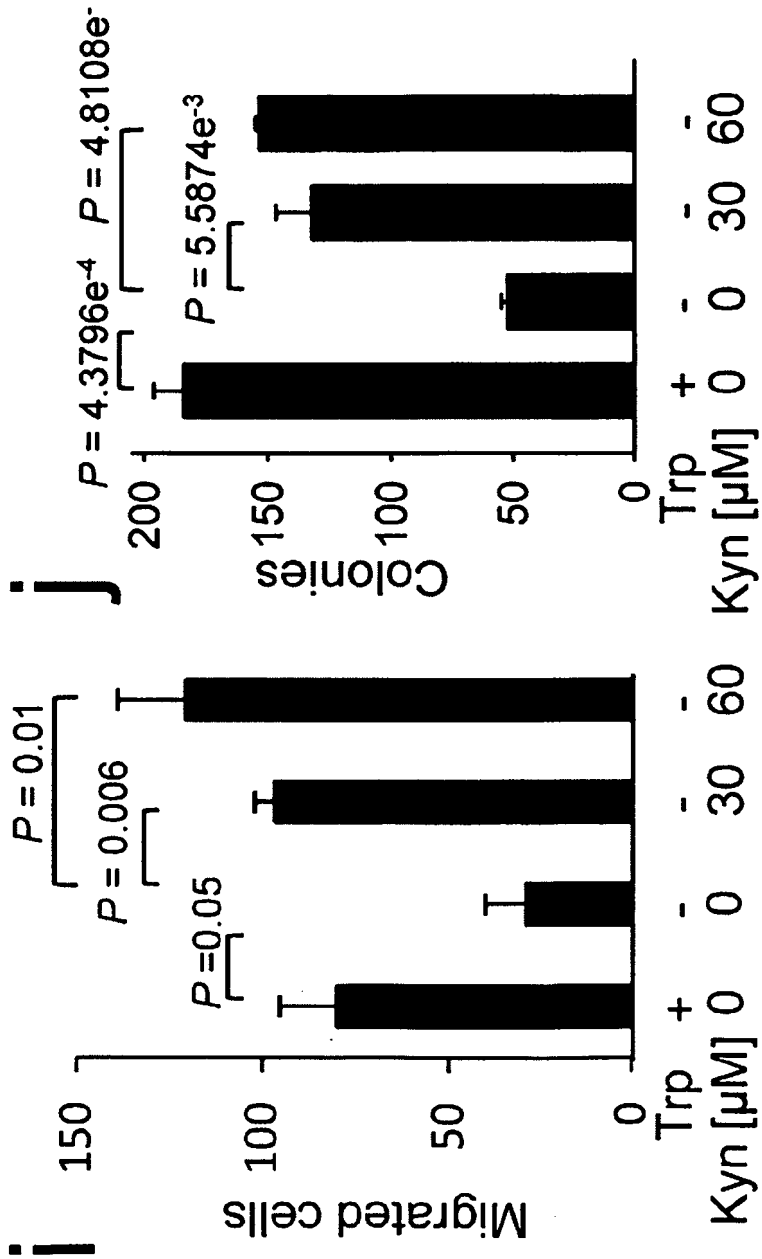

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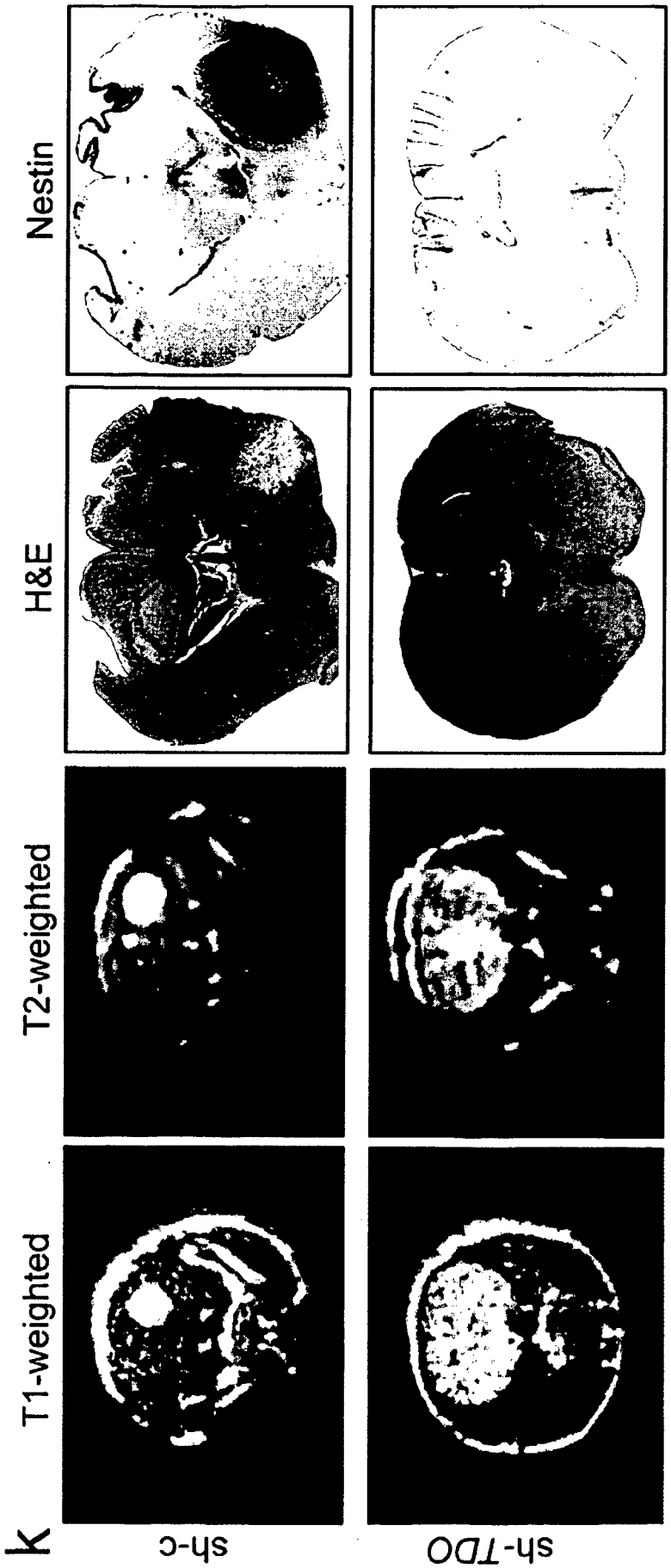

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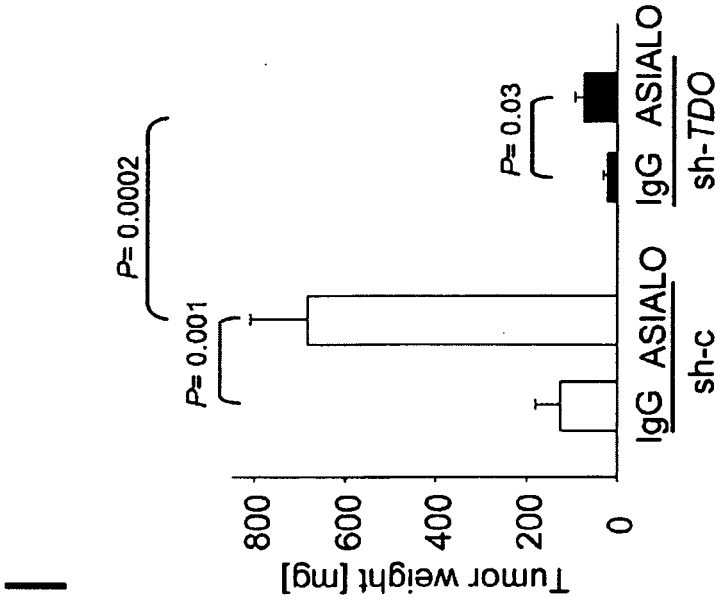

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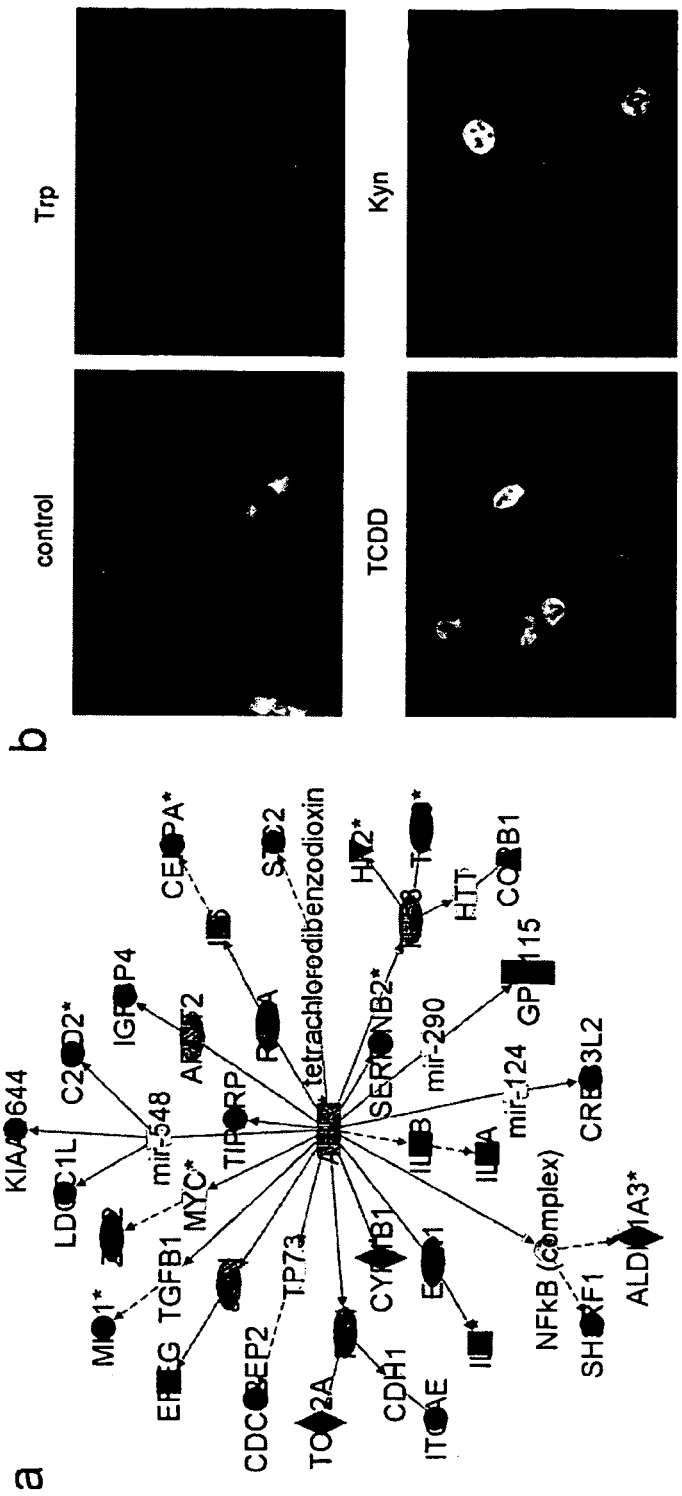
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control
Trp
TCDD
Kyn
a
Tetrachlorodibenzodioxin
mir-548
MYC*
TGFB1
TP73
CDH1
mir-290
mir-124
NFkB (complex)
HTT
TIPARP
IGFBP4

Fig. 3

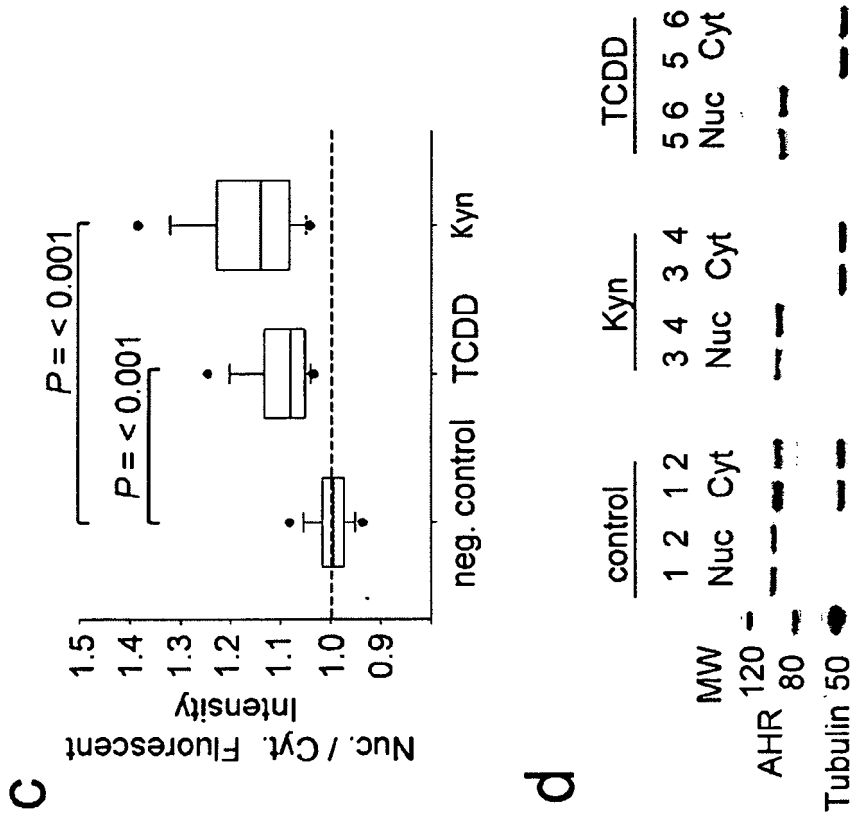


Fig. 3 (continued)

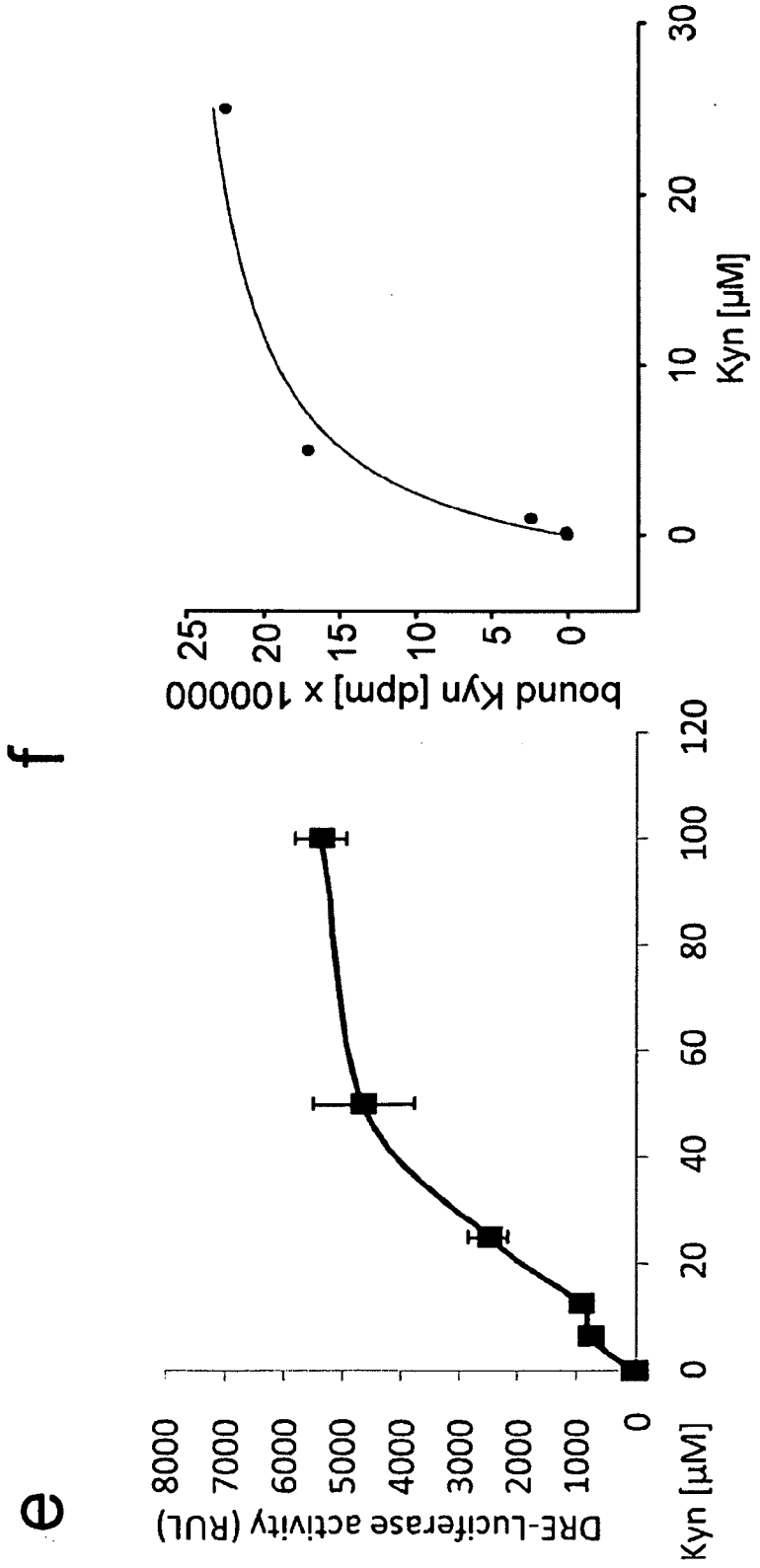


Fig. 3 (continued)

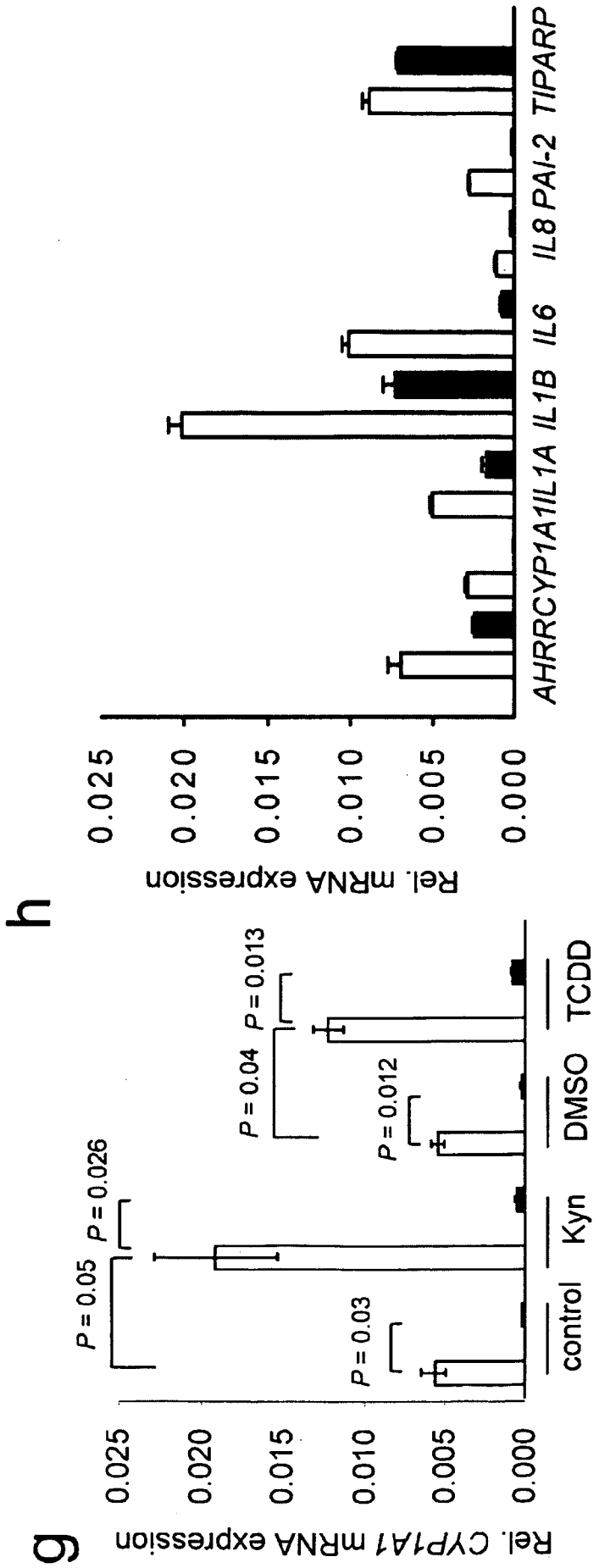


Fig. 3 (continued)

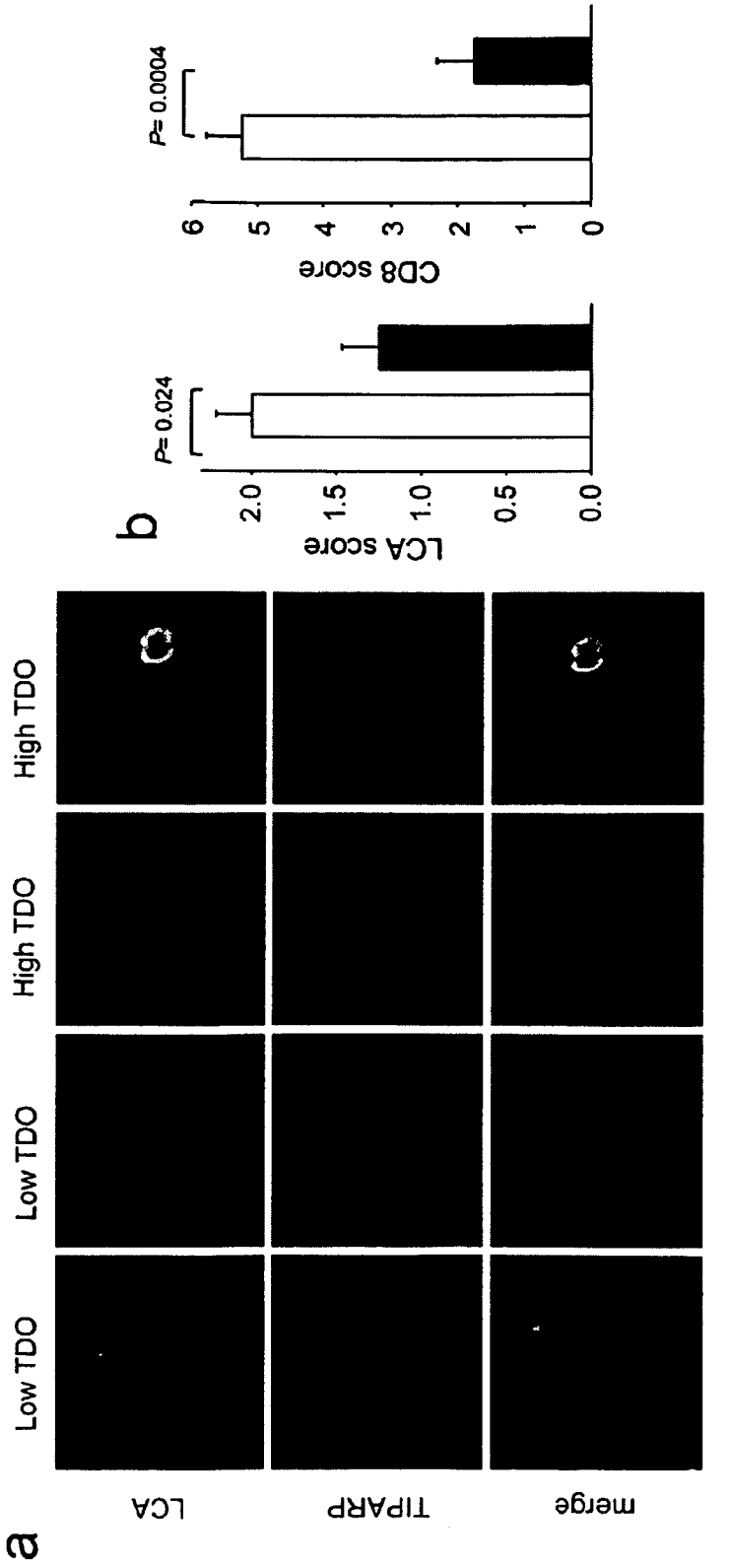
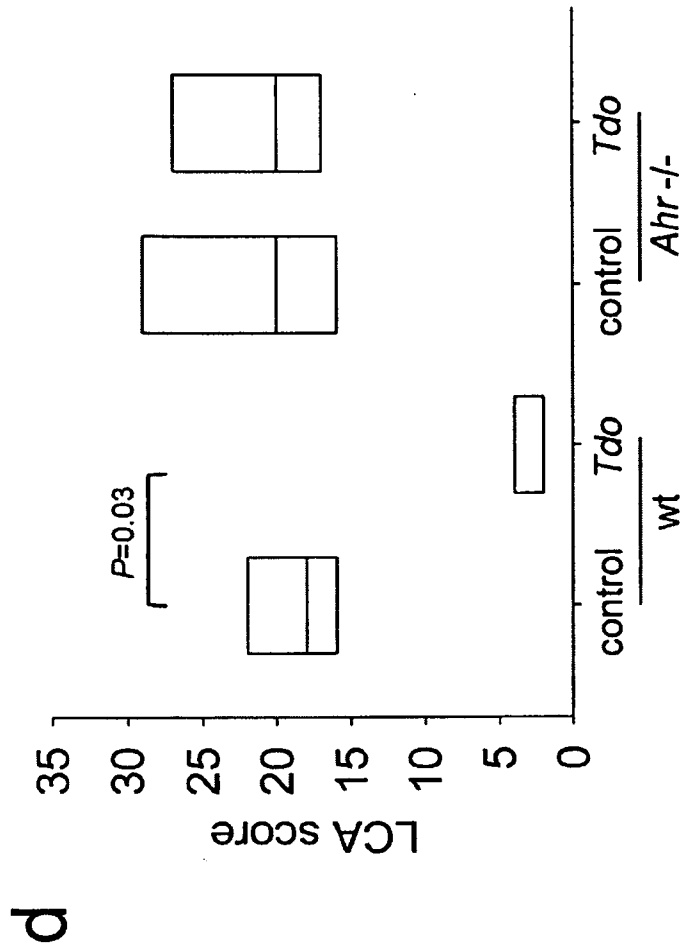

a
LCA
TIPARP
merge
Low TDO
Low TDO
High TDO
High TDO
b
LCA score
2.0
1.5
1.0
0.5
0.0
P= 0.024
CD8 score
6
5
4
3
2
1
0
P= 0.0004

Fig. 4



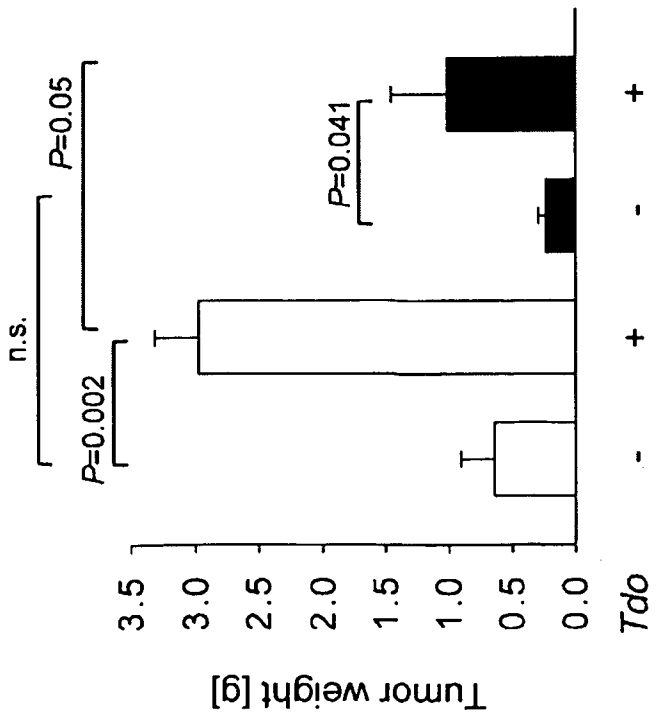
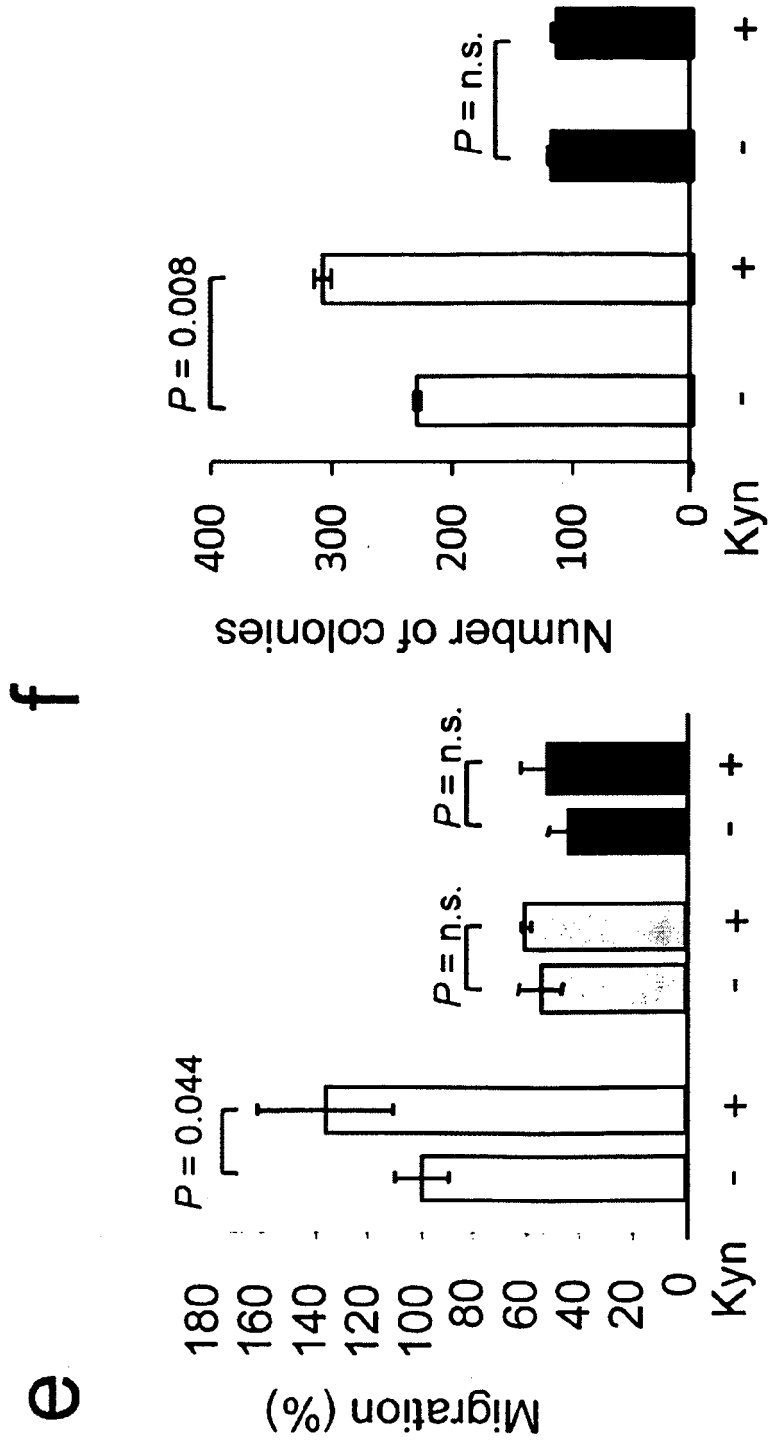


Fig. 4 (continued)



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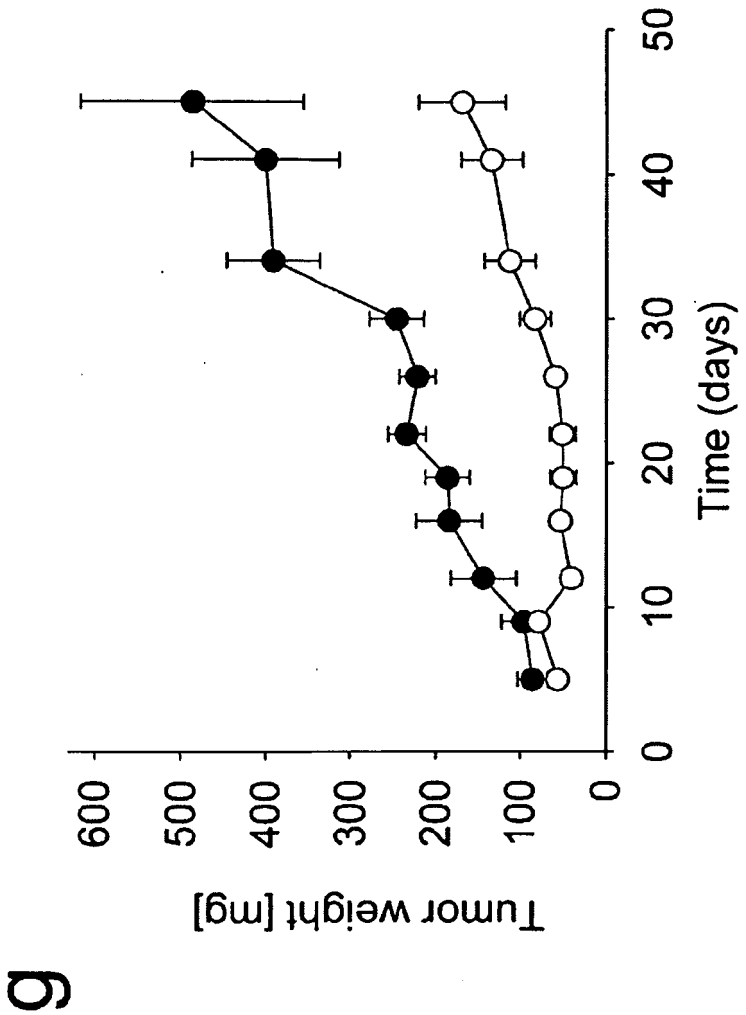
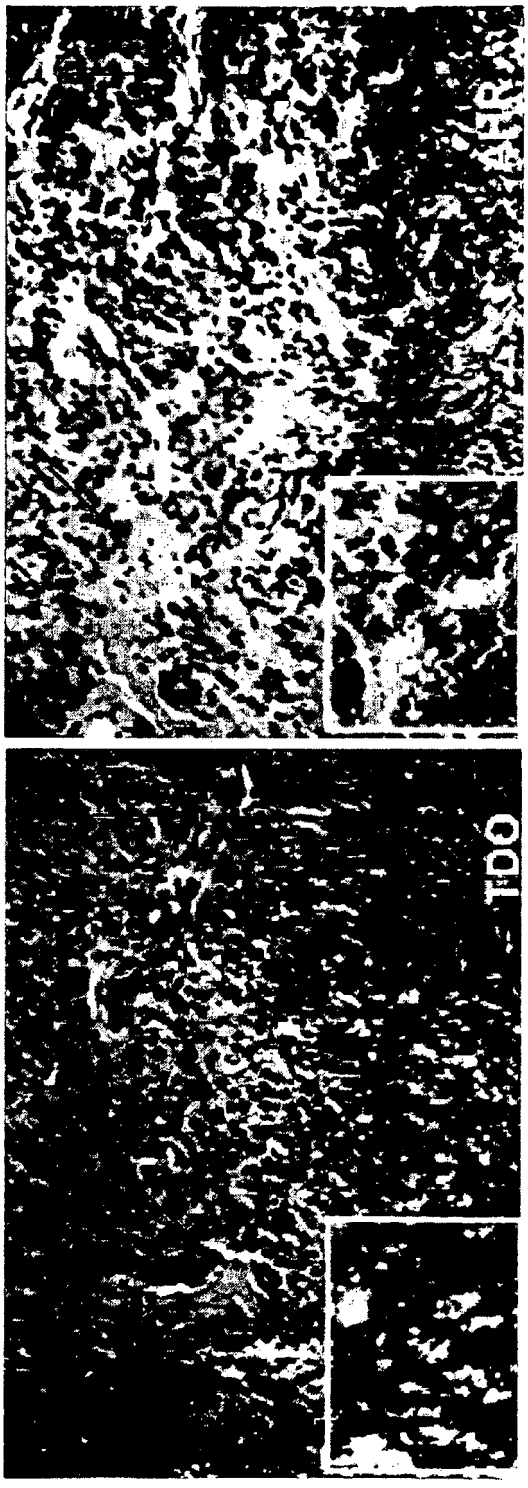


Fig. 4 (continued)



a

Fig. 5

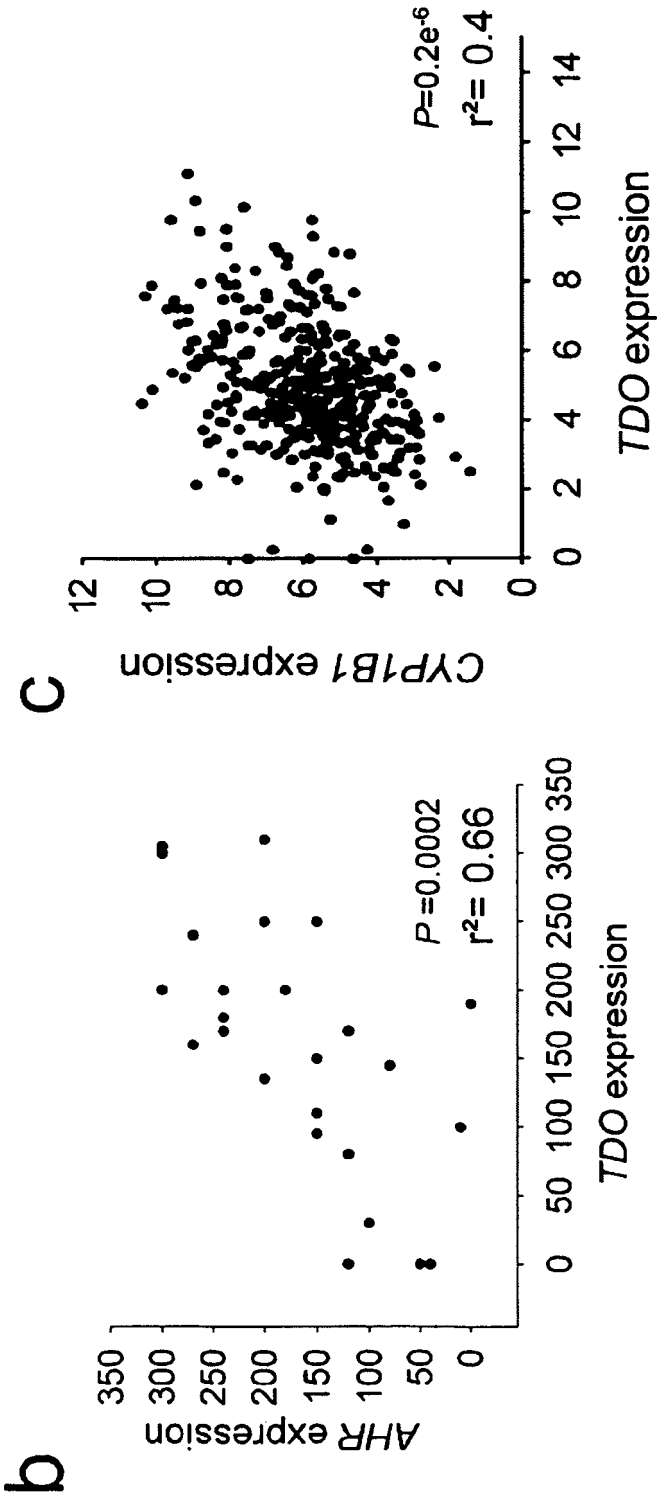


Fig. 5 (continued)

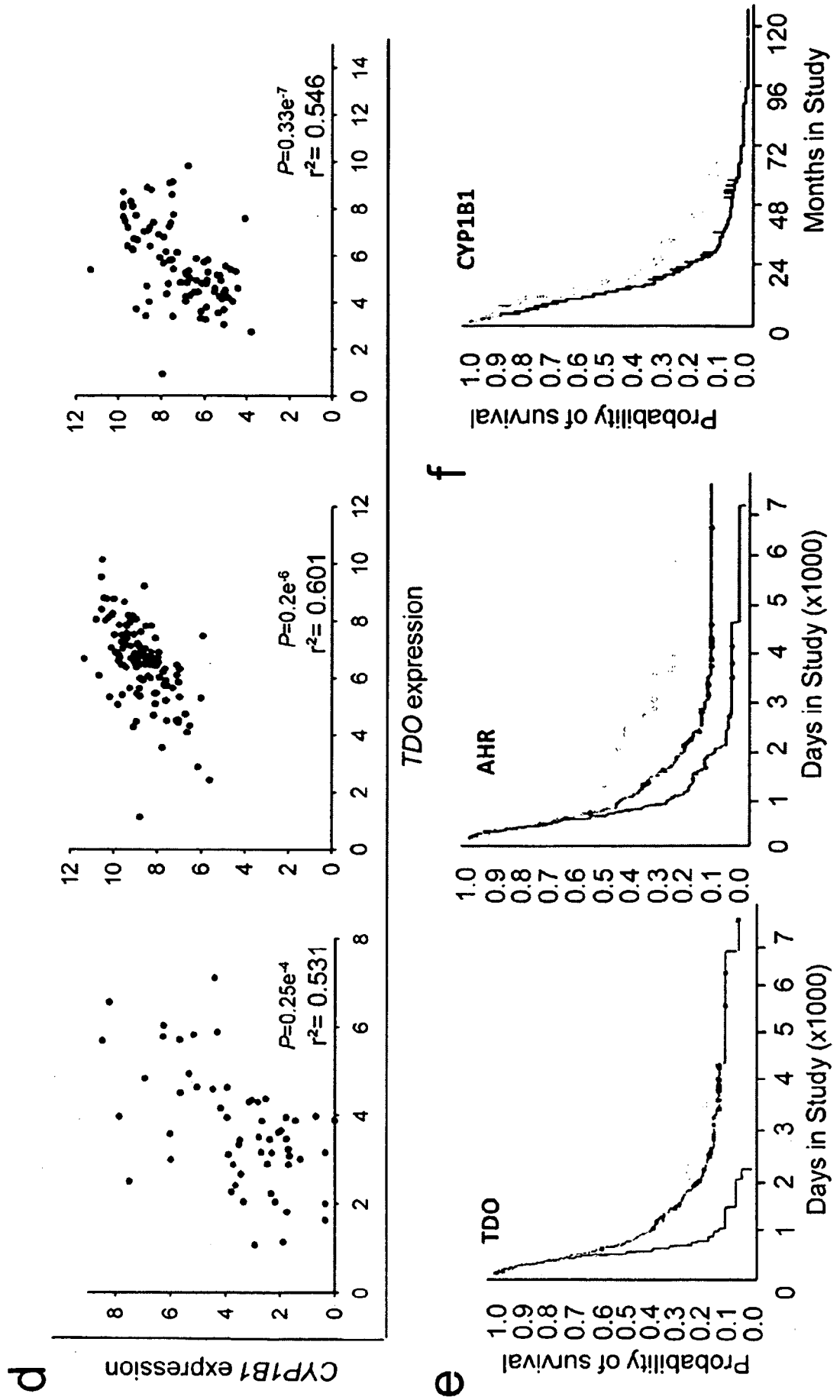


Fig. 5 (continued)

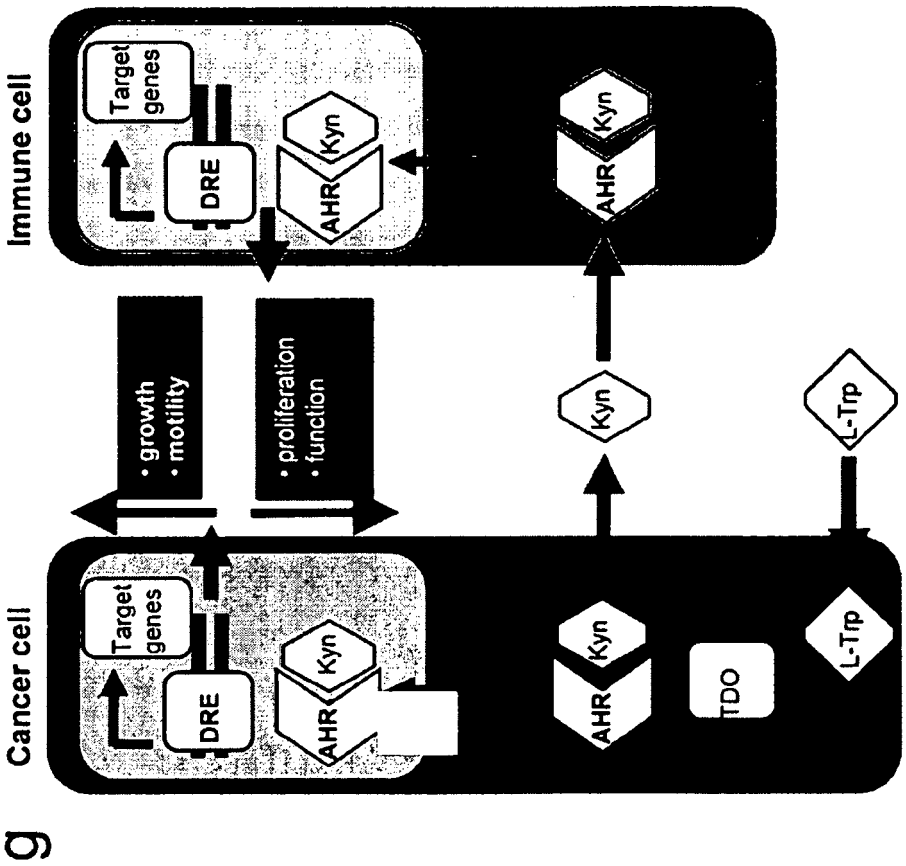


Fig. 5 (continued)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/067504

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/00 A61K31/352 A61K31/415
ADD. A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/128723 A1 (SYMRISE GMBH & CO KG [DE]; HERRMANN MARTINA [DE]; KOCH OSKAR [DE]; VIE) 15 November 2007 (2007-11-15)	1,3,4,8, 13,14
Y	claims 5, 6, 10; p. 7, first full and l. 20 and l. 26-29; bridging pages 4 and 5	1-15
X	S. NAIR ET AL: "UVR Exposure Sensitizes Keratinocytes to DNA Adduct Formation", CANCER PREVENTION RESEARCH, vol. 2, no. 10, 1 October 2009 (2009-10-01), pages 895-902, XP055045565, ISSN: 1940-6207, DOI: 10.1158/1940-6207.CAPR-09-0125	1,3-5, 13,14
Y	abstract; p. 896, col. 1, l. 4-5	1-15

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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 2012	03/12/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Dahse, Thomas

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MILLER M E ET AL: "Benzo-[a]-pyrene increases invasion in MDA-MB-231 breast cancer cells via increased COX-II expression and prostaglandin E2 (PGE2) output", CLINICAL & EXPERIMENTAL METASTASIS ; OFFICIAL JOURNAL OF THEMETASTASIS RESEARCH SOCIETY, KLUWER ACADEMIC PUBLISHERS, DO, vol. 22, no. 2, 1 April 2005 (2005-04-01), pages 149-156, XP019235820, ISSN: 1573-7276, DOI: 10.1007/S10585-005-6536-X	1-5,7, 13-15
Y	title, abstract; Fig. 6	1-15

Y	HANNO BOTHE ET AL: "Epigallocatechin-3-gallate does not affect the activity of enzymes involved in metabolic activation and cellular excretion of benzo[a]pyrene in human colon carcinoma cells", TOXICOLOGY LETTERS, vol. 203, no. 3, 1 June 2011 (2011-06-01), pages 258-264, XP055045397, ISSN: 0378-4274, DOI: 10.1016/j.toxlet.2011.03.026 abstract; penultimate of introduction; Fig. 1	1-15

A	KEH SUNG TSAI ET AL: "Benzo[a]pyrene Regulates Osteoblast Proliferation through an Estrogen Receptor-Related Cyclooxygenase-2 Pathway", CHEMICAL RESEARCH IN TOXICOLOGY, vol. 17, no. 5, 1 May 2004 (2004-05-01), pages 679-684, XP055045487, ISSN: 0893-228X, DOI: 10.1021/tx0499517 abstract	1-15

Y	US 2010/183564 A1 (BOITANO ANTHONY E [US] ET AL) 22 July 2010 (2010-07-22) 0094; 0287	1-15

Y	J.-E. LEE: "3',4'-Dimethoxyflavone as an Aryl Hydrocarbon Receptor Antagonist in Human Breast Cancer Cells", TOXICOLOGICAL SCIENCES, vol. 58, no. 2, 1 December 2000 (2000-12-01), pages 235-242, XP055044250, ISSN: 1096-6080, DOI: 10.1093/toxsci/58.2.235 title, abstract; Fig. 3	1-15

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/067504

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MIZRAHI MEIR ET AL: "ACTIVATION OF THE TRANSCRIPTION FACTOR ARYL HYDROCARBON RECEPTOR INCREASES IL17 AND INHIBITS IL6 SUPPRESSING THE GROWTH OF HEPATOCELLULAR CARCINOMA: A NOVEL METHOD FOR NUCLEAR RECEPTOR DEPENEDENT-TREGS DIRECTED ANTI TUMOR THERAPY", HEPATOLOGY, vol. 50, no. 4, Suppl. S, October 2009 (2009-10), page 1148A, XP009165103, & 60TH ANNUAL MEETING OF THE AMERICAN-ASSOCIATION-FOR-THE-STUDY-OF-LIVE R-DISEASES; BOSTON, MA, USA; OCTOBER 30 -NOVEMBER 03, 2009 ISSN: 0270-9139 the whole document --- AFSHIN MOHAMMADI-BARDBORI ET AL: "Quercetin, Resveratrol, and Curcumin Are Indirect Activators of the Aryl Hydrocarbon Receptor (AHR)", CHEMICAL RESEARCH IN TOXICOLOGY, vol. 25, no. 9, 17 September 2012 (2012-09-17), pages 1878-1884, XP055045458, ISSN: 0893-228X, DOI: 10.1021/tx300169e title, abstract --- CHRISTIANE A. OPITZ ET AL: "An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor", NATURE, vol. 478, no. 7368, 5 October 2011 (2011-10-05), pages 197-203, XP055045387, ISSN: 0028-0836, DOI: 10.1038/nature10491 abstract; figures ---	1-15 1-7, 13-15 1-15

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/067504

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007128723 A1	15-11-2007	CN 101466363 A	24-06-2009
		EP 2023910 A1	18-02-2009
		JP 2009536933 A	22-10-2009
		US 2009208433 A1	20-08-2009
		WO 2007128723 A1	15-11-2007
US 2010183564 A1	22-07-2010	AR 074063 A1	22-12-2010
		AU 2009317898 A1	27-05-2010
		CA 2740589 A1	27-05-2010
		CN 102203096 A	28-09-2011
		CO 6410309 A2	30-03-2012
		CU 20110096 A7	31-01-2012
		EA 201100663 A1	30-12-2011
		EP 2350078 A2	03-08-2011
		JP 2012507554 A	29-03-2012
		KR 20110075042 A	05-07-2011
		MA 32828 B1	01-11-2011
		PE 03622010 A1	27-05-2010
		SM AP201100024 A	11-07-2011
		SV 2011003888 A	05-07-2011
		TW 201028153 A	01-08-2010
		US 2010183564 A1	22-07-2010
		WO 2010059401 A2	27-05-2010

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 104023713 A
(43)申请公布日 2014.09.03

(21)申请号 201280043522.9
(22)申请日 2012.09.07
(30)优先权数据
61/531,861 2011.09.07 US
(85)PCT国际申请进入国家阶段日
2014.03.07
(86)PCT国际申请的申请数据
PCT/EP2012/067504 2012.09.07
(87)PCT国际申请的公布数据
WO2013/034685 EN 2013.03.14
(71)申请人 德国癌症研究中心
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代理人 陈迎春 黄革生
(51)Int.Cl.
A61K 31/00(2006.01)
A61K 31/352(2006.01)
A61K 31/415(2006.01)
A61P 35/00(2006.01)

权利要求书1页 说明书20页
序列表6页 附图24页

(54)发明名称

用于治疗和/或预防天然AHR配体依赖性癌症的手段和方法

(57)摘要

本发明涉及癌症疗法和癌症治疗的领域。特别地，本发明涉及用于治疗和/或预防天然AHR配体依赖性癌症的方法，其包括向患有所述癌症的受试者施用治疗有效量的AHR抑制剂。而且，本发明还涉及AHR抑制剂用于治疗和/或预防天然AHR配体依赖性癌症。

CN 104023713 A

1. 一种用于治疗和 / 或预防天然 AHR 配体依赖性癌症的方法，其包括向患有所述癌症的受试者施用治疗有效量的 AHR 抑制剂。

2. 根据权利要求 1 的方法，其中所述癌症选自：优选为神经胶质瘤的脑肿瘤、黑色素瘤、结直肠腺癌、结肠癌、肾细胞癌、非小细胞肺癌 (NSCLC)、乳癌、肝细胞癌、卵巢癌、头颈癌、膀胱癌、胰腺癌、间皮瘤和小细胞肺癌 (SCLC)。

3. 根据权利要求 1 的方法，其中所述 AHR 抑制剂是小分子化合物。

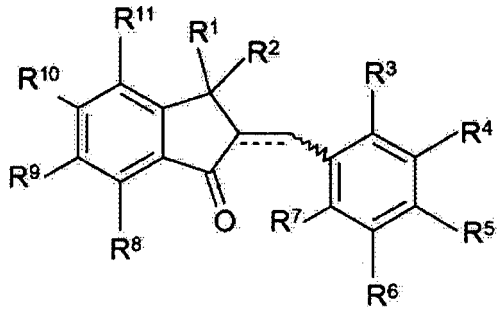
4. 根据权利要求 3 的方法，其中所述的小分子化合物是植物化合物或其衍生物。

5. 根据权利要求 4 的方法，其中所述的植物化合物或其衍生物是黄酮或其衍生物。

6. 根据权利要求 4 的方法，其中所述的黄酮或其衍生物是 3,4- 二甲氧基黄酮、3′ - 甲氧基 -4′ - 硝基黄酮、4′ ,5,7- 三羟基黄酮（芹菜苷配基）或 1- 甲基 -N-[2- 甲基 -4-[2-(2- 甲苯基）二氮烯基] 苯基 -1H- 吡唑 -5- 羧酰胺。

7. 根据权利要求 4 的方法，其中所述的植物化合物或其衍生物是白藜芦醇或其衍生物、表没食子儿茶素或表没食子儿茶素没食子酸酯。

8. 根据权利要求 3 的方法，其中所述的小分子化合物是如以下通式 (I) 表征的化合物：



其中

(i) R^1 和 R^2 彼此独立地是氢或 C_1 至 C_{12} 烷基，

(ii) R^3 至 R^{11} 彼此独立地是氢、C_1 至 C_{12} 烷基、羟基或 C_1 至 C_{12} 烷氧基，和

(iii) 折线代表双键或两个氢。

9. 根据权利要求 1 的方法，其中所述的 AHR 抑制剂是特异地结合并抑制 AHR 蛋白的抗体。

10. 根据权利要求 1 的方法，其中所述的 AHR 抑制剂是 AHR 阻遏蛋白或失活的 AHR 核易位蛋白 (ARNT)。

11. 根据权利要求 1 的方法，其中所述的 AHR 抑制剂是核酸抑制剂。

12. 根据权利要求 11 的方法，其中所述的核酸阻遏物特异地结合编码 AHR 的多核苷酸，且所述核酸阻遏物选自：核酶、反义分子、寡核苷酸抑制剂、适配体、microRNA 和 siRNA。

13. 用于治疗和 / 或预防天然 AHR 配体依赖性癌症的 AHR 抑制剂。

14. 根据权利要求 13 的 AHR 抑制剂，其中所述的 AHR 抑制剂是如权利要求 3 至 12 任一项定义的 AHR 抑制剂。

15. 根据权利要求 13 的 AHR 抑制剂，其中所述癌症是如权利要求 2 定义的癌症。

用于治疗和/或预防天然AHR配体依赖性癌症的手段和方法

[0001] 本发明涉及癌症疗法和癌症治疗的领域。特别地，本发明涉及用于治疗和/或预防天然AHR配体依赖性癌症的方法，其包括向患有所述癌症的受试者施用治疗有效量的AHR抑制剂。而且，本发明还包括用于治疗和/或预防天然AHR配体依赖性癌症的AHR抑制剂。

[0002] 肿瘤微环境是有效肿瘤疗法中的特别挑战，理由是其对肿瘤的恶性具有多种影响(Tennant2010，Nat Rev Cancer10，267)。

[0003] 色氨酸(Trp)代谢是肿瘤微环境重要性的一个例子。在动物模型中已经证实了其作为限制免疫反应的主要内源机制的功能相关性(Munn2007，J Clin Invest117，1147)。

[0004] 特别是，Trp代谢的激活与免疫系统疾病和紊乱相关，所述免疫系统疾病和紊乱如肿瘤免疫、自体免疫、感染性疾病和免疫特权的维持(Opitz2007，Cell Mol Life Sci64，2452)。在肿瘤和肿瘤引流淋巴结中由吲哚胺2,3-双加氧酶1和2(IDO1 / 2)引起的Trp降解抑制了抗肿瘤的免疫反应，并与多种恶性肿瘤中的不良预后相关(Lob2009，Nat Rev Cancer9(6):445)。抑制IDO1 / 2在动物模型中抑制了肿瘤形成，且目前在癌症患者的I / II期临床试验中得到验证(Muller2005，Nat Med11(3):312，Uyttenhove2003，Nat Med9(10)，1269；DiPuccio2010，Expert Opin Ther Pat20，229；Ball2007，Gene396(1)，203；Metz2007，Cancer Res67(15)，7082)。

[0005] 在神经元和肝细胞中参与Trp代谢的其他已知的酶是色氨酸2,3-双加氧酶(TDO)，其也合成Trp降解的第一步(Thackray2008，Biochem Soc Trans36，1120)。TDO还作为肿瘤药物潜在的靶标被报道(WO2010 / 008427)。但是，Trp分解代谢对人肿瘤形成和进展的相关性仍不清楚。

[0006] 犬尿氨酸(Kyn)是Trp的代谢产物，其具有免疫抑制功能。但是，其分子靶标和如何引起该作用的机制仍不清楚。其中报道外源Kyn在树突状细胞和T细胞中激活芳香烃受体(AHR)转录因子(Mezrich2010，J Immunol185，3190；Nguyen2010，Proc Natl. Acad Sci，USA，107，19961)。

[0007] AHR是碱性螺旋-环-螺旋(bHLH)Per-Arnt-Sim(PAS)家族的转录因子，其由异生素如苯并芘和2,3,7,8-四氯二苯并二氧芑(TCDD)激活。在细胞核中AHR与AHR核易位蛋白(ARNT)形成异二聚体，与位于AHR靶基因调控区的二氧芑反应元件(DRE)的核心结合基序相互作用(Reyes1992，Science256，5060；Abel2010，Biol Chem391，1235)。

[0008] 已知AHR参与由例如卤代芳烃诱发的化学致癌。而且，有人报道绿茶提取物能够作为AHR拮抗剂发挥作用，从而能够防止该卤代芳烃的有害作用(Palermo2003，Chem Res Toxicol16，865)。另外，已知AHR基因的组成型表达参与成胶质细胞瘤细胞中的细胞存活(Gramatzki2009，Oncogene28，2593)。

[0009] 鉴于上述情况，提供用于有效治疗其恶性度依赖于如Trp分解代谢的代谢过程的肿瘤的手段和方法尚不存在，但却非常需要这样的手段和方法。

发明内容

[0010] 本发明涉及用于治疗和 / 或预防天然 AHR 配体依赖性癌症的方法，其包括向患有所述癌症的受试者施用治疗有效量的 AHR 抑制剂。

[0011] 在本发明方法的一个优选的实施方案中，所述癌症选自脑的肿瘤（优选为神经胶质瘤）、黑色素瘤、结肠直肠腺癌、结肠癌、肾细胞癌、NSCLC、乳癌、肝细胞癌、卵巢癌、头颈癌、膀胱癌、胰腺癌、间皮瘤和 SCLC。

[0012] 在本发明方法的一个优选的实施方案中，所述 AHR 抑制剂是小分子化合物。

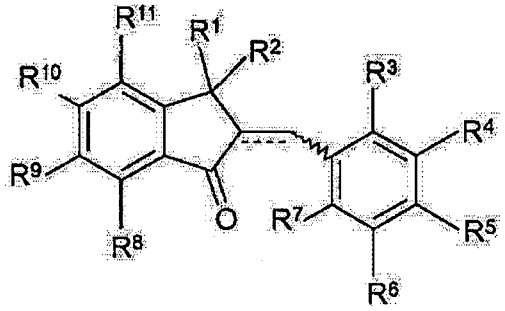
[0013] 在本发明方法的一个优选的实施方案中，所述小分子化合物是植物化合物或其衍生物。

[0014] 在本发明方法的一个优选的实施方案中，所述植物化合物或其衍生物是黄酮或其衍生物。最优选地，所述黄酮或其衍生物是 3,4- 二甲氧基黄酮、3′ - 甲氧基 -4′ - 硝基黄酮、4′ ,5,7- 三羟基黄酮（芹菜苷配基）、或 1- 甲基 -N-[2- 甲基 -4-[2-(2- 甲苯基）二氮烯基] 苯基 -1H- 吡唑 -5- 羧酰胺 (CH223191 ;CAS 号 301326-22-7)。

[0015] 在本发明方法的另一个更优选的实施方案中，所述植物化合物或其衍生物是白藜芦醇 (reveratrol) 或其衍生物、表没食子儿茶素 (epigallocatechin) 或表没食子儿茶素没食子酸酯 (epigallocatechingallate)。

[0016] 在本发明方法的另一个优选的实施方案中，所述小分子化合物是具有以下通式 (I) 特征的化合物：

[0017]



[0018] 其中

[0019] (i) R^1 和 R^2 彼此独立地是氢或 C_1 至 C_{12} 烷基，

[0020] (ii) R^3 至 R^{11} 彼此独立地是氢、C_1 至 C_{12} 烷基、羟基或 C_1 至 C_{12} 烷氧基，和

[0021] (iii) 折线 (the broken line) 代表双键或两个氢。

[0022] 在本发明方法的另一个优选的实施方案中，所述 AHR 抑制剂是特异地结合和抑制 AHR 蛋白的抗体。

[0023] 在本发明方法的另一个优选的实施方案中，所述 AHR 抑制剂是 AHR 阻遏蛋白或失活的 AHR 核易位蛋白 (ARNT)。

[0024] 在本发明方法的另一个优选的实施方案中，所述 AHR 抑制剂是核酸抑制剂。

[0025] 在本发明方法的另一个优选的实施方案中，所述核酸阻遏蛋白特异地结合编码 AHR 的多核苷酸，且所述核酸阻遏蛋白选自：核酶、反义分子、抑制剂寡核苷酸、microRNA 和 siRNA。

[0026] 而且，本发明还包括用于治疗和 / 或预防天然 AHR 配体依赖性癌症的 AHR 抑制剂。

附图说明

[0027] 图 1 表明 TDO 将人脑肿瘤中的 Trp 降解为 Kyn。a，培养 72 小时的人星形胶质细胞 (hAs)、神经胶质瘤细胞系和 GIC(T323) 上清中 Trp（左）和 Kyn（右）的含量，其通过 HPLC 测定 (n=4)。b，TDO mRNA 与人神经胶质瘤细胞 Kyn 释放之间的相关性，其通过定量 RT-RCP 和 HPLC 测定 (n=4)。c，在 TDO 抑制剂 680C91（黑色条棒）或其溶剂（白色条棒；n=4，1、5 和 10 μ M TDOI 的 P 值分别为 P=0.005、0.002 和 0.0009) 存在时培养 48 小时的 U87 神经胶质瘤细胞上清中 Kyn 浓度。d，由 siRNA 基因敲除 TDO（黑色条棒，P 值分别为 P=0.000007，0.0007 和 0.00006)、IDO1（深灰色条棒）或 IDO2（亮灰色条棒）后神经胶质瘤细胞的 Kyn 释放 (n=3)、e，健康脑组织中弱的神经元 TDO 表达（上图）。成胶质细胞瘤 (WHO 分级 IV 级，下图）中的 TDO 表达；红色：TDO 染色；* 坏死；箭头：浸润的脑组织边界。插图：浸润邻近脑组织的单个肿瘤细胞（箭）。放大率：40x，插图 400x（上图）、100x（下图）。f，恶性度渐增的脑肿瘤中 TDO 表达的图 [H 评分](WHO 分级 II-IV；II 级，n=18，III 级，n=15，IV 级，n=35)。g，Ki-67 增殖指数与不同 WHO 分级的神经胶质瘤中 TDO H 评分的相关性 (n=42)。h，由 HPLC 测定的 24 例成胶质细胞瘤患者和 24 例年龄、性别相匹配的健康对照的血清中 Trp（左）和 Kyn（右）浓度。i，健康人脑组织（白色条棒，n=5) 和成胶质细胞瘤组织（黑色条棒，n=5) 中的喹啉酸染色定量。(f) 和 (g) 中的数据分布作为箱图代表，表明第 25 和第 75 个百分点以及中位数，触须线分别代表第 10 和第 90 个百分点。

[0028] 图 2 表明通过神经胶质瘤细胞的 TDO 介导的 Kyn 释放对免疫细胞的旁分泌作用。a，与不同神经胶质瘤细胞系共同培养的 PBMC 的同种异体增殖与神经胶质瘤细胞的 Kyn 释放间的相关性 (n=3)。b，在存在或不存在 100 μ M Kyn 时，与表达 TDO 的对照 U87 神经胶质瘤细胞 (sh-c)、使用稳定的短发夹 RNA 介导的 TDO 基因敲除的 U87 神经胶质瘤细胞 (sh-TDO) 共同培养的 PBMC（黑色条棒）、以及在存在或不存在 100 μ M Kyn 时，单独的 PBMC（白色条棒，n=3) 之间同种异体增值的比较。c，具有 TDO 低表达的人神经胶质瘤切片 (H- 评分 <150，白色条棒，对于 LCA，n=12；对于 CD8，n=10) 和具有 TDO 高表达的人神经胶质瘤切片 (H 评分 ≥ 150，黑色条棒，对于 LCA，n=17；对于 CD8，n=10) 中，染色的 LCA+ 细胞（左图）和 CD8+ 细胞（右图）的定量。d，使用 Tdo（实心圈）或空载体（空心圈）皮下注射至 C57BL / 6N 小鼠胁腹，使用公制卡尺检测稳定转染的 Tdo 缺陷 GL261 鼠神经胶质瘤细胞的生长 (n=6)。通过以下等式计算肿瘤重量：肿瘤重量 (g)=（长 (cm)× 宽 (cm)2)×0.5。e，用神经胶质瘤裂解物再刺激后，皮下带有表达 Tdo 肿瘤的小鼠 T 细胞（黑色条棒）和带有 Tdo 缺陷肿瘤的小鼠 T 细胞（白色条棒）释放的 IFN-γ 的比较，其由 ELISpot 测定 (n=3)。f，由具有表达 Tdo 的 GL261 肿瘤的小鼠的脾细胞裂解的 GL261 鼠神经胶质细胞与那些具有皮下 Tdo 缺陷的 GL261 肿瘤的小鼠的脾细胞裂解的 GL261 鼠神经胶质细胞的比较，其由铬释放测得 (n=4)。g，sh-c（空心圈）和 sh-TDO（实心圈）细胞向胶原蛋白基质迁移距离的定量 (n=3，24、48 和 72h 的 P 值分别是 P=0.004，0.0005 和 0.01)。h，sh-c（白色条棒）和 sh-TDO（黑色条棒）U87 细胞的产克隆细胞的存活 (n=3)。i，在不存在或存在 70 μ M Trp、没有或有 30 μ M 或 60 μ M Kyn 时，U87 神经胶质瘤细胞的基质胶 boyden 小室分析 (Matrigel boyden chamber assay)。j，在不存在或存在 70 μ M Trp、没有或有 30 μ M 或 60 μ M Kyn 时，LN-18 神经胶质瘤细胞的产克隆细胞的存活 (n=3)。k，植入 sh-c（上图）或 sh-TDO（下图）U87 神经

胶质瘤细胞的CD1nu / nu小鼠的代表性头颅MRI、H&E和巢蛋白(nestin)染色。这些影像代表两个独立的实验(n=6)。1,在CD1nu / nu小鼠的胁腹皮下注射sh-c(白色条棒)和sh-TDO(黑色条棒)U87神经胶质瘤细胞的肿瘤重量,该小鼠用为去除NK细胞的抗-去唾液酸(asialo)GMI抗体(ASIALO)处理,或用对照IgG(IgG)处理(n=8)。

[0029] 图3表明Kyn激活AHR。a,U87细胞中Kyn处理8h后最强烈地诱导的25个基因与AHR信号传导的关联(红色:上调,绿色:下调)。b,标记GFP的AHR至小鼠肝癌细胞核的易位,用50μM Kyn、50μM Trp或1nM TCDD处理3h后,并不降解Trp(阴性对照:培养基)。c,指定处理(阴性对照:培养基,阳性对照:1nM TCDD、50μM Kyn)3h后,带有标记GFP的AHR的细胞中细胞核对细胞质荧光强度的比率。数据分布由箱图表示,表明第25和第75个百分点以及中位数,触须线分别代表第10和第90个百分点(P<0.001,先对等级进行单向ANOVA分析,而后进行Dunns'方法)。d,各对照(1,2)、Kyn-处理的(3,4)和TCDD-处理的(5,6)人LN-229神经胶质瘤细胞的两个不同的细胞核和细胞质级分的AHR Western印记。e,用指定Kyn浓度处理的U87神经胶质瘤细胞中二氧芑反应元件(DRE)化学激活的萤光素酶基因表达(n=2)。f,使用来自Ahr正常(Ahr-proficient)和Ahr缺陷小鼠的肝脏细胞溶胶,用指定浓度的L-3H-Kyn进行的放射配体结合实验。通过从Ahr正常的细胞溶胶中测得的放射活性减去Ahr缺陷的细胞溶胶中测得的放射活性计算特异的结合(n=4)。g,与用100μM Kyn、1nM TCDD或对照处理的对照(sh-c,白色条棒)相比,sh-AHR LN-308神经胶质瘤细胞(黑色条棒)中的CYP1A1mRNA表达(n=4)。h,与sh-c U87神经胶质瘤细胞(白色条棒,n=4)相比,sh-TDO(黑色条棒)中AHR靶标基因的mRNA表达。

[0030] 图4表明源自TDO的Kyn的自分泌和旁分泌作用受AHR介导。a,在具有TDO低或高表达的人神经胶质瘤切片中LCA和TIPARP的免疫荧光染色。放大率:400x。b,在具有AHR低表达人神经胶质瘤切片(Histo评分<150,白色条棒,对于LCA,n=10;对于CD8,n=8)和具有AHR高表达人神经胶质瘤切片(Histo评分≥150,黑色条棒,对于LCA,n=12;对于CD8,n=12)中,染色的LCA+细胞(左)和CD8+细胞(右)的定量。c,在Ahr正常(白色条棒)或Ahr缺陷小鼠(黑色条棒,n=6)的胁腹,皮下注射有或无Tdo表达的鼠GL261神经胶质瘤细胞15天后的肿瘤重量。d,如箱图所示,在Ahr正常和Ahr缺陷的小鼠中,在皮下的Tdo正常和Tdo缺陷的GL261肿瘤中,染色的LCA+色疫细胞的定量,其表明第25和第75个百分点以及中位数(n=4)。e,在100μM Kyn存在或不存在时,sh-e LN-308神经胶质瘤细胞(白色条棒)和由两种不同的shRNA(sh-AHR1,灰色条棒和sh-AHR2黑色条棒)基因敲除AHR的LN-308神经胶质瘤细胞的迁移(n=4)。f,存在或不存在100μM Kyn时,sh-c(白色条棒)和sh-AHR(黑色条棒)LN-308神经胶质瘤细胞的克隆发生(n=3)。G使用公制卡尺检测皮下注射到CD1nu / nu小鼠胁腹的AHR-正常(实心圈)和AHR-缺陷的(空心圈)人LN-308神经胶质瘤细胞的生长(n=7)。通过以下等式计算肿瘤重量:肿瘤重量(g)=(长(cm)×宽(cm)2)×0.5。

[0031] 图5表明源自TDO的Kyn在多种人癌症中激活AHR,且AHR的激活预示神经胶质瘤患者的生存。a,在人成胶质细胞瘤组织的连续切片中,TDO表达(红色)和AHR表达(褐色)的相关性。箭表明用于定位的血管。放大率40x,插图200x。b,根据使用斯皮尔曼等级相关计算的TDO和AHR的H评分,在人神经胶质瘤组织中TDO和AHR表达之间的相关性(n=26)。c,在由斯皮尔曼等级相关分析的人成胶质细胞瘤的微阵列数据中TDO和CYP1B1

表达之间的相关性 (n=396)。d,在由斯皮尔曼等级相关分析的人膀胱癌 (左,n=58)、人肺癌 (中间,n=122) 和人卵巢癌 (右,n=91) 的微阵列数据中 TDO 和 CYP1B1 表达之间的相关性。e,来自 Rembrandt 的具有 TDO 或 AHR 高表达 (红色) 的神经胶质瘤患者 (WHO 分级 II-IV 级) 的生存概率与具有这些基因中度表达 (蓝色) 或低表达 (绿色) 的患者的生存概率的比较。统计学分析参见增补的记录 21。f,具有 AHR 靶标基因 CYP1B1 高表达 (红色) 的成胶质细胞瘤患者的生存概率与来自 The Cancer Genome Atlas(TCGA) 网络的成胶质细胞瘤数据组的 CYP1B1 低表达 (绿色) 患者的生存概率的比较 (n=362)。g,强调源自 TDO 的 Kyn 通过 AHR 对癌症细胞和免疫细胞的自分泌和旁分泌作用的概略图。

[0032] 发明详述

[0033] 本发明涉及用于治疗和 / 或预防天然 AHR 配体依赖性癌症的方法,其包括向患有所述癌症的受试者施用治疗有效量的 AHR 抑制剂。

[0034] 用于本文中的术语“治疗”指与未治疗的受试者相比,发生在受治疗的受试者中的癌症的任何改善。所述改善可以是防止癌症的恶化或进展。而且,所述改善还可以是癌症或其伴随症状的改进或治愈。应理解治疗未必对 100% 的待治疗受试者成功。然而,该术语要求治疗对受试者在统计学上显著的部分是成功的 (例如,在定群研究中的群体)。该部分是否是统计学上显著的,可由本领域技术人员使用各种公知的统计学分析工具确定,例如置信区间的确定、p 值的确定、学生 t 检验、Mann-Whitney 检验等,而不需过度劳动。具体见于 Dowdy 和 Wearden, Statistics for Research, John Wiley&Sons, New York1983 中。优选的置信区间是至少 90%、至少 95%、至少 97%、至少 98% 或至少 99%。优选 p 值是 0.05、0.01、0.005 或 0.0001。

[0035] 用于本文中的术语“预防”指避免本文中使用的癌症或其伴随症状的发生。应理解预防指避免在将来的某一时间窗口内发生癌症。所述时间窗口,优选地,在施用本发明的化合物开始,持续至少 1 个月、至少 6 个月、至少 9 个月、至少 1 年、至少 2 年、至少 5 年、至少 10 年或甚至保持在受试者的生理寿命中。应理解预防未必对 100% 的待治疗受试者成功。然而,该术语要求预防对受试者在统计学上显著的部分是成功的 (例如,在定群研究中的群体)。该部分是否是统计学上显著的,可由本领域技术人员使用本文中其他处详细讨论的各种公知的统计学分析工具确定,而不需过度劳动。

[0036] 用于本文中的术语“天然 AHR 配体依赖性癌症”,指依赖于由天然 AHR 配体引起的组成型 AHR 活化的任何恶性赘生物。优选地,所述天然 AHR 配体是犬尿氨酸 (Kyn)。优选地,犬尿氨酸由增加的色氨酸降解酶表达引起的色氨酸降解产生。更优选地,根据本发明的癌症因而与增加的色氨酸 -2-3- 双加氧酶 (TDO) 活性相关。如本文中涉及的 TDO 活性,优选地可以通过测量癌症组织或癌症细胞中存在的犬尿氨酸和 / 或色氨酸浓度评估。而且,还可以通过确定癌症组织或癌症细胞中 TDO 酶含量或编码所述 TDO 酶的转录物含量评估增加的 TDO 活性。可通过基于抗体的技术确定 TDO 酶的含量,如 ELISA,而转录物的含量则通过核酸杂交技术,如 Northern 印记,或核酸扩增技术,如 RT-PCR 确定。用于确定是否存在与癌症相关的增加的 TDO 特别优选的技术描述于本文中以下的实施例中,或公开于 WO2010 / 008427 中,其相应公开的内容在本文中引入作为参考。优选地,所述以上提及的癌症选自 : 脑肿瘤,优选神经胶质瘤、黑色素瘤、结直肠腺癌、结肠癌、肾细胞癌、非小细胞肺癌 (NSCLC)、乳癌、肝细胞癌、卵巢癌、头颈癌、膀胱癌、胰腺癌、间皮瘤、和小细胞肺癌 (SCLC)。可替换地,

也是更优选地，根据本发明的癌症因而是与增加的吲哚胺 2,3- 双加氧酶 1 或 2(IDO1 或 2) 活性相关的癌症。本本文中涉及的优选癌症是本领域公知的；参见，例如 Lob2009，Nat Rev Cancer9(6)，445，其相应公开的内容在本文中引入作为参考。

[0037] 在本发明的意义上，“AHR 抑制剂”是能够直接或间接抑制芳香烃受体 (AHR) 多肽活性的化合物。根据本发明所指的 AHR 多肽是碱性 - 螺旋 - 环 - 螺旋转录因子家族的成员。其是胞质转录因子，通常是失活的，且与一些伴侣蛋白以复合体存在。已经描述了一些能够激活或抑制 AHR 的配体，其中有人工或天然存在的配体。首次发现的配体是合成的，其为卤代芳烃（多氯二苯并二氧芑，如 2,3,7,8 四氯二苯并 - 对 - 二氧芑 (TCDD)，氧芴和联苯）和多环芳烃 (3- 甲基胆蒽，苯并芘，苯并蒽类和苯并黄酮类）的成员。已经鉴定为天然存在的 AHR 配体化合物包括色氨酸衍生物如犬尿氨酸，靛蓝和靛玉红，四吡咯类 (tetrapyroles) 如胆红素，花生四烯酸代谢产物如脂氧素 A4 和前列腺素 G，修饰的低密度脂蛋白，一些膳食类胡萝卜素和 7- 酮胆固醇。当配体结合时，伴侣蛋白解离导致 AHR 易位到细胞核并与 ARNT(AHR 核易位蛋白）二聚化。AHR 和 ARNT 复合体影响基因转录。

[0038] AHR 多肽具有一些对于功能重要的结构域，且其分类为转录因子碱性螺旋 - 环 - 螺旋 /Per-Arnt-Sim(bHLH / PAS) 家族的成员。其 bHLH 基序位于蛋白质的 N 末端。bHLH 超家族成员具有两个功能独特、高度保守的结构域。第一个是碱性区域，其参与转录因子和 DNA 的结合。第二个是螺旋 - 环 - 螺旋 (HLH) 区域，其有助于蛋白 - 蛋白的相互作用。AHR 还包含两个 PAS 结构域，PAS-A 和 PAS-B，其是 200-350 个氨基酸的片段，与果蝇基因 period(Per) 和 single-minded(Sim) 中发现的蛋白结构域具有高度的序列同源性。而且，相似的结构域存在于 ARNT。PAS 结构域支持与包括其他 PAS 结构域的蛋白质的特异的二级相互作用，如与 AHR 和 ARNT 的情况，从而能够形成杂合和纯合的蛋白质复合体。AHR 的配体结合位点包含在 PAS-B 结构域中，其含有一些对配体结合重要的保守残基。特别是，氨基酸 Tyr310、Phe324、His326 和 / 或 Arg352 看来是参与配体结合。最后，富含 Q 的结构域位于蛋白质的 C 末端区域，参与辅助激活因子的募集和反式激活。

[0039] 优选地，AHR 多肽是人 AHR，更优选地，是由 Genbank 登录号 :NM001621.4(GI : 229577137) 所示的多核苷酸编码的人 AHR 或具有如该登录号所示的氨基酸序列的人 AHR。而且，根据本发明，包含之前提及的 AHR 多肽变体。上述包含一个或多个核苷酸取代、缺失和 / 或添加的多核苷酸变体优选地导致其所编码的氨基酸具有一个或多个氨基酸取代、缺失和 / 或添加，即，根据本发明的多肽变体。优选地，多核苷酸变体包含与上述特定的核酸序列相比至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95%、至少 98% 或至少 99% 同一的核酸序列。而且，优选地，多核苷酸变体可以具有编码与上述氨基酸序列相比至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95%、至少 98% 或至少 99% 同一的氨基酸序列的核酸序列。术语“同一的”用于本文中，指序列同一性，其通过确定两个核酸序列或氨基酸序列间相同的氨基酸数目表征，其中比对序列从而获得最高顺序的匹配。可以使用出版的技术或计算机程序中编成的方法计算，如，例如 BLASTP、BLASTN 或 FASTA(Altschul1990，J Mol Biol215,403)。一方面，在氨基酸序列整体上计算同一性百分比，或在较长序列的至少 50% 的核苷酸上计算同一性百分比。为比较不同的序列，技术人员可获得基于多种算法的一系列程序。在本本文中，Needleman and Wunsch 或 Smith and Waterman 算法给出了特别可靠的

结果。为了进行序列比对，可以使用程序PileUp(Higgins1989,CABIOS5,151)或程序Gap和BestFit(Needleman1970,J Mol Biol48:443;Smith1981,Adv Appl Math2,482)，其为GCG软件包(Genetics Computer Group1991,575Science Drive, Madison, Wisconsin, USA53711)的一部分。在本发明的另一方面，使用具有如下设置的程序GAP确定序列整体区域上以上所述的序列同一性值百分比(%)：缺口权重：50，长度权重：3，平均匹配度：10.000，平均错配度：0.000，除非特别指出，其应当通常用作序列比对的标准设置。应理解以上提及的变体仍然基本上表现出与上述AHR同样的特定生物活性。

[0040] 直接抑制AHR活性的化合物，优选是能够物理上与AHR多肽相互作用的化合物，从而抑制该AHR多肽的活性。如果化合物结合AHR的相互作用结构域，或结合其配体结合结构域，则发生所述抑制，从而如本文中其他地方限定的，抑制AHR的生物功能。优选地，抑制剂阻断对犬尿氨酸的配体结合结构域，即，与PAS-B结构域的配体结合结构域或由Tyr310、Phe324、His326和Arg352(氨基酸位置对应于人AHR)形成的配体结合结构域相互作用。可替换地，化合物可引发对AHR多肽的别构效应，并导致生物功能的抑制。间接抑制可由降低或防止AHR多肽转录和/或翻译、从而降低细胞中可利用的AHR多肽含量的化合物引发。AHR抑制剂至少将AHR活性降低至统计学上有意义的程度。当然，优选地，抑制剂将AHR活性降低至低于检测限。可通过本领域公知的试验检测AHR活性的定性和/或定量抑制，优选地，通过本文中以下实施例中公开的试验检测。使用乙氧基-9-羟基异吩噁唑酮-邻-去乙基酶(EROD)试验，通过确定其内源靶标基因CYP1A1的诱导的基因表达，检测AHR活性。可替换地，使用报告基因分析检测AHR活性，其中报告基因的表达由二氧芑反应元件(DRE)依赖性启动子控制。用于测定AHR活性的特别优选的试验在本文中的实施例中详细公开了。

[0041] 优选地，AHR抑制剂是小分子化合物。

[0042] 在本发明的意义上的“小分子化合物”是一种分子量小于10kD、小于5kDa、小于2kDa、小于1kDa或小于500Da的有机分子。优选地，所述小分子不为聚合物。优选地，如根据本发明所指小分子是细胞渗透性的，并能扩散到细胞质中，以结合AHR多肽。如本本文中所提到的，小分子可以人工合成，并且可以包含在用于筛选潜在的AHR抑制剂的化学文库中。可替换地，小分子可以通过提取的方法从天然来源，如组织、细胞或整个生物体获得。特别地，合适的来源是植物、植物组织或微生物。然而，本文也包括AHR小分子抑制剂的其他来源。例如，7-酮胆固醇是人类中显然的AHR竞争性抑制剂(Savouret2001, J. Biol. Chem. 276(5):3054-9)。

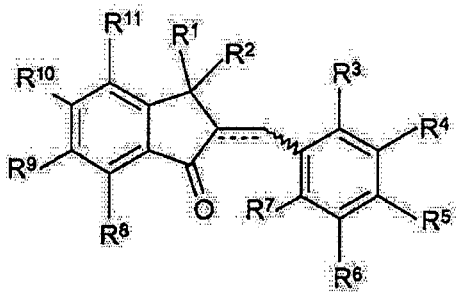
[0043] 在本发明方法的一个优选的实施方案中，所述小分子化合物是植物化合物或其衍生物。

[0044] “植物化合物或其衍生物”用于本文中，是通过提取的方法可从植物、植物组织或植物细胞获得的小分子。通常，小分子植物化合物是代谢产物，如初级，或特别优选地，次级植物代谢产物。在本发明方法的一个更优选的实施方案中，所述植物化合物或其衍生物是黄酮或其衍生物。最优选地，所述黄酮或其衍生物是3,4-二甲氧基黄酮、3′-甲氧基-4′-硝基黄酮、4′,5,7-三羟基黄酮(芹菜苷配基)或1-甲基-NN-[2-甲基-4-[2-(2-甲苯基)二氮烯基]苯基-1H-吡唑-5-羧酰胺。在本发明方法的另一个更优选的实施方案中，所述植物化合物或其衍生物是白藜芦醇(resveratrol)(反式-3,5,

4'-三羟基二苯乙烯）或其衍生物，表没食子儿茶素或表没食子儿茶素没食子酸酯。

[0045] 在本发明方法的另一个优选的实施方案中，其中所述小分子化合物是具有以下通式（I）特征的化合物：

[0046]



[0047] 其中

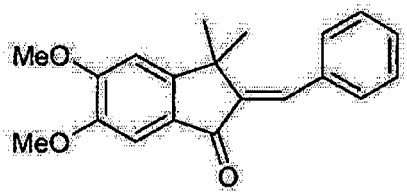
[0048] (i)R^1 和 R^2 彼此独立地是氢或 C_1 至 C_{12} 烷基，

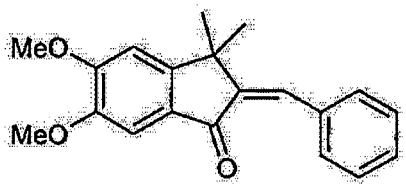
[0049] (ii)R^3 至 R^{11} 彼此独立地是氢、C_1 至 C_{12} 烷基、羟基或 C_1 至 C_{12} 烷氧基，和

[0050] (iii) 折线代表双键或两个氢。

[0051] 特别地，更优选地是具有下式（II）至（V）任一个的化合物：

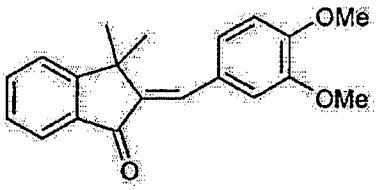
[0052]

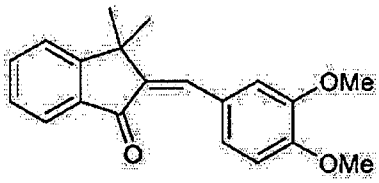




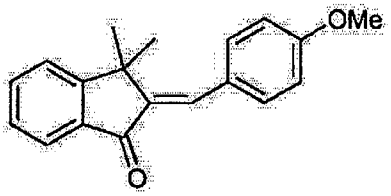
(II)

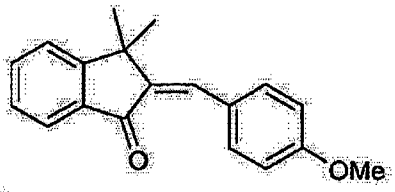
[0053]



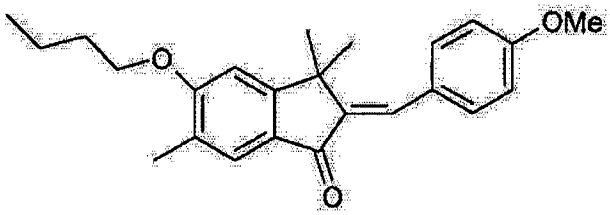


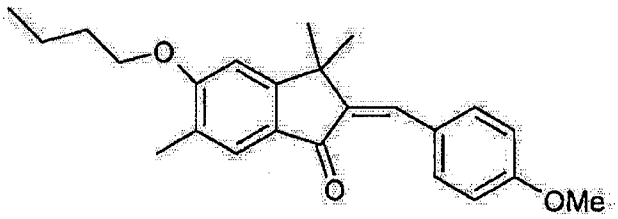
(III)





(IV)





(V)

[0054] 更多用于其生产的更优选化合物和方法公开于 WO2007 / 128723 中，其相应的公开内容引入本文中作为参考。

[0055] 在本发明方法的另一个更优选的实施方案中，所述 AHR 抑制剂是特异地结合并抑制 AHR 蛋白的抗体。

[0056] 如本文中其他地方特定的，术语“抗体”用于本文中，指所有类型的特异地结合 AHR 多肽、并抑制 AHR 活性的抗体。优选地，本发明的该抑制性抗体特异地结合位于配体结合结构域的 AHR 多肽中的表位。可替换地，抗体通过其抑制 AHR 活性的结合表位可以位于 AHR 的 DNA 结合结构域，或位于负责与 ARNT 多肽相互作用的结构域。合适的结构域在本文中其他地方描述了。优选地，本发明的抗体是单克隆抗体、多克隆抗体、单链抗体、嵌合抗体或任何该抗体的片段或衍生物。包含在术语抗体中的这样的片段或衍生物用于本文中，包括双

特异抗体、合成的抗体、Fab、F(ab)$_2$Fv 或 scFv 片段、或这些抗体中任何一个的化学修饰的衍生物。在本发明抗体的上下本文中使用的特异的结合，指抗体不与其他多肽交叉反应。可使用公知技术检测特异的结合。

[0057] 一般地，可使用例如 Harlow and Lane" Antibodies, A Laboratory Manual"，CSH Press, Cold Spring Harbor, 1988 中描述的方法获得抗体或其片段。可通过包括将小鼠骨髓瘤与源自致免疫哺乳动物（优选地，致免疫小鼠）的脾细胞融合的技术制备单克隆抗体（**Köhler** 1975, Nature256, 495, and Galfr61981, Meth. Enzymol. 73, 3）。优选地，将具有上述表位的免疫原性肽施加到哺乳动物。根据宿主种属，可使用佐剂增强免疫反应。这样的佐剂包括，优选地，弗氏佐剂、矿物凝胶，如氢氧化铝、和表面活性物质，如溶血卵磷脂、普流罗尼多元醇、聚阴离子、肽、油乳剂、匙孔血蓝蛋白和二硝基苯酚。

[0058] 在本发明方法的另一个实施方案中，上述 AHR 抑制剂是 AHR 蛋白阻遏蛋白或失活的 AHR 核易位蛋白 (ARNT)。

[0059] 术语“AHR 阻遏蛋白 (AHRR)”用于本文中，指一般认定的负性调节 AHR 和 AHR / ARNT 复合体活性的肿瘤抑制基因。优选地，本文中涉及的编码 AHRR 多肽的多核苷酸以及 AHRR 多肽的氨基酸序列本身如 Genbank 登录号 BC151852(GI :156229770) 所示。而且，根据本发明的 AHRR 多肽可以是以上提及的特定多核苷酸或多肽的变体。上述包含一个或多个核苷酸取代、缺失和 / 或添加的多核苷酸变体优选地导致其所编码的氨基酸具有一个或多个氨基酸取代、缺失和 / 或添加，即，根据本发明的多肽变体。优选地，多核苷酸变体包含与上述特定的核酸序列相比至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95%、至少 98%或至少 99%同一的核酸序列。而且，优选地，多核苷酸变体可以具有编码与上述氨基酸序列相比至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95%、至少 98%或至少 99%同一的氨基酸序列的核酸序列。在本文中的其他地方详细公开了如何计算两个给定序列之间的序列同一性。

[0060] 如以上讨论的，本领域技术人员可根据前述特定的多核苷酸或氨基酸序列或其变体设计 ARNT 多肽的失活版本，而不需过度劳动。而且，这些失活的 ARNT 多肽或编码其的多核苷酸可被本领域公知的方法导入待治疗癌症细胞中。特别地，根据本发明，可以考虑将通过病毒表达系统的基因转移作为编码失活 ARNT 的多核苷酸的传递系统。适合的技术是本领域公知的（见上）。

[0061] 术语“AHR 核易位蛋白 (ARNT)”用于本文中，指 AHR 转录因子的结合蛋白。具体参见本说明书的其他部分。本文中涉及的作为 AHR 抑制剂的 ARNT 多肽是仍然能够与 AHR 相互作用的多肽，但是其阻止了核易位、或引导 AHR / ARNT 复合体被细胞的蛋白降解机制降解。如何设计该修饰的抑制性 ARNT 多肽是本领域技术人员公知的。优选地，本文中涉及的编码（未修饰的）ARNT 多肽的多核苷酸如 Genbank 登录号 :NM001197325.1(GI :309747070) 所示。优选地，所述多核苷酸编码具有如 Genbank 登录号 :（蛋白）NP001184254.1(GI : 309747071) 所示氨基酸序列的多肽。而且，根据本发明的 ARNT 多肽可以是以上提及的特定 ARNT 多核苷酸或多肽的变体。上述包含一个或多个核苷酸取代、缺失和 / 或添加的多核苷酸变体优选地导致其所编码的氨基酸具有一个或多个氨基酸取代、缺失和 / 或添加，即，根据本发明的多肽变体。优选地，多核苷酸变体包含与上述特定的核酸序列相比至少 40%、

至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95%、至少 98%或至少 99%同一的核酸序列。而且，优选地，多核苷酸变体可以具有编码与上述氨基酸序列相比至少 40%、至少 50%、至少 60%、至少 70%、至少 75%、至少 80%、至少 85%、至少 90%、至少 95%、至少 98%或至少 99%同一的氨基酸序列的核酸序列。在本文中的其他地方详细公开了如何计算两个给定序列之间的序列同一性。

[0062] AHRR 多肽或编码其的多核苷酸可被本领域公知的方法导入待治疗癌症细胞。特别地，根据本发明，可以考虑将通过病毒表达系统的基因转移作为编码失活 ARNT 的多核苷酸的传递系统。适合的技术是本领域公知的，在例如 Gardlik2005, Med Sci Monit. 11(4) : RA110-21 ;Salmons1993, Hum Gene Ther. 4(2) :129-41 中描述了。

[0063] 在本发明方法的另一个优选的实施方案中，其中所述 AHR 抑制剂是核酸抑制剂。

[0064] “核酸抑制剂”用于本文中，指如适配体的核酸分子，其通过以类似于对上述抗体所描述的方式结合多肽来抑制 AHR 多肽活性，或者指由于与编码 AHR 多肽的多核苷酸互补而结合所述多核苷酸的核酸分子，其抑制多核苷酸的转录或翻译。例如，抑制性核酸可通过干扰 AHR 基因的正确转录作为三螺旋形成寡核苷酸发挥作用。而且，抑制性核酸可以是核酶，其特异地结合并降解 AHR 转录物。可替换地，其可以是能够结合、降解转录物或至少抑制其有效翻译的反义（核酸）、siRNA、或 microRNA。后一类型的抑制性核酸的特征在于其通常包含与 AHR 转录物中的序列互补的核酸序列。该互补序列应当足够长，且应当包含足够数目的匹配核苷酸，从而允许与细胞中转录物的特异杂交。当被本文中其他地方所指的基因转导系统传递后，该核酸抑制剂能够在癌症细胞中表达。优选地，抑制性核酸分子能够在表达控制序列的控制下被表达。因而，能够通过表达控制序列调节 RNAi 的调控，以抑制靶标基因的表达，所述表达控制序列能被外源刺激、如 tet 操纵子调节，而 tet 操纵子的活性被四环素或热诱导型启动子或在肿瘤特异或组织特异的启动子的控制下调节。但是，核酸抑制剂还能被基于脂质体的传递系统传递。

[0065] 因而，在本发明方法的另一个更优选的实施方案中，所述核酸抑制剂选自核酶、反义分子、抑制性寡核苷酸、适配体、microRNA 和 siRNA。

[0066] 根据本发明的“核酶”是包含与 AHR 转录物互补的序列的 RNA 分子。而且，核酶包含能够在 AHR 转录物中引起磷酸二酯键水解的核酸序列。根据本发明所指的核酶可以是所谓的锤头状核酶、发夹核酶或 VS 核酶。核酶技术是本领域公知的，本领域技术人员能够设计和应用合适的核酶，而无需过度劳动 ;参见，例如，Khan2006, Clin. Chim. Acta367(1-2) : 20-27 ;Kalota2004, Cancer Biology & Therapy3 :14-12。

[0067] “反义分子”用于本文中，指与 AHR 转录物互补的治疗性反义 RNA 或能够结合 AHR 转录物的吗啉代寡核苷酸。包括吗啉代寡核苷酸应用的反义技术是本领域公知的，参见，例 如 Kalota2004, Cancer Biology&Therapy3 :14-12 ;Morcos2007, Biochem Biophys Res Commun358(2) :521-7。

[0068] 抑制性寡核苷酸用于本文中，优选地，指能够结合靶标基因组 DNA 特定的区域、从而实现基因沉默（所谓的三螺旋形成寡核苷酸）的小双链 DNA 分子，或者指作为诱饵发挥作用、而阻隔靶标基因转录特异地需要的转录因子的寡核苷酸。这些技术已经成功地用于体内，而且在某种程度上在治疗中已经获得结果（也参见 Kalota2004, Cancer Biology&Therapy3 :14-12.）。

[0069] 术语“适配体”用于本文中，指特异地结合 AHR 多肽的核酸适配体。通过使用，例如通过指数富集的配体系统进化 (SELEX) 技术，可生成适配体的池 (pool)。能将选择步骤用于那些特异地结合 AHR 多肽的适配体。在特异地结合的适配体中，阻断配体结合的那些适配体，或阻断相互作用结构域的那些适配体，因而能被鉴定为本发明意义上适合的适配体。用于生成适配体的技术是本领域公知的；参见，例如 Tuerk1990, Science. Aug3；249(4968)：505-510；or Ellington1990, Nature. Aug30；346(6287)：818-822。

[0070] 在本发明意义上的“microRNA”指单链 RNA 分子，其与 AHR 转录物中包含的核酸序列至少部分互补。microRNA 通常具有大约 19 至 26 个核苷酸长度。microRNA 作为前体合成，即所谓的 pri-microRNA，所述 pri-microRNA 具有发夹结构和形成发夹茎的两个互补的自互补区域。自互补核酸序列之一是 microRNA。pri-microRNA 具有大约 70 个核苷酸的长度，其在靶标细胞中被加工成成熟 microRNA。成熟 microRNA 能够通过影响与其杂交的待转录的 mRNA 的翻译或稳定下调基因表达。如何设计 microRNA 以及其 pri-microRNA 前体是本领域技术人员公知的。特别地，内源 pri-microRNA 分子的自互补区域被自互补区域对取代，所述自互补区域对包含至少部分地与 AHR 转录物互补的一个自互补区域。microRNA 技术描述于，例如，Bartel2009, Cell136(2)：215-33, Trang2008, Oncogene27Suppl2：S52-7or Li2009, The AAPS journal11(4)：747-57 中。

[0071] 根据本发明所指的“短发夹 RNA(shRNA)”具有与上述 pri-microRNA 相似的结构。但是，shRNA 通常在长度上比较短。更优选地，根据本发明所指的作为 AHR 抑制剂的 shRNA 是包含如 SEQ ID NO：1 至 4 任一个所示的核酸序列的核酸分子或是基本上由如 SEQ ID NO：1 至 4 任一个所示的核酸序列组成的核酸分子。shRNA 的设计和应用是本领域公知的，描述于 McIntyre2006, BMC Biotechnol. 6：1or Cao2005, J Appl Genet. 46(2)：217-25 中。

[0072] 术语“小干扰 RNA(siRNA)”指为双链 RNA 剂的核酸分子，其与 AHR 转录物的一部分互补，并能够碱基配对。siRNA 通过特异地指导宿主细胞中的酶而发挥作用，从而切割靶标 RNA。凭借 siRNA 序列的特异性，以及其与 RNA 靶标的同源性，siRNA 能够引起靶标 RNA 链的切割，从而失活靶标 RNA 分子。优选地，足以调节 RNAi 的 siRNA 包含如下核酸序列，其含有靶标基因反向重复片段和目标基因编码区域（或其部分）。siRNA 的互补区域允许 siRNA 足以与靶标 RNA 杂交，从而调节 RNAi。在哺乳动物中，siRNA 是大约 19-25 个核苷酸的长度。siRNA 序列需要足够长以使得 siRNA 和靶标 RNA 通过互补碱基对的相互作用在一起。siRNA 的长度优选大于或等于 10 个核苷酸，且足够长以与靶标 RNA 稳定地相互作用；特别是 15-30 个核苷酸、更特别地是 15 至 30 间的任意整数个核苷酸、最优选 15、16、17、18、19、20、21、22、23、24、25、26、27、28、29 和 30。足够长，意指大于或等于 15 个核苷酸的寡核苷酸，其长度足以提供预期条件下的需要的功能。稳定的相互作用，意指小的干扰 RNA 与靶标核酸的相互作用（例如，通过在生理条件下与靶标中的互补核苷酸形成氢键）。一般而言，该互补程度是 siRNA 与 RNA 靶标之间的 100％的互补，但是如果需要，可以少于 100％，优选 91％、92％、93％、94％、95％、96％、97％、98％或 99％。例如，21 个碱基中的 19 个碱基可以碱基配对。在一些例子中，当需要在不同等位基因变体之间选择时，为了从其他等位基因序列中有效区分靶标序列，需要 100％与靶标基因互补。涉及使用 RNAi 在生物体中，包括在线虫、果蝇、植物和哺乳动物中沉默基因的方法，是本领域公知的（参见，例如，Fire1998, Nature391：806-811；Fire1999, Trends Genet. 15, 358-363；WO2001 / 29058；WO2009 /

932619)。

[0073] 最后，一般而言，本发明中还涉及AHR抑制剂，优选如上本文所定义的AHR抑制剂，用于治疗和/或预防与增加的色氨酸-2,3-双加氧酶(TDO)活性相关的癌症。

[0074] 迄今为止，由Trp降解引起的癌症相关的免疫抑制被主要归因于癌症细胞和肿瘤引流淋巴结中IDO的酶活性。因而，IDO抑制目前被评估为临床试验中治疗癌症的治疗策略，尽管其对人癌症细胞有一些脱靶效应。在本发明的研究中表明TDO强烈地表达于癌症中，同样地能够产生免疫抑制的Kyn。在IDO阴性神经胶质瘤细胞中，TDO看起来是组成型Trp降解的主要决定因素，表明TDO代表了神经胶质瘤治疗中的新治疗靶点。实际上，最近开发了可口服利用的TDO抑制剂。由于我们描述了组成型Trp降解通过作用于肿瘤细胞自身对维持癌症恶性表型的重要性，TDO的抑制可能不仅恢复了抗肿瘤免疫反应，还作用于肿瘤细胞固有的恶性表型。出现证据指向AHR的肿瘤促进作用。AHR的激活促进癌症细胞的克隆发生和侵袭。具有组成型活性的AHR的转基因小鼠自发形成肿瘤，AHR抑制剂(AHRR)是多种人癌症中的肿瘤抑制剂。Ahr-缺陷小鼠的异常表型指向内源AHR配体的存在。尽管不同的内源产生的代谢产物，如花生四烯酸代谢产物、胆红素、cAMP、色胺和6-甲酰基吲哚并[3,2-b]咔唑(6-formylindolo[3,2-b]carbazole)(FICZ)被表明是AHR激动剂，其在病理生理的环境，如癌症或免疫激活的环境中的功能还没有被有说服力地证实。因而，寻找AHR的内源配体仍在进行。

[0075] 根据本发明，通过表明Trp分解代谢导致AHR的激活，(公开了)参与癌症进展的这两个重要途径，并提供了与产生足够量的功能相关的内源AHR配体有关的人适应症的病理生理证据。本发明研究的结果揭示了原代免疫细胞与转化的癌症细胞对AHR介导信号的不同反应，这与使用经典外源AHR配体、TCDD和3-MC的多种毒理学研究一致。暴露于异生素导致细胞和体液免疫反应的严重抑制，同时还促进致癌作用，诱导肿瘤生长。这些AHR作用中细胞特异的差异可能取决于不同地调节AHR信号传导的各因子的表达，如AHRR，以及定型对AHR激活的反应的细胞特异的转录因子串话的表达。Kyn介导的AHR激活可能不仅与癌症发生有关。例如，由激动剂型配体激活的小鼠和人AHR诱导调节性T细胞。有趣的是，在没有外源配体时，Ahr-缺陷小鼠遭受恶化的CNS自体免疫，而Trp代谢产物抑制CNS自体免疫，证明Trp分解代谢的激活代表了通过AHR限制炎症的内源性反馈环。实际上，外源Kyn通过AHR参与小鼠免疫细胞的调节。在对炎症刺激反应时也由IDO产生足以激活AHR的Kyn浓度。在更广的意义上，显著数量的恶性肿瘤大多数来自于慢性感染和炎症区域，而肿瘤微环境中的Trp分解代谢被激活并维持局部免疫抑制。因而，在对炎症刺激反应时由Kyn产生的AHR激活可能构成之前没有认识的连接炎症和癌发生的路径。

[0076] 本说明书中引用的所有文献，包括其整体公开的内容和本说明书特别提及的内容，在本文中引做参考。

实施例

[0077] 现在本发明将描述以下实施例，但是，其并不能限制本发明的范围。

[0078] 实施例1:材料和一般方法

[0079] 细胞和试剂

[0080] 细胞系的来源和培养条件在增补的方法中详细描述了。所有的神经胶质瘤初

始细胞 (GIC) 建立于新鲜切除的肿瘤，并在第一代期间使用。常规检测所有细胞的细菌污染。使用不含 Trp 的 RPMI1640(Promocell) 和渗吸的 FBS(Invitrogen) 在不含 Trp 的条件下培养细胞。L-Trp 和 L-Kyn 来自 Sigma-Aldrich。干扰素 -γ(IFN-γ) 来自 Immunotools(Friesoythe, 德国)。TCDD 和 3- 甲基胆蒽 (3-MC) 来自 Sigma-Aldrich，3,4- 二甲氧基黄酮 (3,4-DMF) 来自 Alfa Aesar(Karlsruhe, 德国)。TDO 抑制剂 ((E)-6- 氟 -3-[2-(3- 吡啶) 乙烯基]-1H- 吲哚)680C91 在哌啶存在时通过缩合 6- 氟吲哚 -3- 甲醛和吡定 -3- 乙酸而合成。

[0081] 小鼠

[0082] C57BL / 6N 和 CD-1nu / nu 小鼠购自 Charles River(Sulzfeld，德国)。Ahr- 缺陷小鼠 (B6.129-AHRtm1Bra/J) 由 Charlotte Esser(Düsseldorf, 德国) 善意提供。与 Ahr- 缺陷小鼠年龄匹配的 C57BL / 6N 来自 Harlan Laboratories(Rossdorf，德国)。

[0083] TDO 表达分析

[0084] 通过免疫组化分析人肿瘤中的 TDO 表达。使用 TDO 基因敲除或过度表达确定其与 Trp 降解的相关性。由 HPLC 测量细胞培养物上清、人血清和异种移植物组织中的 Trp 和 Kyn。使用混合白细胞反应、铬释放、肿瘤组织中的 ELISpot 和免疫细胞染色法评估 TDO 活性的免疫作用。应用细胞周期分析、基质胶和球状体侵袭试验、划痕试验、球形成试验和克隆发生试验分析 TDO 活性的自分泌作用。所有动物操作遵循设立的实验动物研究指南，并经管理机构许可。将有或没有稳定的 TDO 基因敲除的人神经胶质瘤细胞同位移植至 CD1nu / nu 小鼠，将这些细胞皮下注射到不含 NK 或野生型的 CD1nu / nu 小鼠，将鼠 Tdo- 正常和 Tdo- 缺陷的 GL261 细胞皮下注射到同系的 C57BL / 6N 小鼠，以分析体内 TDO 活性的自分泌和旁分泌作用。进行 Kyn 处理的人神经胶质瘤细胞的微阵列分析，以鉴定由 Kyn 激活的信号传导路径。AHR 易位分析、DRE 萤光素酶试验和放射性配体结合试验确认了由 Kyn 可激活 AHR。药理学抑制和 AHR 稳定的基因敲除 (体外和体内) 证实了 Kyn 的作用是 AHR 依赖的。使用将 Tdo 正常和 Tdo 缺陷肿瘤细胞注射到 Ahr+ / + 和 Ahr-/- 小鼠，解决了宿主作用对 TDO 介导癌症的促进的贡献。最后，使用人肿瘤组织的染色、微阵列数据和临床数据分析了 TDO 是否激活人癌症中的 AHR，以及其如何影响生存。

[0085] 由高效液相色谱法 (HPLC) 分析 Trp 和 Kyn 浓度

[0086] 使用具有光电二极管阵列 (PDA) 检测和 Lichrosorb RP-18 柱 (250mm x4mm ID，5μm，Merck，Darmstadt，德国) 的 Beckman HPLC 进行 HPLC 分析。测定 $3x10^5$ 个细胞的培养基中 Kyn 和 Trp 的浓度。在获得知情同意后，从 24 例成胶质细胞瘤患者 (10 例女性、14 例男性、年龄中间值 54.5 岁) 和 24 例年龄、性别匹配的健康对照 (10 例女性、14 例男性、年龄中间值 53.5 岁) 获得人血清，分析 Trp 和 Kyn 浓度。为了测定 U87 异种移植物中的 Kyn 浓度，切下 U87 肿瘤、称重、立刻在液氮中冰冻、加工。

[0087] 定量 (q)RT-PCR

[0088] 使用 Qiagen RNeasy 试剂盒分离总 RNA，使用 Applied Biosystems 逆转录试剂盒 (Foster City，CA，USA) 合成 cDNA。使用 SYBR Green PCR Mastermix(both Applied Biosystems)、在 ABI7000 热循环仪器中进行 QRT-PCR。所有的引物被基因组 DNA 上至少一个内含子分开，以排除扩增基因组 DNA。通过包含非 RT 对照、通过模板删节、以及通过溶解曲线和凝胶分析检查 PCR 反应。为每个基因制备标准曲线。通过比较阈值确定基因表达的

相对定量。所有结果相对 GAPDH 归一化。

[0089] siRNA 实验

[0090] 为了基因敲除 IDO1(INDO)，IDO2 和 TDO(TDO2)，使用 Dharmacon RNA Technologies(Lafayette，CO，USA) 的 SMART- 池 siRNA。靶标序列如下所示：

[0091] 人 INDO(Genbank 登录号 NM_002164)：

[0092] 5’-UCACCAAAUCCACGAUCAUUU-3’(SEQ ID NO:5)；

[0093] 5’-UUUCAGUGUUCUUCGCAUAUU-3’(SEQ ID NO:6)；

[0094] 5’-GUAUGAAGGGUUCU GGGAAUU-3’(SEQ ID NO:7)；

[0095] 5’-GAACGGGACACUUUGCUAAUU-3’(SEQ ID NO:8)

[0096] 人 IDO2(Genbank 登录号 NM_194294)：

[0097] 5’-CAAACUUCCUCAAUUGAUU-3’(SEQ ID NO:9)；

[0098] 5’-UUGGAAAGCUAUCACAUAU-3’(SEQ ID NO:10)；

[0099] 5’-GAGUAUGGCUUUCUUCUUC-3’(SEQ ID NO:11)；

[0100] 5’-GCACCCAGUUGAAGUUUAA-3’(SEQ ID NO:12)

[0101] 人 TDO2(Genbank 登录号 NM_005651)：

[0102] 5’-UCAUAAGGAUUCAGGCUAA-3’(SEQ ID NO:13)；

[0103] 5’-AGUGAUAGGUACAAGGUAU-3’(SEQ ID NO:14)；

[0104] 5’-GGAUUUAACUUCUGGGGAA-3’(SEQ ID NO:15)；

[0105] 5’-GCGAAGAAGACAAAUCACA-3’(SEQ ID NO:16)

[0106] TDOAshRNA 正义：

[0107] 5’-GGAAAGAACTCCAGGTTTATTCAAGAGATAAACCTGGAGTTCTTTCC-3’(SEQ ID NO:17)

[0108] TDOAshRNA 反义：

[0109] 5’-CCTTTCTTGAGGTCCAAATAAGTTCTCTATTTGGACCTCAAGAAAGG-3’(SEQ IDNO:18)

[0110] TDOBshRNA 正义：

[0111] 5’-TCATAAGGATTCAGGCTAATTCAAGAGATTAGCCTGAATCCTTATGA-3’(SEQ ID NO:19)

[0112] TDOBshRNA 反义：

[0113] 5’-AGTATTCCTAAGTCCGATTAAGTTCTCTAATCGGACTTAGGAATACT-3’(SEQ ID NO:20)

[0114] ON-TARGET plus siCONTROL 非靶标池 (D-001810-10-05，Dharmacon) 和不含 siRNA 的转染被用作阴性对照。使用购自 Invitrogen 的脂质体 RNAiMAX 转染细胞。由 qRT-PCR 分析基因敲除效率。

[0115] 稳定的基因敲除细胞

[0116] 使用 FUGENE HD 转染试剂 (Roche，Mannheim，德国)，用表达 sh-TDO 的 pSUPER. puro 质粒 (OligoEngine，Seattle，WA，USA) 或干扰对照 (scrambled control) 转染 U87 人神经胶质瘤细胞。转染 72 小时后，将培养基交换为含有 5μg/ml 嘌呤霉素 (AppliChem GmbH) 的 DMEM。如果没有另外的说明，使 sh-TDOA。为了在 LN308 神经胶质细胞中基因敲除 AHR，从 Clontech(CA，USA) 购买了 pSingle-tTS-shRNA 载体。使用 XhoI / HindIII 克隆位点，将编码所需 shRNA 序列的带有 XhoI / HindIII 悬垂的退火 ds 寡核苷酸克隆至载体。用于对照 / AHR 或包含 XhoI / HindIII 悬垂的 TDO shRNA 沉默的短发夹序列如下所示：

[0117] 干扰 shRNA 反义寡核苷酸：

[0118] 5'-AGCTTGGATCCAAAAAAGTACTTCCACCTCAGTTGGCTCTCTTGAAGCCAACTGAGGTGGAAGTACC-3'(SEQ ID No :21),

[0119] 干扰 shRNA 正义寡核苷酸:

[0120] 5 ' -TCGAGGTACTTCCACCTCAGTTGGCTTCAAGAGAGCCAACTGAGGTGGAAGTACTTTTTTGGATCCA-3'(SEQ ID NO :22),

[0121] AHR shRNA 反义寡核苷酸:

[0122] 5'-AGCTTGGATCCAAAAAAGCGTTTACCTTCAAACTTTATCTCTTGAATAAAGTTTGAAGGTAAACGCC-3'(SEQ ID NO :1),

[0123] AHR shRNA 正义寡核苷酸:

[0124] 5'-TCGAGGCGTTTACCTTCAAACTTTATTCAAGAGATAAAGTTTGAAGGTAAACGCTTTTTTGGATCCA-3'(SEQ ID NO :2),

[0125] AHR shRNA 反义寡核苷酸 (Dharmacon siRNA#6,AHRAHR siRNA 的 Smart 池):

[0126] 5'-AGCTTGGATCCAAAAAAGGAACTCAAGCTGTATGGTATCTCTTGAATACCATACAGCTTGAGTTCCC-3'(SEQ ID NO :3),

[0127] AHR shRNA 正义寡核苷酸 (Dharmacon):

[0128] 5'-TCGAGGGAACTCAAGCTGTATGGTATTCAAGAGATACCATACAGCTTGAGTTCCTTTTTTGGATCCA-3'(SEQ ID NO :4).

[0129] 将重组载体转染至 LN-308 和 LN-18 神经胶质细胞,用 1mg/ml 新霉素 (Sigma-Aldrich) 选择克隆的转化体。使用 2μg / ml 强力霉素 (Sigma-Aldrich) 诱导基因敲除,诱导后 72 修饰分析细胞。如果没有另外的说明,使 sh-AHR1。

[0130] 稳定的过表达

[0131] 使用 FUGENE HD 试剂 (Roche),用表达 Tdo cDNA(NM_019911) 的 pcDNA3.1(-) (Invitrogen) 或空载体转染 GL261 细胞。使用 1mg / ml 新霉素 (Sigma-Aldrich) 选择克隆的转换体。

[0132] 组织标本和免疫组化

[0133] 孵育切成 3μm 的切片,使用 Ventana BenchMark XT® 免疫染色 (Ventana),用各自的抗体加工切片。为了进行 TDO 染色的定量分析,使用光学显微镜 (Olympus BX51),在代表性的放大率视野 (200x) 下,在组织切片上评估染色的肿瘤细胞百分比和染色强度。

[0134] 免疫荧光染色

[0135] 为了进行免疫荧光染色,在 Ventana 细胞调节器中,经 30 分钟热介导的抗原修复后,用兔抗 TIP ARP(1 : 50) 和小鼠抗 LCA(1 : 50),在 4℃下过夜孵育具有 TDO 低表达的神经胶质瘤切片和 TDO 高表达的神经胶质瘤切片。然后,施用驴抗兔 AlexaFluor568(1 : 500, Invitrogen) 和驴抗小鼠 DyLight488(1 : 100,Jackson ImmunoResearch,West Grove,PA, USA) 次级抗体 5 小时。在 Olympus BX-50 显微镜下 (Olympus GmbH, Hamburg,德国)、使用 Zeiss Axiocam MRm(Zeiss, Jena,德国) 拍摄显微照片。

[0136] 混合白细胞反应 (MLR)

[0137] 在 96 孔平板上、在含有 10 % FBS、100U / mi 青霉素和 100μg/ml 链霉素的 RPMI1640 中接种神经胶质瘤细胞。接种 24 小时后,加入作为刺激物的 $2x10^5$ 个经辐射的 (30Gy)PBMC 和来自不相关供体、作为反应物的 $2x10^5$ 个 PBMC。进行 6 天的 MLR,用 [3H]- 甲

基胸苷 (PerkinElmer, Waltham, MA, USA) 脉冲培养物，持续至少 18 小时。收获细胞，通过闪烁计数测定放射性核素摄取。用六个不相关供体的 PBMC 供体重复实验。

[0138] 报告试验

[0139] 如增补的方法中详细描述的进行双萤光素酶 / 海肾 (renilla) 试验。为了进行 DRE 驱动的对不同 Trp 代谢产物反应的报告基因活性分析，使用 pGL3- 启动子表达质粒和对照质粒 pRL-SV40、表达海肾萤光素酶 Dual 萤光素酶 (Promega, Heidelberg, 德国)。

[0140] 侵袭试验

[0141] 在基质胶试验中，使用微网格通过在 5 个独立的显微镜高倍视野中计数跨膜迁移的细胞数评估细胞侵袭，并将其表示为相对于对照的侵袭百分比。在球状体试验中，每个球状体覆盖区域的显微照片是在移植后 0、24、48 和 72 小时拍摄的。对于定量分析，用 ImageJ 测定在 24 小时的时间间隔测定指定时间点的侵袭的神经胶质瘤细胞所覆盖的平均面积，并与在 0 小时的面积相比。

[0142] 铬释放测定

[0143] 使用标准的 51 铬释放试验 (增补的方法) 分析免疫细胞细胞毒性的抑制。特异性裂解的百分比如下计算 :[实验的 51Cr 释放 - 最小释放] / [最大释放 - 最小释放]x100。该实验用至少 4 个不同的 PBMC 供体进行。

[0144] 酶联免疫斑点技术试验 (ELISpot)

[0145] 从健康 C57BL / 6N 小鼠骨髓分离树突状细胞 (DC)，并培养在含 20ng / ml GM-CSF(Immunotools) 的 RPMI1640 中，5 天。从患肿瘤的小鼠中移除脾脏，并通过 40-μm 的细胞过滤器研磨。红细胞被裂解，使用 pan T 细胞分离试剂盒 II(Miltenyi GmbH) 通 MACS 分离 T 细胞。在包被抗 IFNγ 抗体 (Mabtech AB, Nacka Strand, Sweden) 的 ELISpot 平板 (Millipore) 上接种 $2x10^5$ 个 DC，并用在 PBS 中反复冻融循环产生的 10μgGL261 裂解液脉冲 4 小时，而后加入 $1x10^5$ 个 T 细胞。36 小时后，使用生物素化的抗 IFNγ 抗体、链霉亲和素 -ALP 和 BCIP / NBTPLUS(Mabtech) 检测产生 IFNγ 的 T 细胞，并使用 ImmunoSpot Analyzer(Cellular Technology Limited, Shaker Heights, OH, USA) 定量。

[0146] AHR 易位的检测

[0147] 为了检测 AHR 易位，将每孔 7000 个带有 GFP 标记的 AHR 的 Tao BpRclc 细胞暴露于 50μM Kyn 或 50μM Trp，在含 3.7% 甲醛的 PBS 中固定，使其在 0.1% Triton X100 中透化，与 1μg / ml Hoechst33342(Invitrogen) 孵育，并在 BD Pathway™ Imager855 上、使用 20xU-Apo340 物镜 (Olympus, NA0.75) 在非共焦模式下成像。使用 Attovision 软件 (BD Biosciences) 进一步分析荧光强度。另外，通过免疫印迹比较 LN-229 神经胶质瘤细胞的细胞核和细胞质中 AHR 蛋白含量。

[0148] 用 3H- 标记的 Kyn 的放射性配体结合试验

[0149] 从 Quotient Bioresearch(Radiochemicals)Ltd. (Cardiff, UK) 获得具有 11Ci / mmol 比活性的 L-3H-Kyn。使用来自 Ahr- 正常和 Ahr- 缺陷小鼠的小鼠肝脏细胞溶胶进行与 L-3H-Kyn 的结合试验。特异性结合定义为 Ahr- 正常和 Ahr- 缺陷胞液间放射活性的差异。

[0150] 动物实验

[0151] 所有动物操作遵循设立的实验动物研究指南，并是管理机构许可的。人神经胶质

瘤细胞皮下注射或立体定向地移植到六只 6-12 周龄无胸腺小鼠 (CD1nu / nu) 的右侧纹状体，并监控。在肿瘤细胞注射两天前，开始每两周的腹腔注射兔抗去唾液酸 GM1 抗体 (Wako Chemicals,Duesseldorf,德国)，以去除 NK 细胞。对照注射兔 IgG(Calbiochem,Darmstadt,德国)。为了诱导体内的 AHR 基因敲除，在含蔗糖的饮用水中，以 2mg / ml 的浓度向小鼠施用强力霉素。将鼠神经胶质瘤细胞皮下注射至 6-12 周龄野生型 C57BL6 / N 小鼠或 AHR- / -C57 / B16 小鼠右侧胁腹。

[0152] 磁共振成像 (MRI)

[0153] 在传统的全身 1.5T MRI 扫描仪上 (Symphony,Siemens,Erlangen,德国) 使用定制开发的发送 / 接受小动物线圈进行图 3g 中所示的 MRI 扫描。

[0154] 微阵列

[0155] 用 100μM Kyn 处理 U87 神经胶质瘤细胞 8 小时或 24 小时，而后收集细胞，使用 RNAeasy- 试剂盒 (Qiagen) 分离 RNA。如在增补的方法中详细描述的，将 RNA 进行微阵列分析。对四种处理 (8 小时、24 小时、Kyn 处理的、未处理的) 的每一个进行两个微列阵杂交，计算在 Kyn 处理样品对未处理样品中基因表达的平均 log2 比率。进一步的数据分析在增补的方法中详述了。从分子脑瘤数据资源库 (the REpository for Molecular BRAin Neoplasia DaTa(REMBRANDT)) 获得临床样品的微阵列和临床数据 (增补的方法)。使用 R2 微阵列分析的 Kaplan-Meier 分析模块和可视化平台 (http : / / r2.amc.nl) 进行 The Cancer Genome Atlas(TCGA) 网络中未处理的原代成胶质细胞瘤 (n=362) 数据组中的存活分析。

[0156] 统计学分析

[0157] 数据表示为平均值 ±s.e.m。使用学生 t 检验 (SigmaPlot) 进行显著性分析。P 值 <0.05 被认为是显著的。由斯皮尔曼等级相关 (SPSS，IBM，Somers，NY,USA) 分析 Ki67 和 TDO 的相关性。由斯皮尔曼等级相关 (Sigmaplot) 分析 TDO 和 AHR(图 6c) 以及 TDO 和 CYP1B1(图 6d) 之间的相关性。先对等级进行单向 ANOVA 分析 (p=<0.001)，随后进行 Dunn's 方法的分析 (p<0.05)，来分析核荧光强度。

[0158] 实施例 2 :由 TDO 产生的 Kyn 自分泌和旁分泌作用

[0159] 根据本发明的研究，人癌症细胞系的筛选揭示了人脑肿瘤细胞，即神经胶质瘤细胞系和神经胶质瘤初始的细胞 (GIC)、而不是人星形胶质细胞中，组成型 Trp 降解和高微摩尔含量 Kyn 的释放 (图 1a)。令人惊奇地，IDO1 和 IDO2 并不引起脑肿瘤中组成型 Trp 分解代谢。相反地，显著表达在肝脏并被认为调节系统 Trp 浓度的色氨酸 2-3- 双加氧酶 (TDO)，在人神经胶质瘤细胞中强烈地表达，并与 Kyn 的释放相关 (图 1b)。药理学抑制或 TDO 的基因敲除阻断了神经胶质瘤细胞释放 Kyn，而基因敲除 IDO1 和 IDO2 并没有作用 (图 1c，d)，因此，确认了 TDO 是人神经胶质瘤细胞中主要的 Trp 降解酶。在人脑肿瘤标本中，TDO 蛋白水平随恶性度而增加，并与增殖指数相关 (图 1e-g)。如之前所描述的 (Miller2004，Neurobiol Dis15(3)，618，健康人脑在神经元中仅表现弱的 TDO 染色 (图 . 1e)。TDO 表达不仅局限于神经胶质瘤，但是也可以在本文中其他地方所述的其他类型的肿瘤中检测到，包括肝细胞癌、结肠癌、乳癌、NSCLC、卵巢癌、恶性黑色素瘤 (脑转移) 和肾细胞癌。

[0160] 在神经胶质瘤患者血清中检测到降低的 Trp 浓度 (图 1h)。这些可能没有翻译成增加的 Kyn 水平 (图 1h)，因为 Kyn 被其他细胞摄取，并代谢成喹啉酸。实际上，在表达 TDO 的成胶质细胞瘤组织中检测到喹啉酸的积聚 (图 1i)。Kyn 抑制同种异体 T 细胞增生。同种

异体 T 细胞增殖与由神经胶质瘤来源的 TDO 形成的 Kyn 成反比（图 2a）。神经胶质瘤细胞中 TDO 的基因敲除（增补的图 4c、d；增补的记录 9）复原了同种异体 T 细胞增殖，而向 TDO 基因敲除细胞中添加 Kyn 防止了 T 细胞增殖的复原（图 2b）。Kyn 浓度依赖地抑制 T 细胞受体刺激的 CD4+ 和 CD8+T 细胞的增殖（增补的图 4e）。另外，TDO 基因敲除导致增强了由同种异体反应 PBMC 产生的神经胶质瘤细胞的裂解（增补的图 4f）。最后，与 TDO 低表达的人神经胶质瘤切片相比，在 TDO 高表达的人神经胶质瘤切片中观察到白细胞共同抗原（LCA）阳性和 CD8+ 免疫细胞的浸润降低（图 2c），这表明 TDO 引起的 Kyn 形成可能抑制抗肿瘤免疫反应。在具有免疫能力的小鼠体内实验中证明了，与 TDO 缺陷的对照小鼠相比，表达 TDO 的肿瘤生长较快，并呈现较高的增殖指数（图 2d）。正如以下事实所证明的，肿瘤中的 TDO 活性抑制体内的抗肿瘤免疫反应：与带有 TDO 缺陷肿瘤的小鼠相比，带有表达 TDO 肿瘤的小鼠中，肿瘤特异性 T 细胞引起的干扰素 - γ（IFN- γ）的释放降低，且脾细胞引起的肿瘤细胞裂解（图 2e，f）。

[0161] 接下来分析了 Kyn 对神经胶质瘤细胞的自分泌作用。尽管在对照和具有 TDO 基因敲除的神经胶质瘤之间没有检测到细胞周期进展中的差异，但是，TDO 基因敲除降低了游动性和产克隆细胞的存活（图 2g，h）。其是由 Kyn 调节的，因为外源添加 Kyn 恢复了 Trp 不存在时的游动性和产克隆细胞的存活（图 2i，j），这表明 Kyn 增加了恶性神经胶质瘤细胞的游动性。在 GIC 中对 Kyn 反应增强了球形成。最后，当 TDO 基因敲除肿瘤正位移植到缺乏功能性 T 细胞的裸鼠脑时，肿瘤形成被破坏（图 2k）。

[0162] 实施例 3：TDO 介导的 N 细胞抑制

[0163] 为了分析 TDO 介导的抗肿瘤 NK 细胞反应的抑制（其在裸鼠中是有功能的），是否能够解释 TDO 基因敲除肿瘤形成的破坏，比较了在 NK 细胞存在或不存在时的皮下肿瘤生长。去除 NK 细胞增强了对照和 TDO 基因敲除肿瘤的生长，但是并没有使 TDO 基因敲除肿瘤的生长恢复到对照水平（图 2l），这表明由组成型 TDO 活性产生的 Kyn、在不存在功能性抗肿瘤 T 细胞和 NK 细胞反应时、以自分泌的方式增强了人神经胶质瘤细胞的恶性表现型。

[0164] 实施例 4：经由 AHR 的 Kyn 活性的分子机制

[0165] 为了更好地理解构成 Kyn 对神经胶质瘤细胞自分泌作用的分子机制，进行了 Kyn 处理的神经胶质瘤细胞的微阵列分析，其揭示了由 Kyn 对 AHR 反应基因的广泛诱导（图 3a）。途径分析表明在 U87 细胞中、在 8 小时和 24 小时由 Kyn 处理最强烈诱导的 25 个基因全部是直接地或间接地被 AHR 调节（图 3a）。

[0166] 恶性神经胶质瘤细胞系以及 GIC 组成型地表达 AHR，并且在两种不同的神经胶质瘤细胞系中确认了由 Kyn 上调的 AHR 靶标基因。Kyn 导致 AHR 在 1 小时后易位至细胞核中，因而，表明了 Kyn 对 AHR 的即时作用（图 3b、c）。一致地，Kyn 激活的肿瘤细胞的 Western blot 分析表明降低的细胞质定位与增加的 AHR 细胞核积聚并行，与 TCDD 诱导的是可比较的（图 3d）。

[0167] Kyn 浓度依赖地诱导神经胶质瘤细胞中的 DRE 萤光素酶活性，EC50 是 36.6 μM（图 3e）。在一组 Trp 代谢物中，AHR 激活唯一对应着 Kyn。乙氧基 -9- 羟基异吩噁唑酮 - 邻 - 去乙基酶（EROD）实验确认了功能性 AHR 靶标基因细胞色素 P450、家族 1、亚家族 A、多肽 1（CYP1A1）的诱导，其针对 Kyn 的 EC50 是 12.3 μM。使用来自 Ahr- 正常和 Ahr- 缺陷小鼠的小鼠肝脏细胞溶胶的放射性配体结合试验证明 Kyn 结合 AHR 的 KD（app）≈ 4 μM（图

3f)。对 Kyn 反应引起的 AHR 激活和 AHR 调节基因表达的上调被 AHR 拮抗剂 3,4-DMF 或 AHR 基因敲除抑制(图 3g),表明 Kyn 是 AHR 特异性的激动剂。相同或相似的 AHR 残基参与对 Kyn、TCDD 和 3- 甲基胆蒽 (3-MC) 的结合,这由 3,4-DMF 抑制所有 3 种配体激活 AHR 这一事实确认。重要地,神经胶质瘤细胞产生的内源性 Kyn 足以激活 AHR,因为 TDO 的基因敲除降低了 AHR 调节基因的表达(图 3h)。由于在 U87 异种移植中检测到平均 Kyn 浓度是 37.01+ / -13.4μM(n=6),在体内也能够到达足以激活 AHR 的 Kyn 浓度。与由 TDO 来源的 Kyn 激活 AHR 一致地,仅在表达 TDO 的人神经胶质瘤切片中观察到 LCA+ 免疫细胞中 AHR 靶标基因 TIPARP 的表达(图 4a)。

[0168] 为了确定 TDO 是否通过 AHR 影响抗肿瘤免疫反应,分析了人神经胶质瘤切片中免疫细胞的浸润与其 AHR 表达的关联。实际上,与 AHR 低表达的人神经胶质瘤切片相比,AHR 高表达的人神经胶质瘤切片中 LCA+ 和 CD8+ 免疫细胞的浸润降低了(图 4b)。为了分析宿主 AHR 表达对肿瘤生长的贡献,比较了 Ahr- 正常和 Ahr 缺陷小鼠中有 TDO 表达和没有 TDO 表达的鼠肿瘤的生长。当与 Ahr 正常小鼠比较时,Ahr 缺陷小鼠中表达 TDO 的肿瘤的生长减慢了(图 4c),表明 AHR 介导的宿主作用增强了肿瘤生长。肿瘤细胞中 LCA+ 免疫细胞染色揭示了 TDO 表达降低了 Ahr 正常小鼠中 LCA+ 免疫细胞的浸润,但这不发生在 Ahr 缺陷小鼠(图 4d),表明通过 AHR 的 TDO 介导的抗肿瘤免疫反应的抑制对增强表达 Tdo 的肿瘤生长的宿主作用有贡献。另外,尽管在 Ahr 正常小鼠中,与不表达 Tdo 的肿瘤相比,Tdo 表达强烈地增强了肿瘤生长,然而,在 Ahr 缺陷小鼠中观察到同样的作用,虽然在非常低的程度上(图 4c)。由于鼠神经胶质瘤细胞表达功能性的 AHR,这些结果证实了 Ahr 缺陷小鼠中由 TDO 介导的肿瘤生长的增强是由于 TDO 对肿瘤细胞自身的自分泌作用。这一观点得到了以下事实的支持 :AHR 基因敲除后,Kyn 不能诱导人神经胶质瘤细胞的游动性(图 4e)。而且,在 AHR 基因敲除的神经胶质瘤细胞中,对 Kyn 反应的产克隆细胞的存活的增加消失了(图 4f)。最后,体内实验证明了在人神经胶质瘤细胞中诱导的 AHR 基因敲除抑制免疫减弱小鼠中的肿瘤生长(图 4g),这强调了 AHR 信号传导对 Trp 降解的自分泌作用的重要性。

[0169] 随后,我们研究了 TDO 来源的 Kyn 是否激活人脑肿瘤组织中的 AHR。实际上,TDO 表达与人神经胶质瘤组织中的 AHR 表达以及 AHR 靶标基因的表达相关(图 5a、b、c),表明神经胶质瘤细胞中组成型 TDO 表达产生了足够量的 Kyn,以激活 AHR。为了解决 TDO-Kyn-AHR 信号传导途径是否还在除神经胶质瘤以外的其他癌症中被激活的问题,我们分析了不同人肿瘤实体的微阵列数据。有趣的是,不仅在神经胶质瘤中 TDO 表达与 AHR 靶标基因 CYP1B1 的表达相关(图 5c),而且其还在 B 细胞淋巴瘤、Ewing 肉瘤、膀胱癌、宫颈癌、结肠直肠癌、肺癌和卵巢癌中相关(图 5d)。这一发现表明 TDO-Kyn-AHR 途径不局限于脑肿瘤,看起来其是癌症的共同特性。Rembrandt 数据库分析揭示了 TDO、AHR 或 AHR 靶标基因 CYP1B1 高表达的神经胶质瘤患者 (WHO 分级 II-IV) 的总存活与具有这些基因的中度或低表达的患者相比是降低的(图 5e)。最后,在成胶质细胞瘤 (WHO 分级 IV) 患者 14 中,发现受胶质瘤细胞中 TDO 来源的 Kyn 调节的 AHR 靶标 CYP1B1、IL1B、IL6 和 IL8 的表达(图 3h) 能够预测存活,甚至不依赖于 WHO 分级(图 5f),因而进一步强调了 AHR 激活对于神经胶质瘤恶性表现型的重要性。总之,这些数据表明了内源性肿瘤来源的 Kyn 以自分泌 / 旁分泌的方式激活 AHR,促进肿瘤的进展(图 5g)。

[0001]

序列表

<110> 德国癌症研究中心

<120> 用于治疗和/或预防天然 AHR 配体依赖性癌症的手段和方法

<130> DK10702PC

<150> US 61/531,861
<151> 2011-09-07

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<170> PatentIn version 3.5

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[0003]

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[0004]

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[0005]

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<213> 人工序列

[0006]

<220>
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<212> DNA
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<220>
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aagtacc 67

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<213> 人工序列

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<223> 干扰 shRNA 正义寡核苷酸

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ggatcca 67


b
犬尿氨酸 [μM]
TDO mRNA 表达的相关性
r²=0.93
0.0
0.02
0.04
0.06
0
5
10
15
20
25
30
35
40
45
U373
U251
U87
T323
T98G
LN-308
LN-18
A172
hAs
犬尿氨酸 [μM]
0
10
20
30
40
50
60
a
色氨酸 (%)
0
20
40
60
80
100

图 1

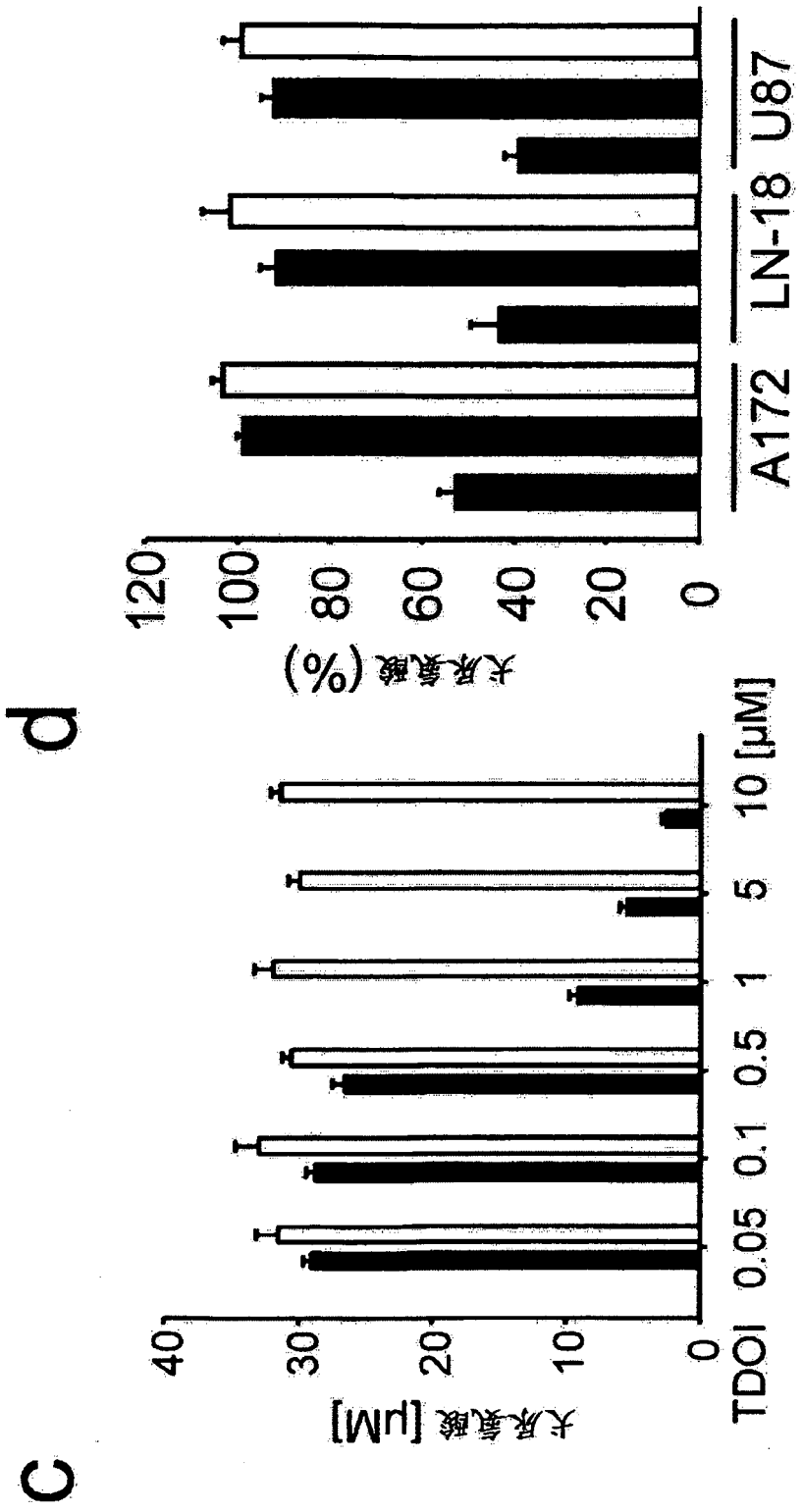

d
犬尿氨酸 (%)
120
100
80
60
40
20
0
A172
LN-18
U87
C
犬尿氨酸 [μM]
40
30
20
10
0
TDOI
0.05
0.1
0.5
1
5
10 [μM]

图 1（续）

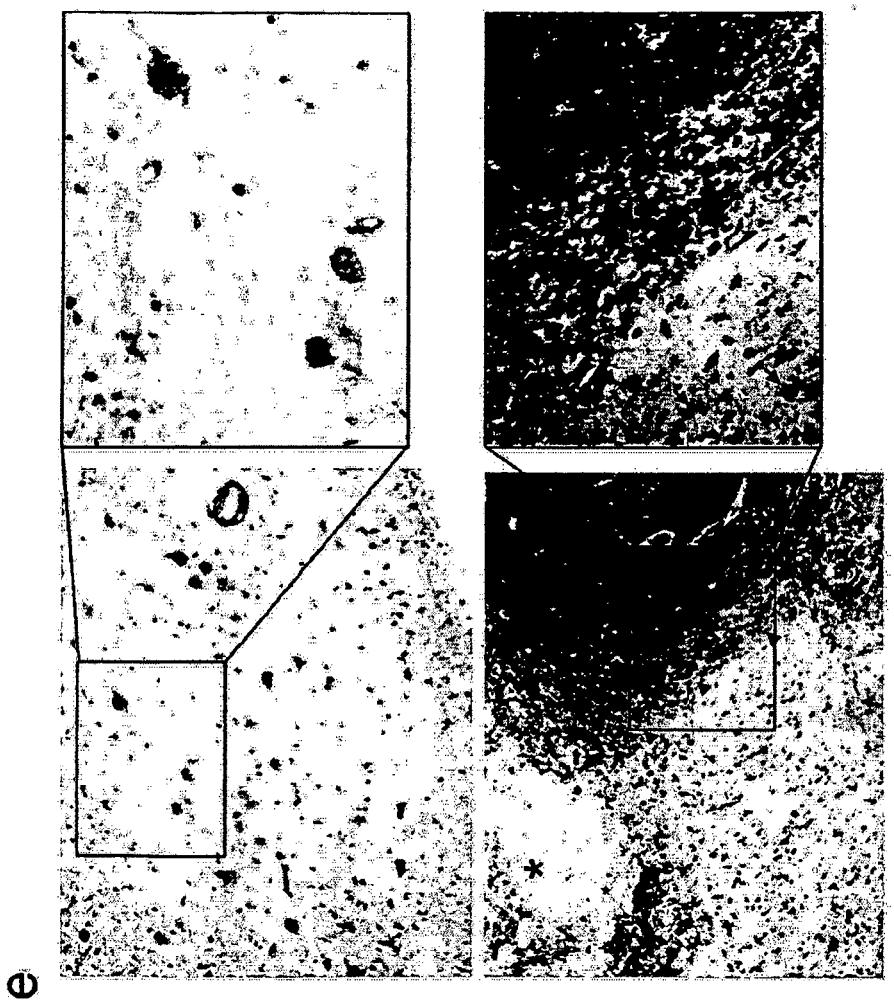


图 1(续)

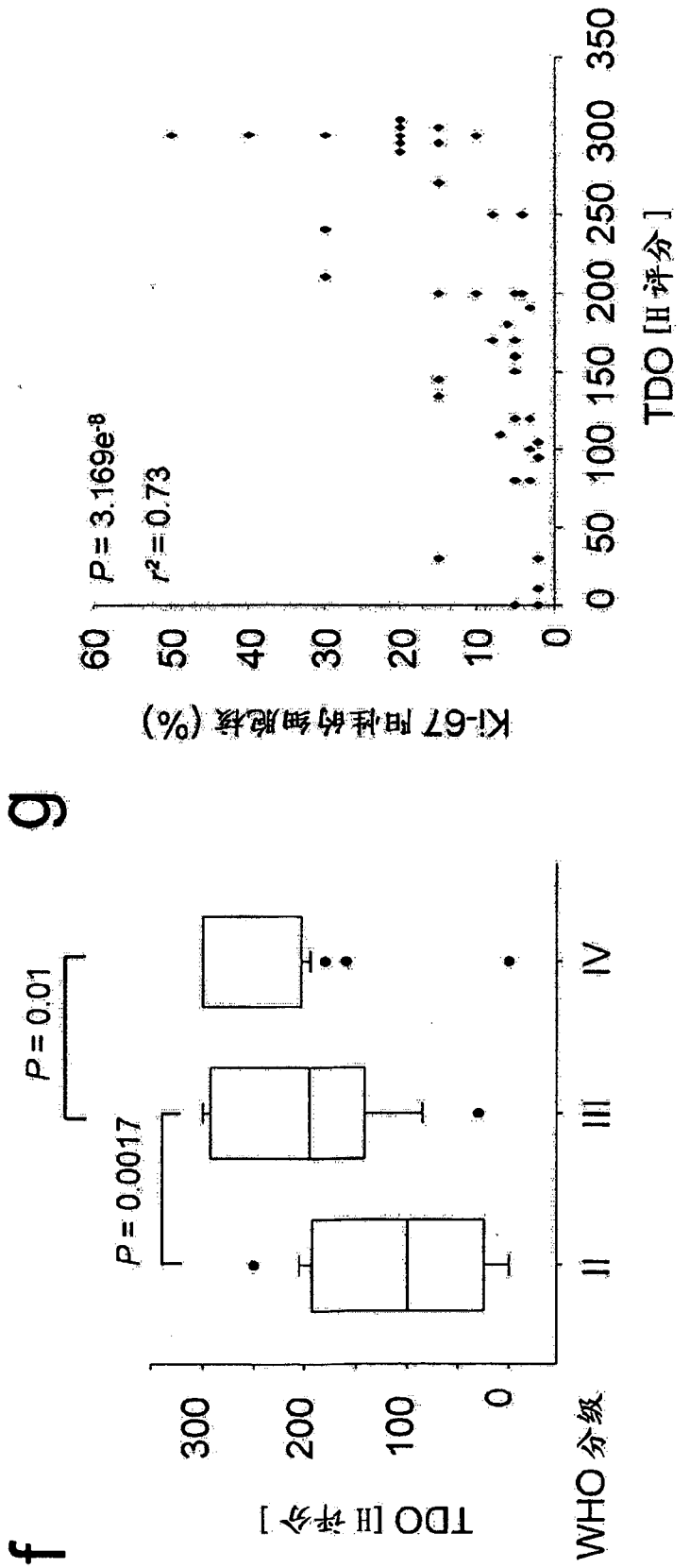

g
Ki-67 阳性的细胞核 (%)
60
50
40
30
20
10
0
P = 3.169e-8
r² = 0.73
0 50 100 150 200 250 300 350
TDO [H 评分]
f
TDO [H 评分]
300
200
100
0
P = 0.01
P = 0.0017
II
III
IV
WHO 分级

图 1（续）

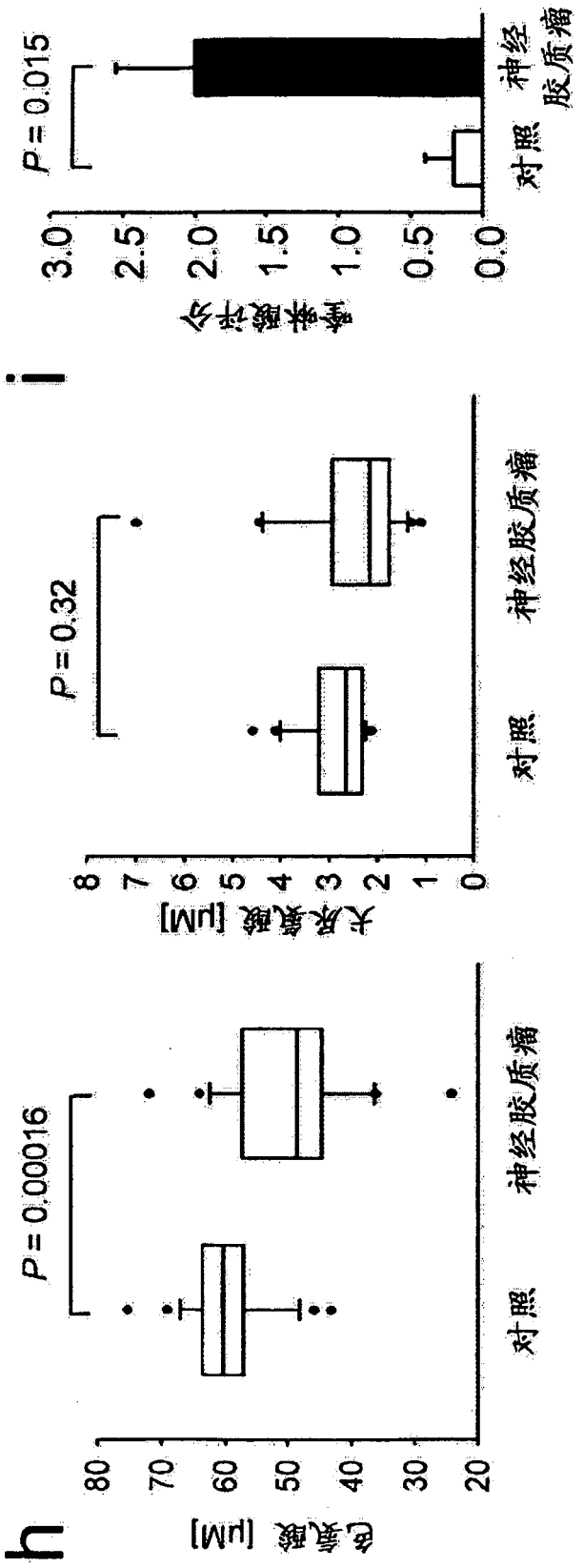

h
色氨酸 [μM]
80
70
60
50
40
30
20
P = 0.00016
对照
神经胶质瘤
i
犬尿氨酸 [μM]
8
7
6
5
4
3
2
1
0
P = 0.32
对照
神经胶质瘤
j
脊髓酸评分
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P = 0.015
对照
神经胶质瘤

图 1(续)

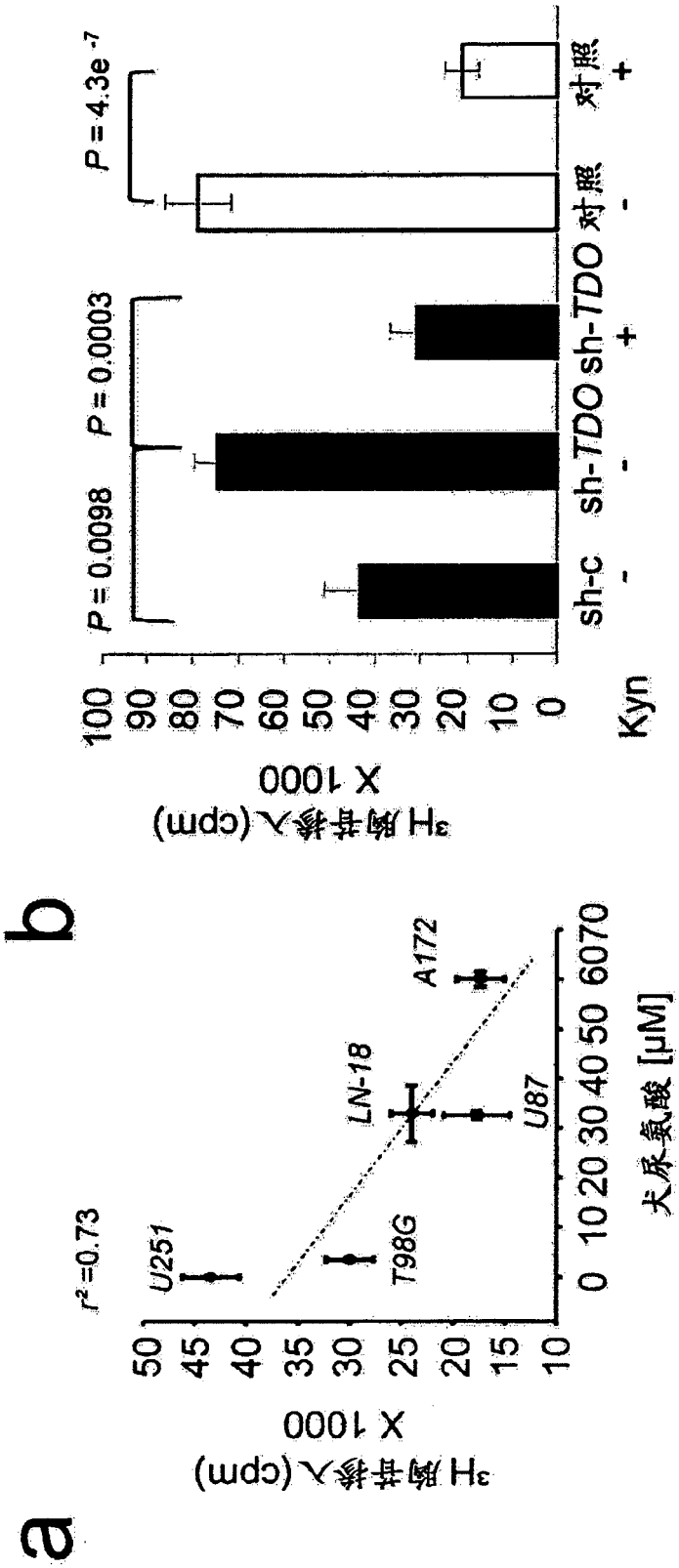

b
3H 胸苷掺入(cpm)
X 1000
100 90 80 70 60 50 40 30 20 10 0
P = 0.0098
P = 0.0003
P = 4.3e-7
sh-c
sh-TDO
sh-TDO 对照
对照
Kyn
a
3H 胸苷掺入(cpm)
X 1000
50 45 40 35 30 25 20 15 10
r² =0.73
U251
T98G
LN-18
U87
A172
0 10 20 30 40 50 60 70
犬尿氨酸 [μM]

图 2

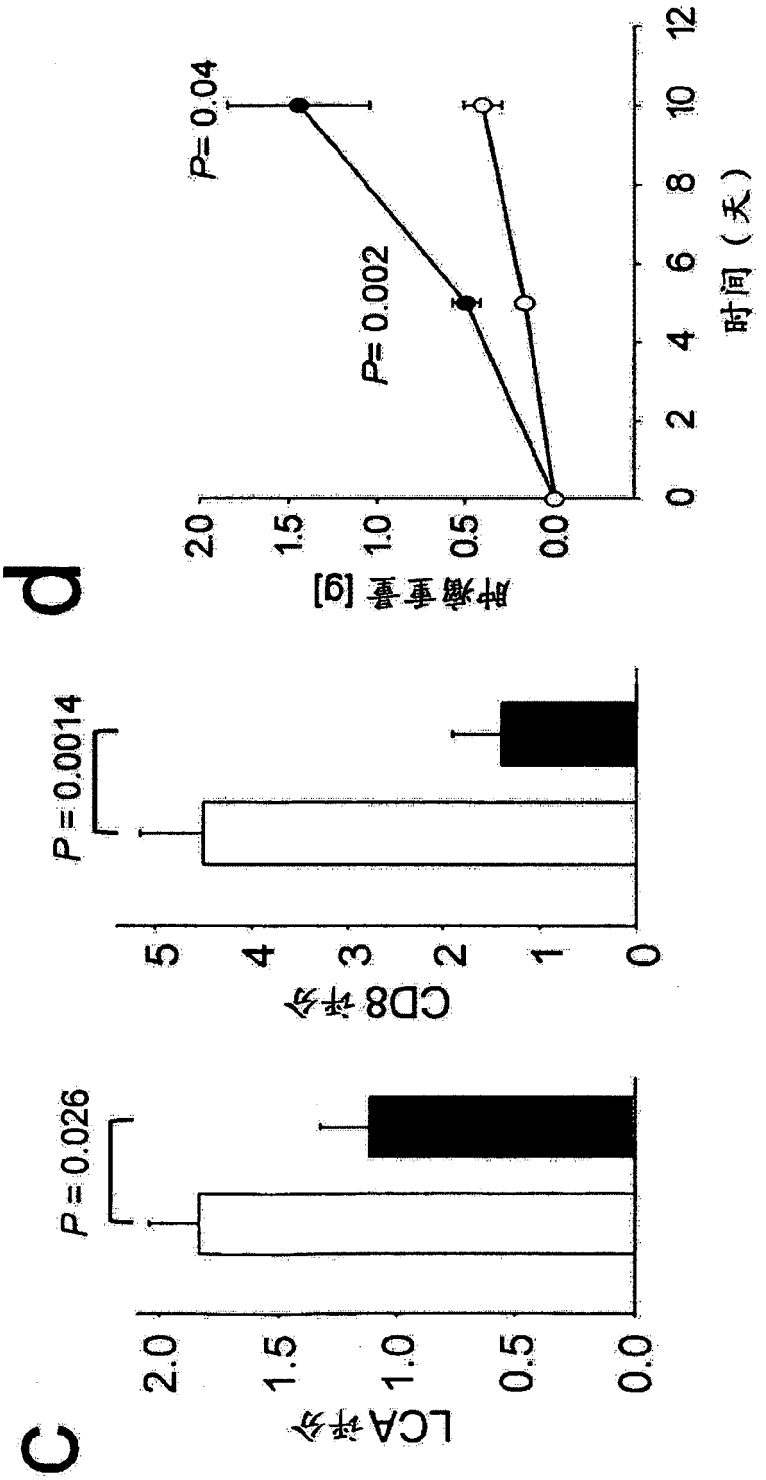

d
肿瘤重量 [g]
2.0
1.5
1.0
0.5
0.0
P= 0.04
P= 0.002
0 2 4 6 8 10 12
时间（天）
CD8 评分
P = 0.0014
5 4 3 2 1 0
C
LCA 评分
P = 0.026
2.0 1.5 1.0 0.5 0.0

图 2（续）

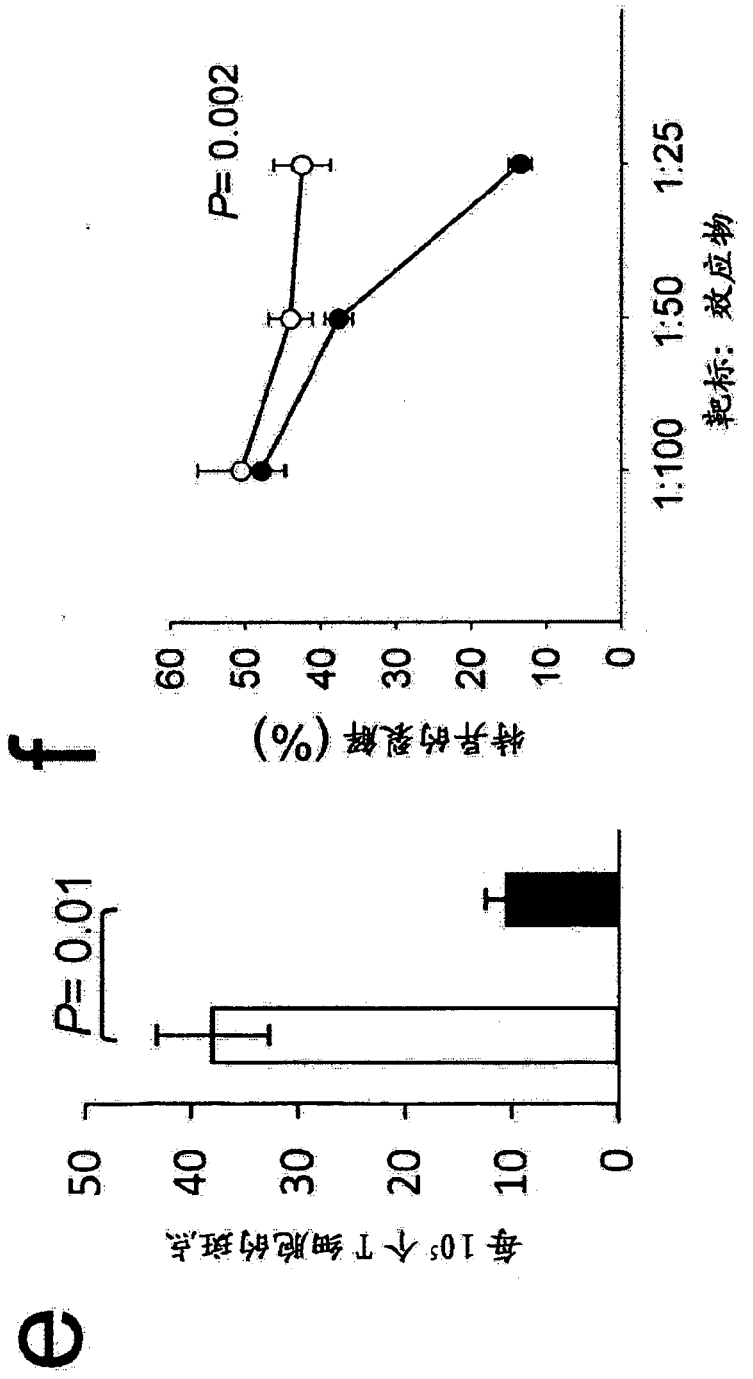

f
特异的裂解 (%)
60
50
40
30
20
10
0
P= 0.002
1:100
1:50
1:25
靶标：效应物
e
每 10⁵ 个 T 细胞的斑点
50
40
30
20
10
0
P= 0.01

图 2（续）

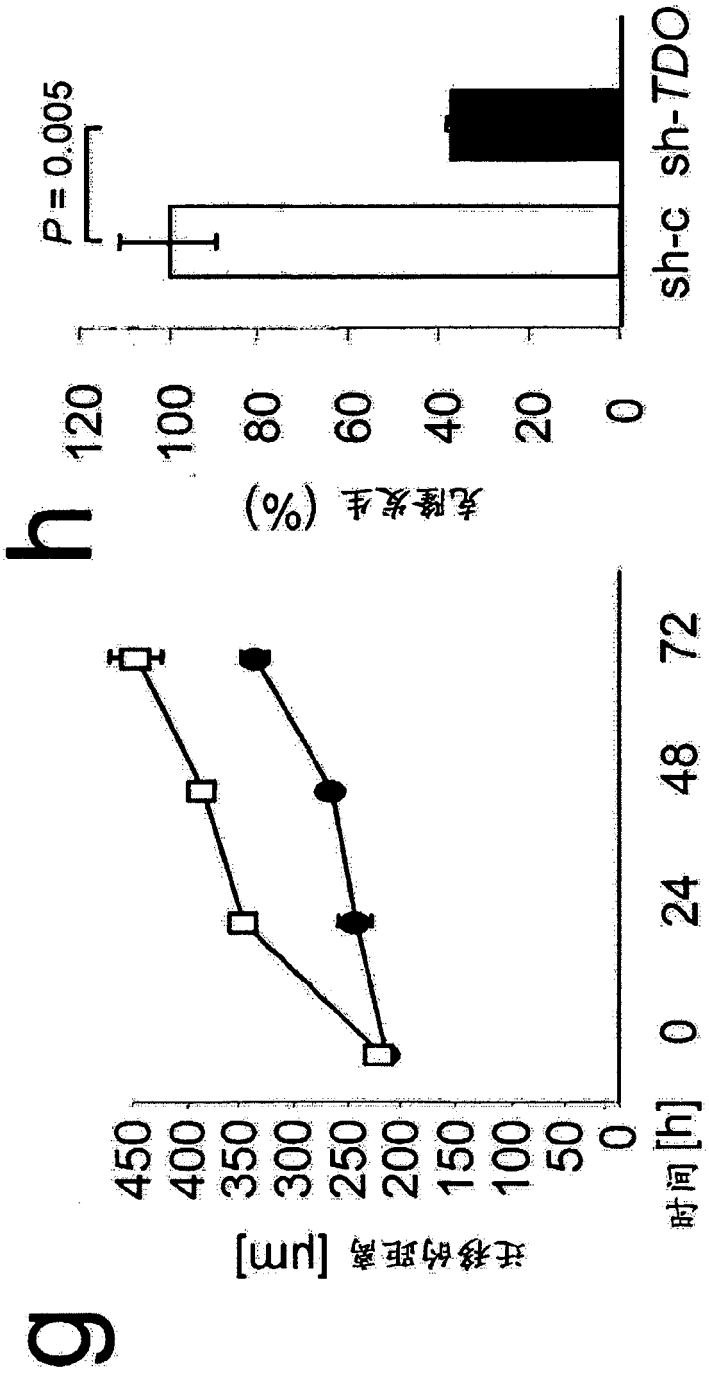

h
左降发生 (%)
120
100
80
60
40
20
0
P = 0.005
sh-c
sh-TDO
g
迁移的距离 [μm]
450
400
350
300
250
200
150
100
50
0
0
24
48
72
时间 [h]

图 2(续)

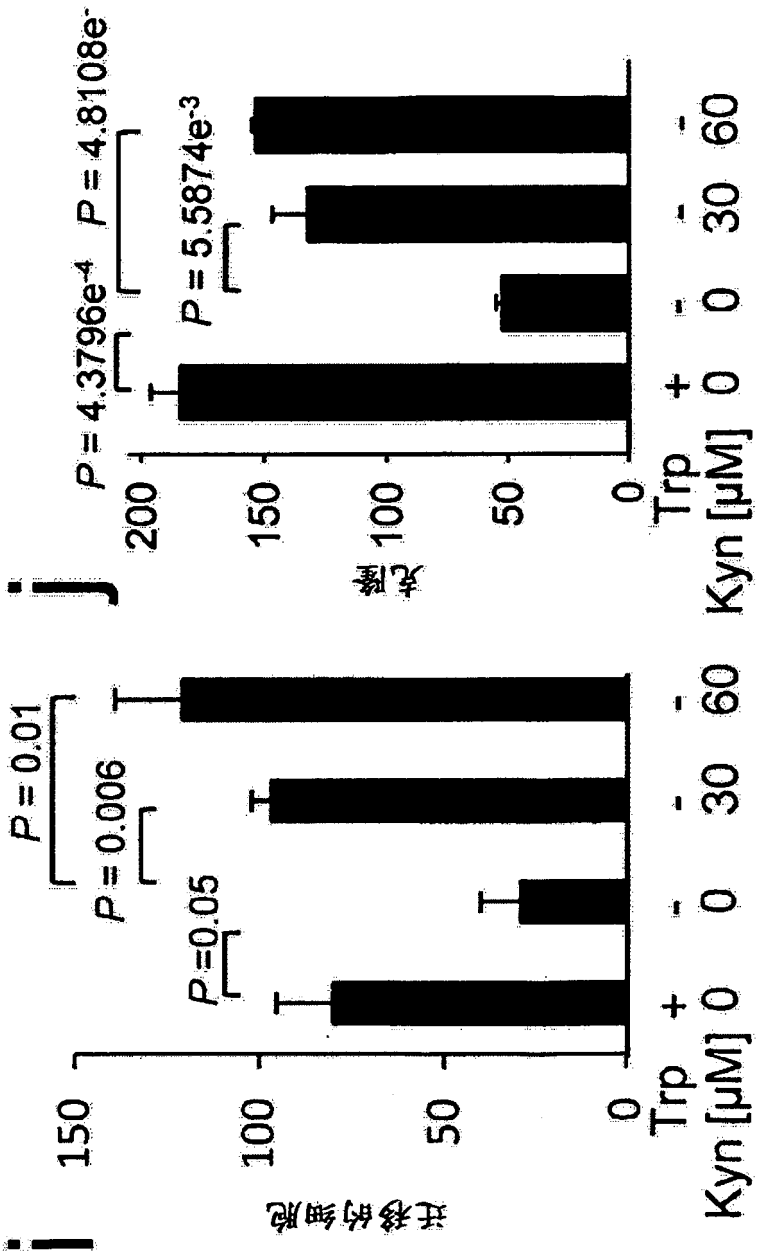

i
迁移的细胞
150
100
50
0
P = 0.01
P = 0.006
P=0.05
Trp + 0 - 30 -
Kyn [μM] 0 - 0 - 60
j
光度
200
150
100
50
0
P = 4.3796e-4
P = 4.81086e-
P = 5.5874e-3
Trp + 0 - 30 -
Kyn [μM] 0 - 0 - 60

图 2(续)

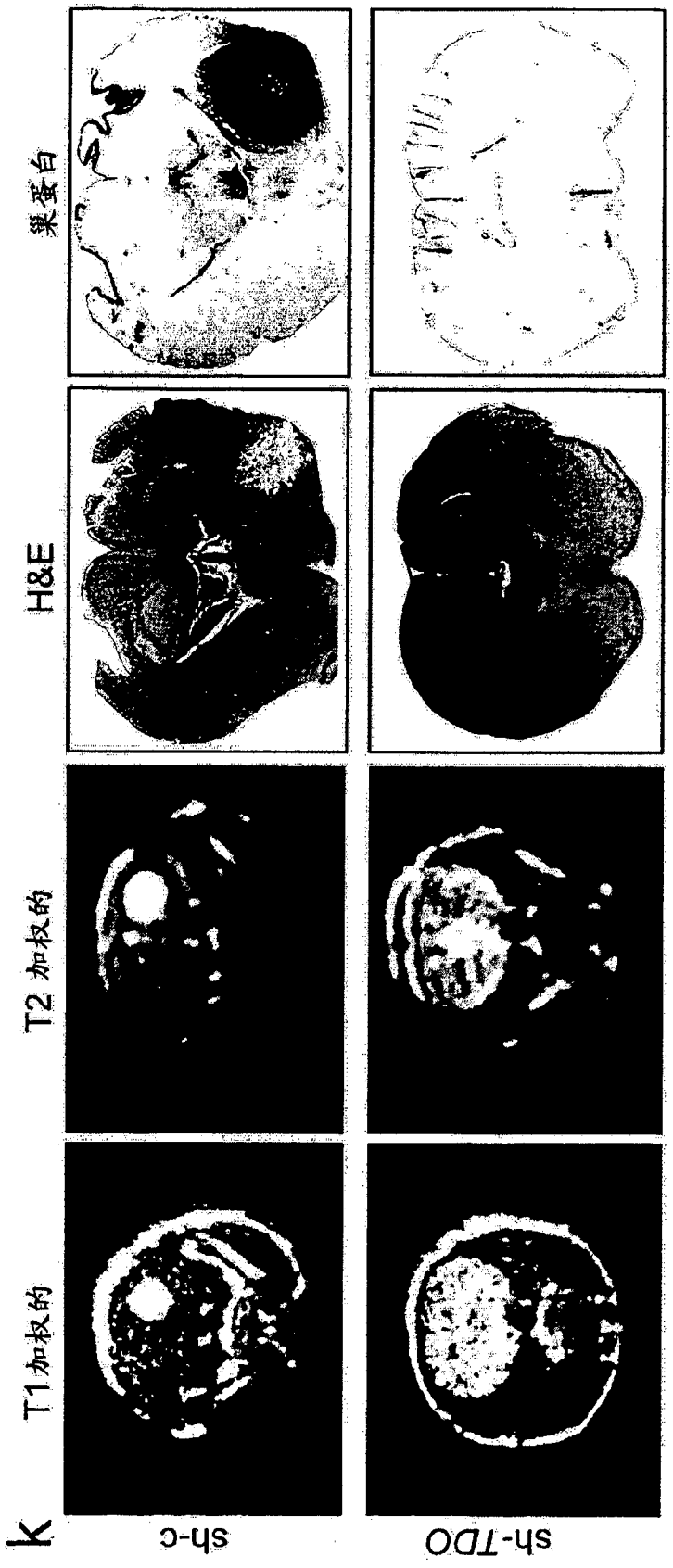


图 2(续)

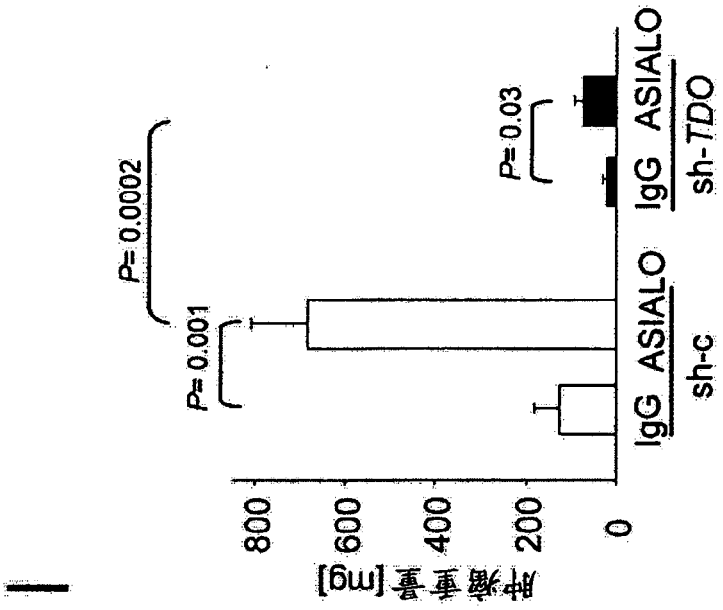

P= 0.0002
P= 0.001
P= 0.03
IgG ASIALO
sh-c
IgG ASIALO
sh-TDO
0
200
400
600
800
肿瘤重量 [mg]

图 2（续）

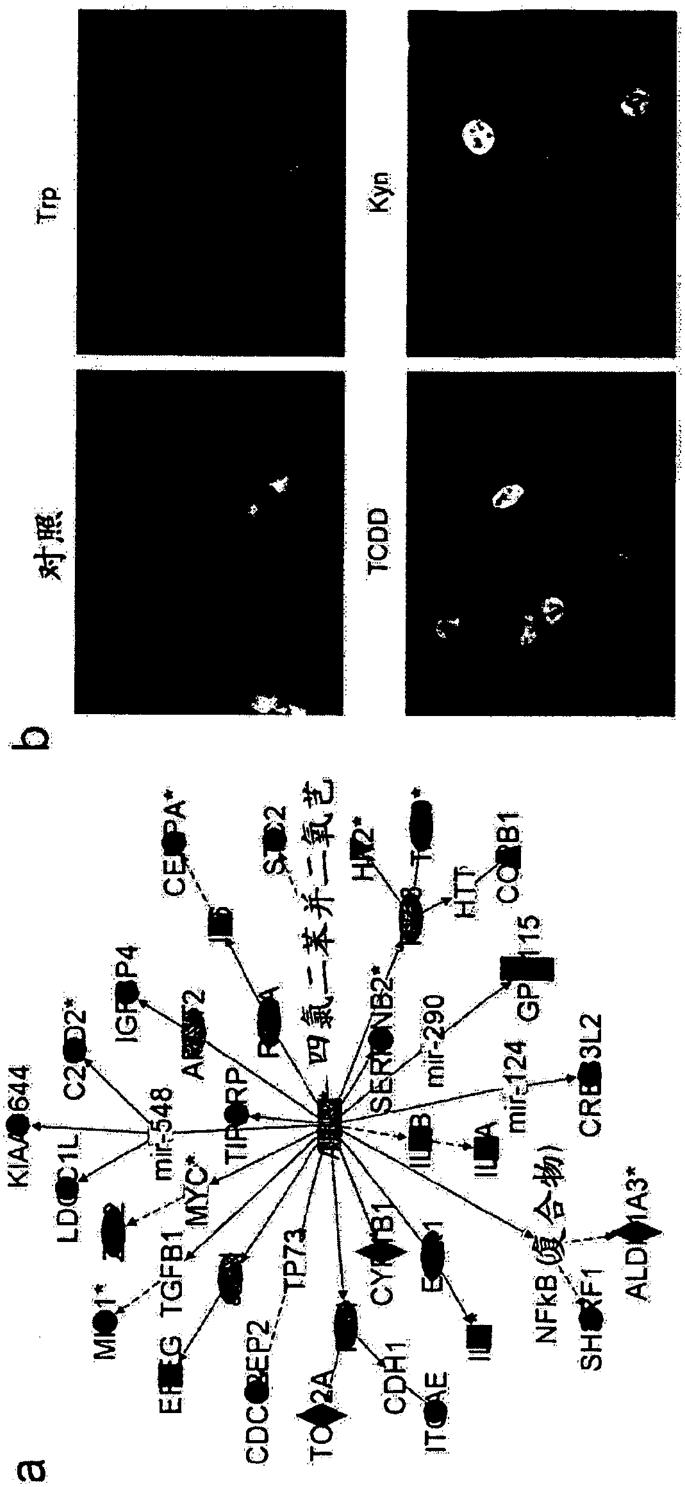

a
b
Trp
Kyn
对照
TCDD
四氯二苯并二氧芑
mir-548
mir-290
mir-124
MYC*
TGFB1
TP73
CDH1
HTT
NFkB(复合物)
IGFBP4
C2CD2*
ALDH1A3*

图 3

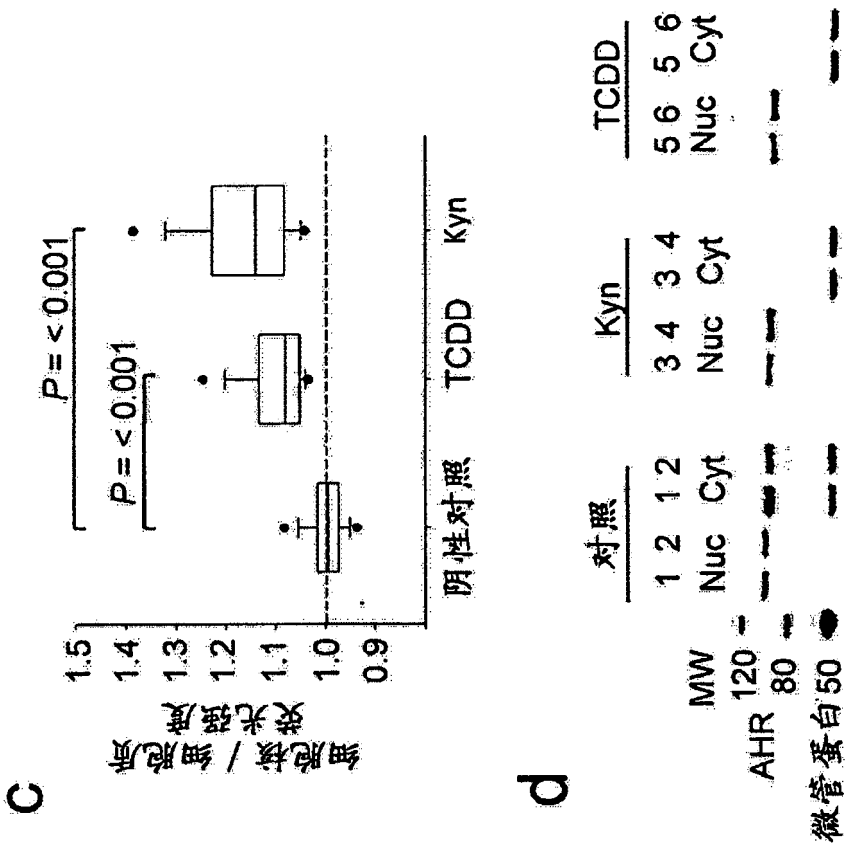

C
细胞核 / 细胞质
荧光强度
1.5
1.4
1.3
1.2
1.1
1.0
0.9
P = < 0.001
P = < 0.001
阴性对照
TCDD
Kyn
d
对照
Kyn
TCDD
1 2 1 2
3 4 3 4
5 6 5 6
Nuc Cyt
Nuc Cyt
Nuc Cyt
MW
AHR 120
80
微管蛋白 50

图 3（续）

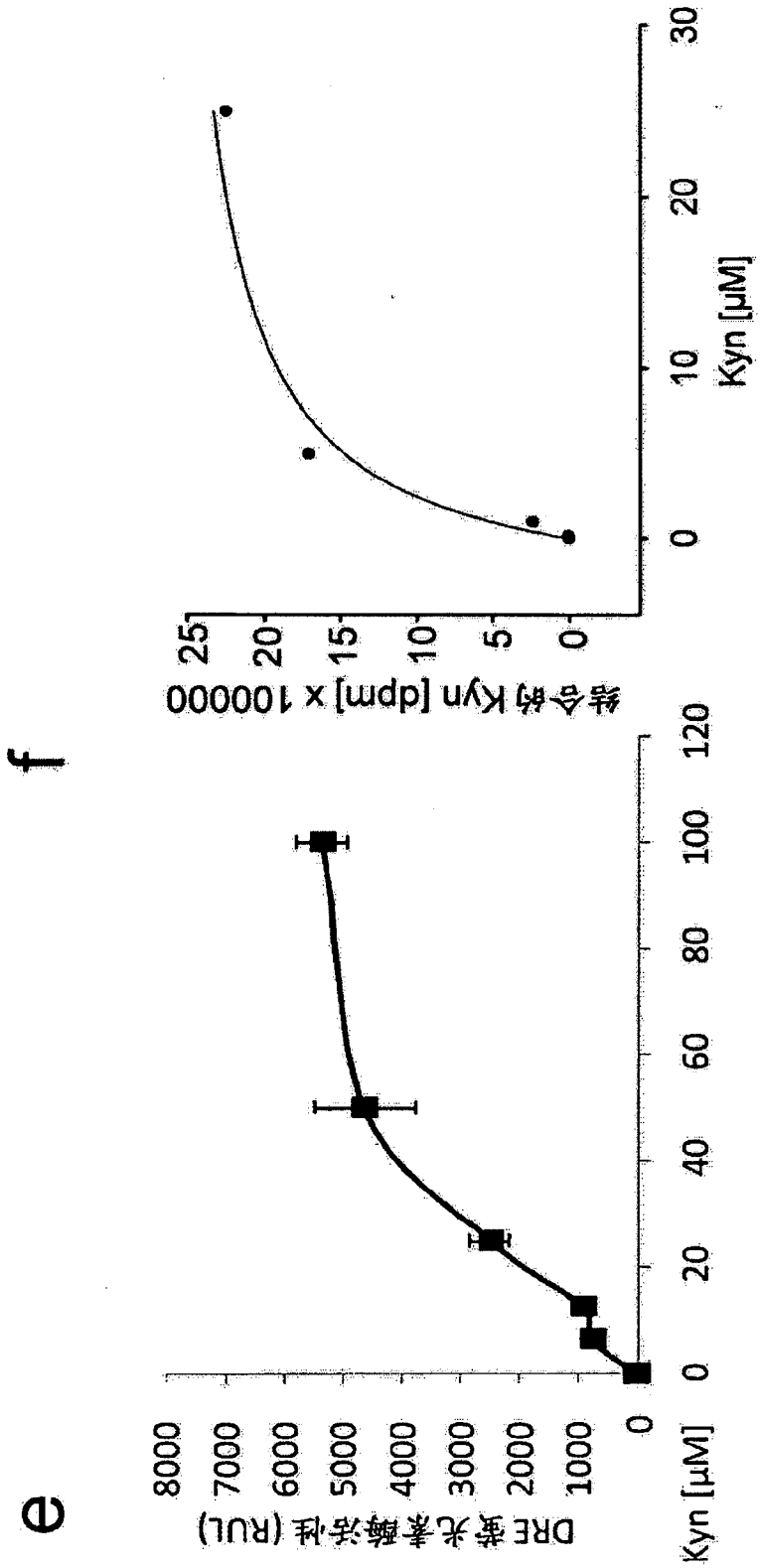

f
结合的 Kyn [dpm] x 100000
25
20
15
10
5
0
0
10
20
30
Kyn [μM]
e
DRE 萤光素酶活性 (RUL)
8000
7000
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
120
Kyn [μM]

图 3(续)

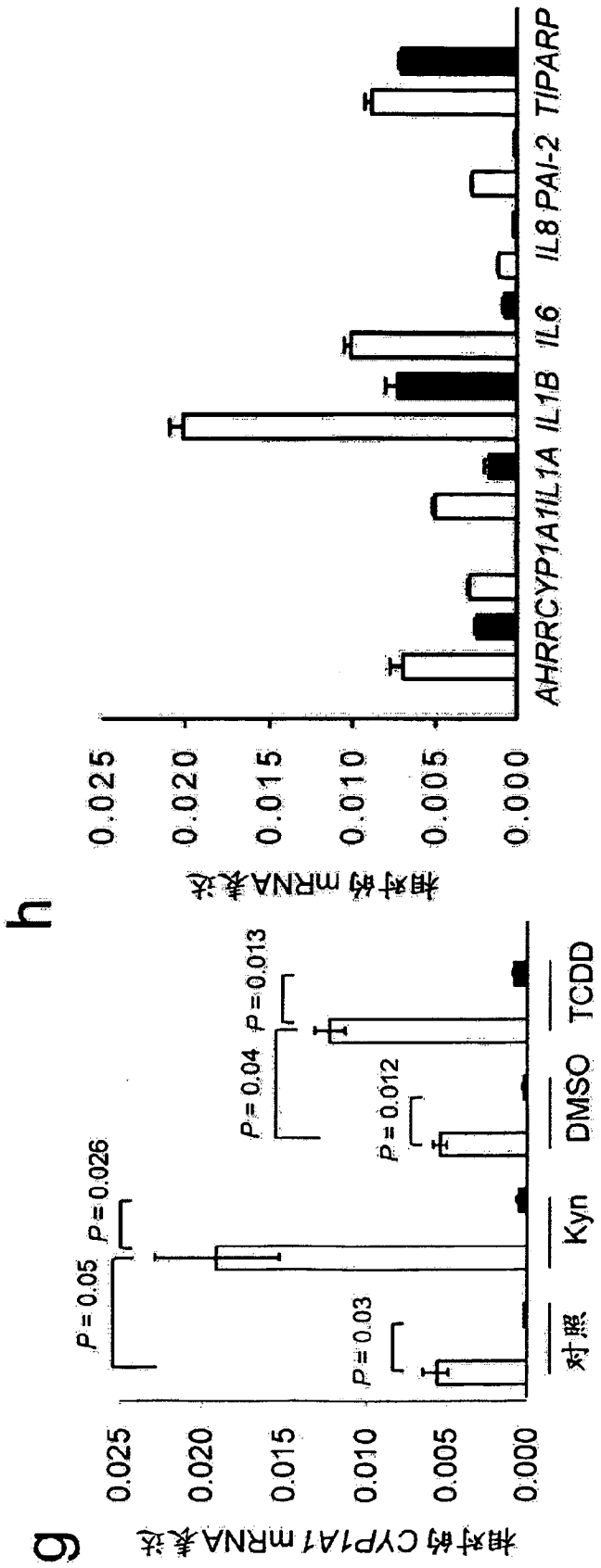

g
相对的 CYP1A1 mRNA 表达
0.025
0.020
0.015
0.010
0.005
0.000
P = 0.05
P = 0.026
P = 0.03
P = 0.04
P = 0.013
P = 0.012
对照
Kyn
DMSO
TCDD
h
相对的 mRNA 表达
0.025
0.020
0.015
0.010
0.005
0.000
AHRR CYP1A1 IL1A IL1B IL6 IL8 PAI-2 TIPARP

图 3（续）

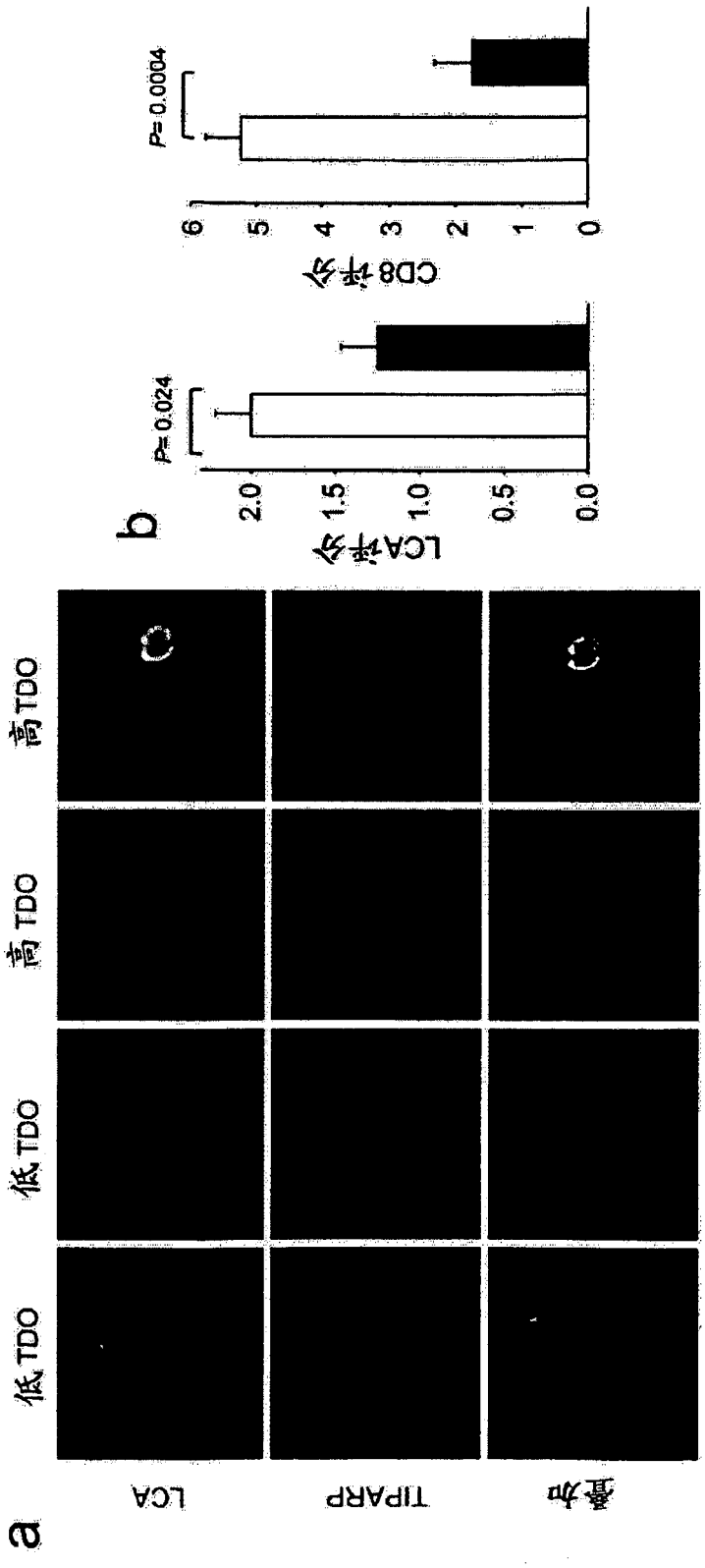
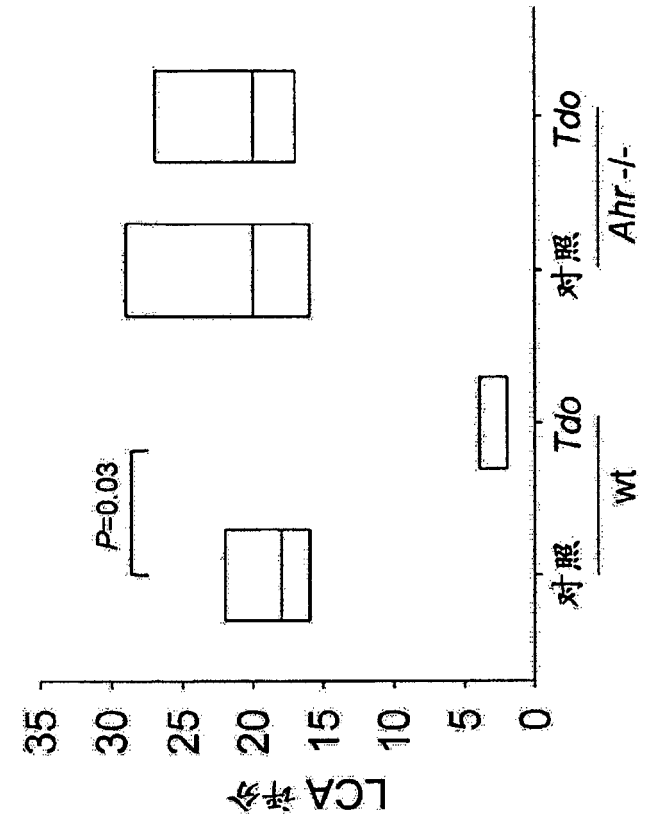

a
LCA
TIPARP
叠加
低 TDO
低 TDO
高 TDO
高 TDO
b
LCA评分
2.0
1.5
1.0
0.5
0.0
P= 0.024
CD8评分
6
5
4
3
2
1
0
P= 0.0004

图 4


d
LCA 浓度
35
30
25
20
15
10
5
0
P=0.03
对照
Tdo
wt
对照
Tdo
Ahr-/-

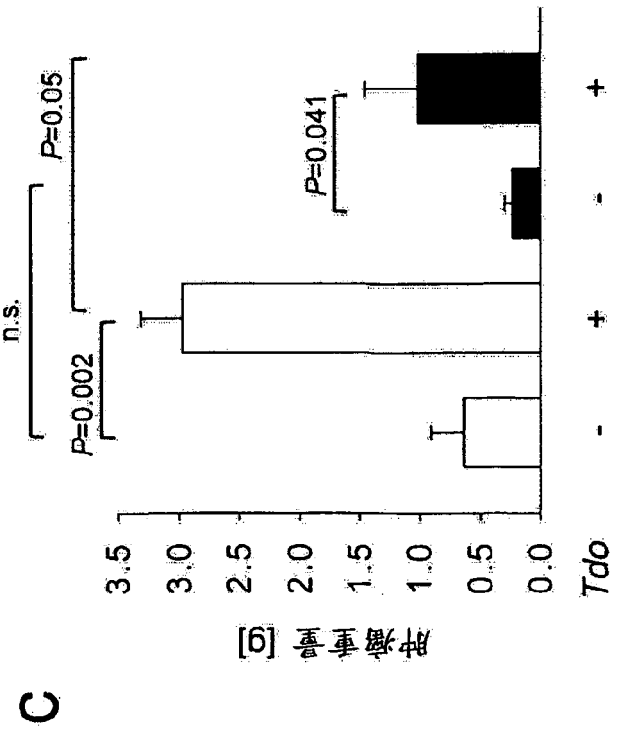

C
肿瘤重量 [g]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
n.s.
P=0.002
P=0.05
P=0.041
-
+
-
+
Tdo

图 4（续）

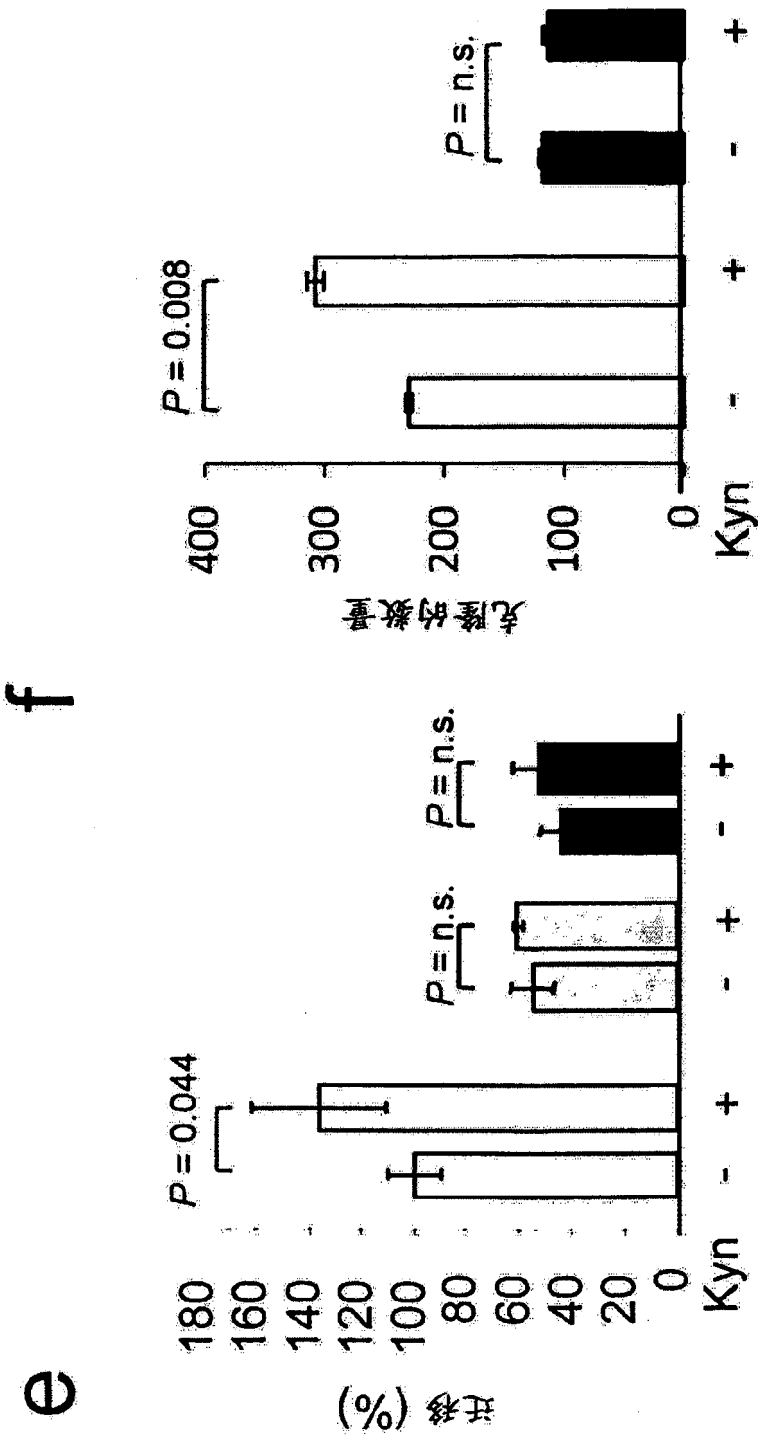
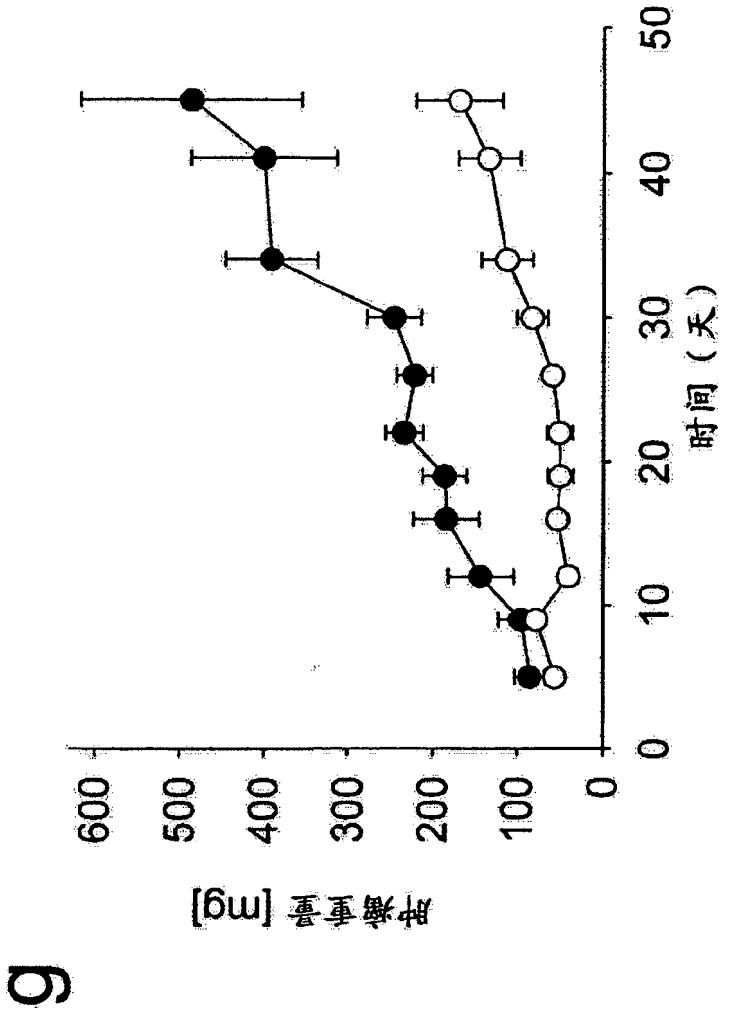
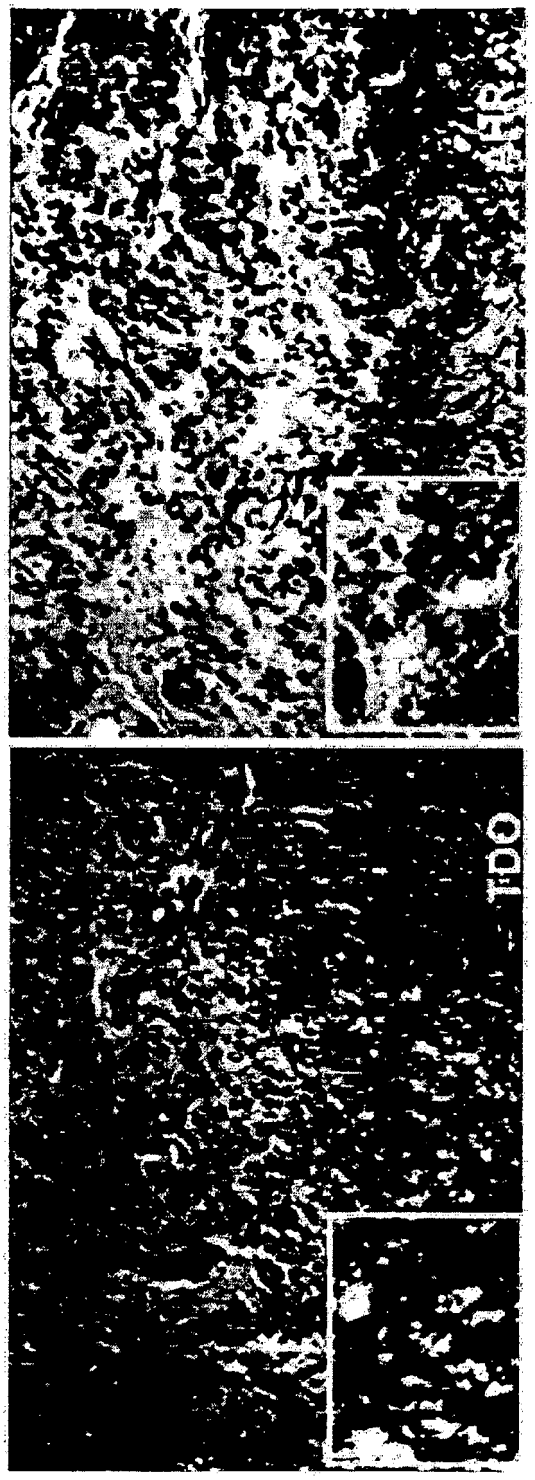

f
e
P = 0.008
P = n.s.
P = 0.044
P = n.s.
P = n.s.
Kyn
+
-
0
100
200
300
400
20
40
60
80
100
120
140
160
180
迁移 (%)
去除的数量

图 4(续)



g

图 4（续）



a

图 5

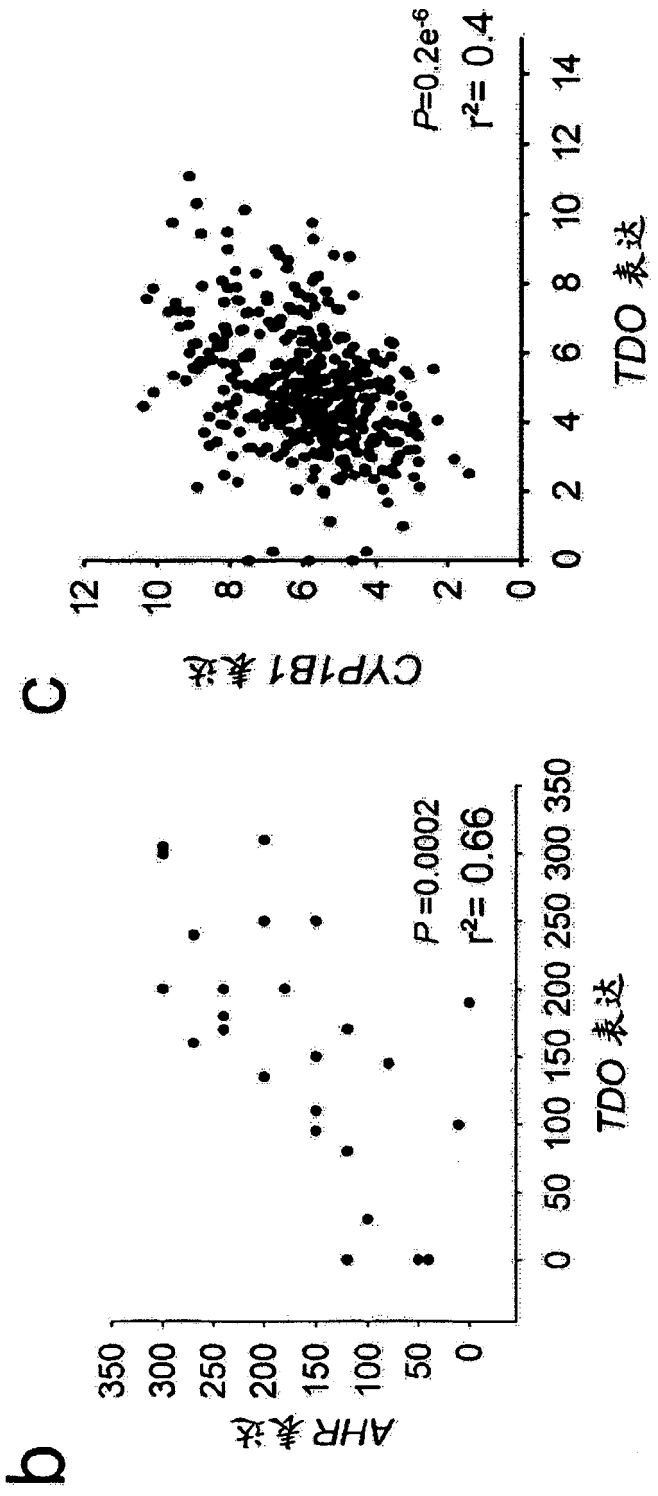


图 5（续）

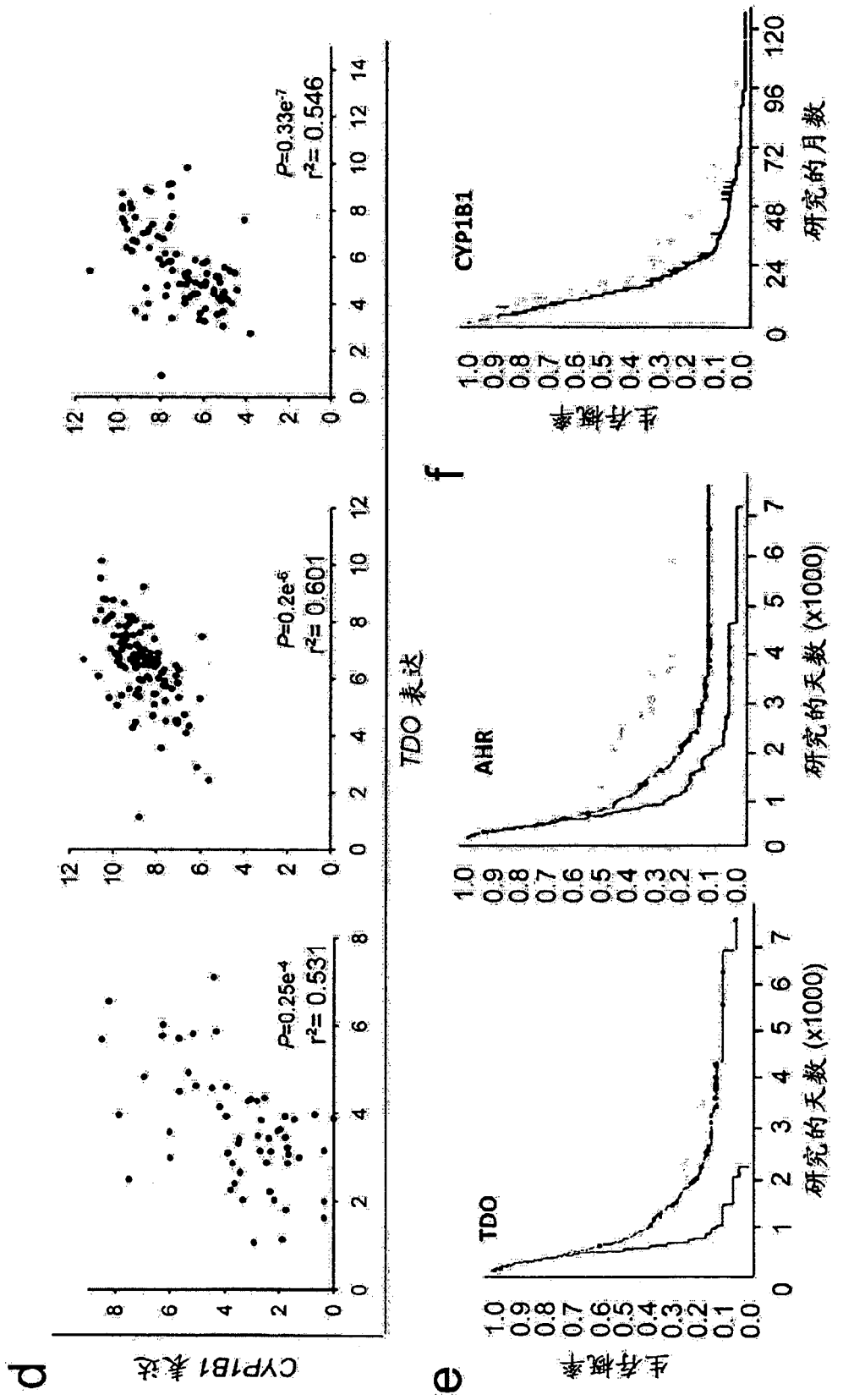

d
CYP1B1 表达
TDO 表达
P=0.25e-4
r²= 0.531
P=0.2e-6
r²= 0.601
P=0.33e-7
r²= 0.546
e
生存概率
研究的天数 (x1000)
TDO
AHR
f
生存概率
研究的月数
CYP1B1

图 5(续)

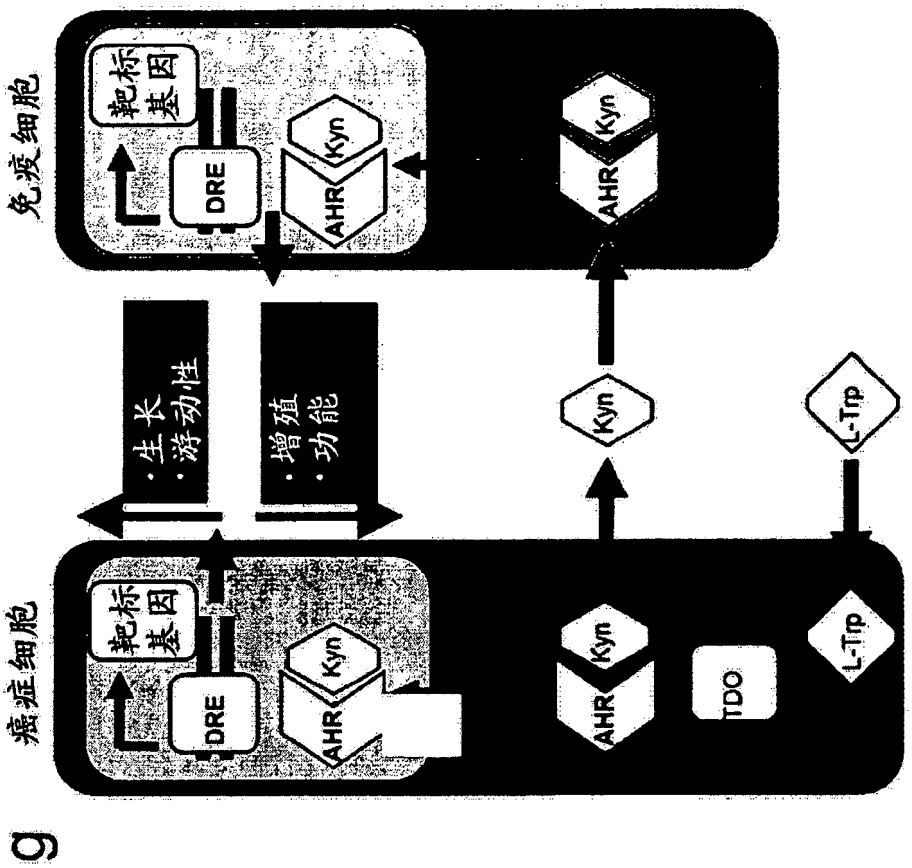

免疫细胞
靶标基因
DRE
Kyn
AHR
Kyn
AHR
·生长
·游动性
·增殖
·功能
Kyn
L-Trp
癌症细胞
靶标基因
DRE
Kyn
AHR
Kyn
AHR
TDO
L-Trp
g

图 5(续)

Abstract

The present invention relates to the field of cancer therapeutics and treatment of cancer. In particular, it relates to a method for treating and/or preventing a natural AHR ligand-dependent cancer comprising administering to a subject suffering from said cancer a therapeutically effective amount of an AHR inhibitor. Moreover, contemplated is a AHR inhibitor for use in treating and/or preventing a natural AHR ligand-dependent cancer.