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Correlazioni molecolari fra angiogenesi e neurodegenerazione

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Tesi di dottorato

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Introduzione

Neurodegenerazione

Col termine neurodegenerazione si intende una condizione patologica caratterizzata da disfunzione e/o morte di cellule neuronali a livello cerebrale e del midollo spinale. La neurodegenerazione è alla base di varie patologie quali il morbo di Parkinson, di Alzheimer, di Huntington, la ALS, la sclerosi multipla. Le malattie neurodegenerative sono associate ad una elevata morbilità e mortalità, e per il loro trattamento sono disponibili strategie terapeutiche scarsamente efficaci.

Il morbo di Alzheimer (AD), in particolare, è la principale causa di demenza senile nelle persone anziane, è una complessa sindrome neurodegenerativa, che porta con progressione inesorabile ad una perdita delle capacità cognitive per la quale non esiste ad oggi una cura efficace. Ad oggi, infatti sono disponibili solo alcuni farmaci sintomatici, mentre mancano sia farmaci in grado di far regredire il processo di neurodegenerazione sia efficaci strumenti diagnostici che consentano una diagnosi negli stadi primari Il progressivo allungamento di vita della popolazione fa supporre che l'AD possa raggiungere proporzioni epidemiche entro i prossimi venti anni, il che rende essenziale lo sviluppo di nuove strategie terapeutiche e di strumenti diagnostici affidabili per la diagnosi precoce di questa malattia. L'opinone emergente è che alla base del processo eziopatologico ci sia una disregolazione cerebrovascolare, che è una caratteristica non solo di patologie prettamente cerebrovascolari, come l'ictus, ma anche di condizioni neurodegenerative come l'AD. Molti studi neuropatologici hanno descritto, infatti, alterazioni morfologiche nei capillari cerebrali ed una riduzione di flusso sanguigno nei distretti cerebrali dei pazienti AD comunque l'importanza patogenetica di queste alterazioni vascolari è difficile da interpretare perché non è

chiaro se si tratta di una consequenza o una causa delle disfunzioni neuronali e del processo neurodegenerativo.

Angiogenesi

Con il termine angiogenesi s'intende l'insieme di processi funzionali che portano alla formazione di nuovi vasi sanguigni a partire da vasi preesistenti. Le cellule endoteliali che costituiscono lo strato interno di tutti i vasi, sono centrali nel fenomeno angiogenico (Conway et al., 2001). Esse sono coinvolte, seguendo un programma geneticamente determinato, mediato da specifici fattori e recettori, in una serie di eventi quale proliferazione, differenziazione, migrazione ed organizzazione morfologica in stretta connessione con il tessuto circostante. La formazione di un vaso passa quindi attraverso diversi stadi ben definiti, caratterizzati da modificazioni dell'endotelio e della matrice extracellulare. Nella prima tappa dell'angiogenesi si verifica una "destabilizzazione" dei vasi preesistenti dovuta ad un aumento della permeabilità vasale, ed una perdita delle connessioni tra le cellule endoteliali. Segue quindi una seconda fase, di migrazione e proliferazione delle cellule endoteliali nel punto del tessuto dove è necessaria la formazione di nuovi vasi. Nel corso di tale fase vengono liberati enzimi proteolitici che alterano la densità della matrice extracellulare allo scopo di facilitare l'attività migratoria delle cellule endoteliali. Il terzo stadio dell'angiogenesi corrisponde alla differenziazione delle cellule endoteliali, ed è caratterizzata da arresto della proliferazione cellulare e formazione di capillari primitivi. L'ultima tappa di maturazione funzionale è caratterizzata dal reclutamento di cellule periendoteliali di supporto, quali periciti e cellule muscolare lisce, nonché alla riorganizzazione delle interazioni cellulari (Bussolino 1998).

Angiogenesi e neurodegenerazione

L'angiogenesi - lo sviluppo di nuovi vasi sanguigni - è un punto cruciale per la formazione e rimodellamento del sistema nervoso e la protezione di esso dalla malattia. Ricerche recenti hanno migliorato la nostra comprensione di come il cervello ed altri tessuti coltivano i nuovi vasi sanguigni nelle circostanze normali e patologiche. Alcuni fattori dell'angiogenesi, particolarmente il VEGF (fattore di crescita endoteliale vascolare) sono noti per avere un coinvolgimento nella proliferazione neuronale (neurogenesi), nella prevenzione o nella protezione da danni neuronali (neuroprotezione) e nella patogenesi dell' infarto e della malattia di Alzheimer .

Studi epidemiologici evidenziano che farmaci anti angiogenici apportano benefici ai pazienti con la malattia di Alzheimer (AD), indicando che la mancata regolazione vascolare svolga un ruolo critico nell'AD, che è quindi una malattia "angiogenesi-dipendente" (Harris 2005). Inoltre, i fattori di crescita angiogenica, VEGF e bFGF sono anche compresi fra quelli la cui sintesi è stimolata dall'A-beta (Folkman 2007), e sono anche coinvolti nella plasticità neuronale a causa della loro attività gliotrofica/neurotrofica (McAuslan 1980,Hu 1998). Lo studio della relazione tra A-beta e il processo angiogenico (Glesne DA 2006,Gaggelli 2006) può chiarire l'eziopatologia del processo AD.

Sia l'AD, come pure altri disordini neurodegenerativi, sono da considerarsi malattie neurovascolari, infatti negli ultimi anni, è emerso il concetto dell'unità neurovascolare come nuovo paradigma per lo studio sia della fisiologia che della patologia a carico del SNC. Questo concetto propone che il focus puramente neurocentrico non sia sufficiente ed enfatizza che tutte le cellule dell'unità neurovascolare cioè i componenti vascolari, i neuroni e la glia, devono essere esaminati in un contesto integrato. Il *signaling* e

l'accoppiamento cellula-cellula fra questi compartimenti differenti costituiscono la base per una normale funzionalità.

Una comunicazione disordinata e un accoppiamento neuro-vascolare perturbato dà il via a disfunzioni e alla malattia.

Oltre ad un'associazione fisica nello stesso microambiente, queste cellule interagiscono l'una con l'altra attraverso meccanismi di feedback positivi e negativi legati anche alle richieste metaboliche (Pogue A 2004).

La neurogenesi e l'angiogenesi hanno rivelato molte affinità fra questi processi. Queste includono comuni di fattori di crescita e loro recettori, segnali simili per la proliferazione cellulare, destino delle cellule, formazione di contorni e spazio fisico comune come conseguenza di un modello parallelo di sviluppo anatomico (Cantara 2004).

L'angiogenesi è un processo complesso a più fattori la cui disomeostasi può essere causa di diverse malattie con decorso acuto o cronico. Essa ha suscitato l'interesse di molti studiosi che sono coinvolti nello studio di vari processi patologici, dal cancro alle malattie neurodegenerative. Quindi, uno dei principali obiettivi di questo studio riguarda la ricerca di correlazioni e di fattori comuni tra le malattie neurodegenerative croniche ed i disordini vascolari, per evidenziare il ruolo ancora sottostimato dell'angiogenesi ed individuare possibili interventi terapeutici che hanno come bersaglio primario il processo angiogenico e quindi le cellule endoteliali.

Ipossia e angiogenesi

Il termine ipossia indica uno stato di insufficiente disponibilità di ossigeno nell'ambiente cellulare; tale condizione può interessare l'intero organismo in situazioni di bassa concentrazione dell'ossigeno nell'ambiente circostante (come ad esempio accade ad altitudine elevata) o può instaurarsi in una delimitata area di tessuto come

conseguenza di un'ischemia o di crescita neoplastica (Jeong et al., 2011). Considerata la vitale importanza dell'ossigeno per il metabolismo cellulare aerobico, in tutti gli organismi animali si sono evoluti complessi meccanismi omeostatici al fine di contrastare l'ipossia. Specifici sensori dell'ossigeno erano già stati descritti nei batteri e nel lievito, ma solo recentemente si è incominciato a far luce sui sistemi di regolazione attuati dagli organismi superiori, uomo incluso. Nonostante sia nota l'esistenza di alcune cellule specializzate nella valutazione dei livelli di ossigeno, tutte le cellule di mammifero sono in grado di reagire a una condizione locale di ipossia mediante l'attivazione di una cascata trascrizionale che coinvolge numerose vie metaboliche. Questo fenomeno si attua in poche ore ed è fondamentale per tutto l'organismo, tanto da essere coinvolto in numerosi processi fisiologici e patologici 11 (Gariboldi et al 2010). Numerose evidenze sperimentali e l'analisi approfondita della patofisiologia delle malattie vascolari e neoplastiche hanno permesso di individuare nella disponibilità di ossigeno un importante elemento regolatore dell'angiogenesi, sebbene i sistemi di adattamento siano complessi e ancora non del tutto chiari (Cassavaugh and Lounsbury, 2011). La comprensione dei meccanismi che regolano i processi cellulari promossi dall'ipossia potrebbero quindi assumere un ruolo molto importante nello sviluppo di strategie terapeutiche volte a contrastare importanti patologie come ad esempio retinopatie proliferative e tumori (Mazzone, 2010). Numerose ricerche indicano che l'ipossia induce un incremento dell'espressione di VEGF in diversi tipi cellulari, incluse le cellule endoteliali umane (Avouac et al., 2008; Cho et al., 2008; Liu et al., 2009; Nilsson et al., 2004Takata et al., 2008; Zheng et al., 2008). L'ipossia può inoltre modulare l'espressione dei recettori del VEGF sebbene i dati riportati nelle cellule endoteliali umane siano talvolta molto in contrasto tra loro, sia per quanto riguarda VEGFR-1 (Avouac et al., 2008; Cho et al., 2008; Gerber et al., 1997; Nilsson et al.

2004) che VEGFR-2 (Avouac et al., 2008; Takata et al., 2008; Waltenberger et al., 1996).

Fattori di trascrizione coinvolti nell'angiogenesi

E' stato fatto molto lavoro per chiarire il ruolo dei fattori di trascrizione nel regolare geni target coinvolti nell'angiogenesi. A questo proposito, il fattore inducibile da ipossia HIF-1 ed il trasduttore del segnale e attivatore della trascrizione STAT3, sono particolarmente interessanti perché la loro attivazione è accoppiata alla regolazione di VEGF (Chen and Han, 2008; Fong, 2009). HIF-1 è un fattore di trascrizione eterodimerico costituito dalle subunità α e β. La subunità HIF-1β è costitutivamente espressa mentre l'espressione e l'attività della subunità HIF-1α sono strettamente controllate dalle concentrazioni cellulari di ossigeno (fig 4)

Infatti, in condizioni di livelli di ossigeno normali, HIF-1α viene rapidamente idrossilata dalle prolil idrossilasi, enzimi che necessitano di ossigeno e ferro per poter esercitare la propria attività, inducendo il legame di questa subunità con la proteina di Von Hippel-Lindau, una E3-ubiquitina ligasi che ne determina l'avvio alla degradazione da parte del proteasoma. In condizione di ipossia HIF-1α sfugge a questa degradazione a causa dell'inattivazione delle idrossilasi ed inizia ad accumularsi nel citoplasma della cellula. Quando la sua concentrazione citoplasmatica raggiunge un livello critico, trasloca nel nucleo dove dimerizza con la subunità HIF-1β (Takahashi and Shibuya, 2005). Una volta avvenuta la dimerizzazione tra le subunità α e β all'interno del nucleo, HIF-1 si lega all'elemento responsivo all'ipossia (HRE) presente nel promotore o nelle regioni enhancer di geni inducibili dall'ipossia determinandone l'attivazione (Jewell et al., 2001). HIF-1 promuove la trascrizione di oltre 70 geni bersaglio (Semenza, 2004) a seconda della tipologia di cellula (Kelly et al., 2003) o di organo (Schneider et al., 2009). HIF-1 è in grado di regolare vari processi fisiologici

tra cui l'eritropoiesi, la glicolisi e l'angiogenesi, che sono le principali risposte adattative all'ipossia (Jiang et al., 1997). In particolare, HIF-1 è il principale fattore responsabile dell'espressione e secrezione di VEGF da parte delle cellule in condizioni ipossiche e gioca un ruolo chiave in diverse patologie caratterizzate da anomalo sviluppo dei vasi sanguigni quali disordini neurodegenerativi (Pagés and Pouysségur, 2005).

Per cui è sembrato interessante studiare eventuali correlazioni molecolari fra i 2 fenomeni.
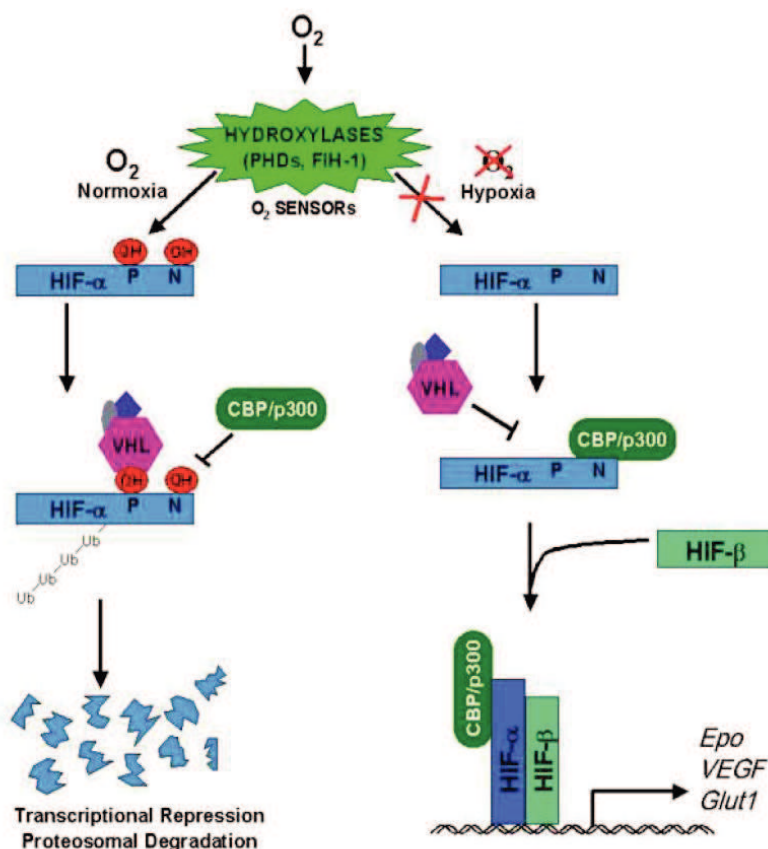

Fig. 4. Cambiamenti della concentrazione cellulare di ossigeno vengono trasdotti nel nucleo come cambiamenti nell'attività di HIF-1. A sinistra della figura, in condizioni di normossia, HIF-1α è idrossilato da PHD (prolil-idrossilasi), in una reazione che richiede ossigeno, con conseguente legame alla E-3 ubiquitina ligasi VHL che ne determina l'avvio verso il proteasoma. In condizioni di ipossia (destra dell'immagine) l'attività di PHD è inibita, HIF-1α si accumula, dimerizza con HIF-1β, si lega al DNA e attiva la trascrizione di geni bersaglio. Tratto da pagina web università Adelaide 2007.

Lenalidomide

La Lenalidomide, è un analogo della talidomide con proprietà anti-neoplastiche, anti-angiogeniche, pro-eritropoitiche e immunomodulatorie. Lenalidomide appartiene alla classe di sostanze degli IMiDs®, abbreviazione di Immunomodulatory Drugs, ossia sostanze farmaceutiche in grado di modulare il sistema immunitario. Gli IMiDs® sono composti derivati dal talidomide che, a parità o superiorità di efficacia, presentano effetti collaterali sensibilmente più contenuti.
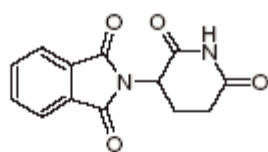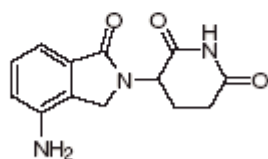

Fig. 1. Chemical structure of thalidomide.

Fig. 2. Chemical structure of lenalidomide.

Meccanismo d'azione

L'azione di Revlimid® (lenalidomide) si esplica con molteplici meccanismi che agiscono simultaneamente, includendo proprietà immunomodulanti, antiproliferative ed antiangiogenetiche (Fig.3).

Effetti immunomodulanti

Lenalidomide inibisce la produzione di citochine proinfiammatorie, tra cui il fattore della necrosi tumorale alfa (TNF-α), l'interleuchina-1 beta (IL-1β), l'interleuchina 6 (IL-6) e l'interleuchina 12 (IL-12) da parte di cellule mononucleate di sangue periferico (PBMC) stimolate da lipopolisaccaride (LPS) (Marriott 2001) La Lenalidomide inoltre, in cellule PBMC stimolate da LPS, aumenta la produzione della citochina

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antinfiammatoria interleuchina 10 (IL-10) inibendo di conseguenza l'espressione della ciclo-ossigenasi 2 (COX-2) ma non la sua azione enzimatica.(Muller 1996).

L'azione della lenalidomide aumenta la produzione delle citochine quali l'interferone gamma (IFN-γ) e l'interleuchina 2 (IL-2), incrementando pertanto la proliferazione mediata da IL-2 dei linfociti T e l'azione citotossica cellulare, compresa la distruzione delle cellule tumorali mediata da NK . La Lenalidomide può aumentare la segnalazione mediata da TCR sia in assenza che in presenza di tali segnali secondari, favorendo in questo modo le risposte immunitarie (Parve 2004).

La Lenalidomide è anche in grado di modulare il legame con le cellule stromali del midollo osseo (BMSC), riducendo così la produzione delle citochine pro-angiogenetiche e di quelle che stimolano la crescita e sopravvivenza tumorale, come il VEGF, fattore di crescita vascolare endoteliale e l'IL-6.(Corral 1999).

Azione antiproliferativa e pro-apoptotica

La lenalidomide ha dimostrato di possedere effetti antitumorali diretti, tra cui la capacità di arrestare la crescita cellulare nella fase G1 e di innescare il processo apoptotico caspasi-dipendente.(Haslett 2003) Essa inibisce la proliferazione di varie linee cellulari di tumori ematopoietici in vitro, tra cui quelli con difetti citogenetici del cromosoma 5 e le cellule di mieloma multiplo MM. Si pensa che l'inibizione del fattore stimolante la crescita tumorale IL-6 sia uno dei meccanismi mediante i quali la lenalidomide esplica la propria azione antiproliferativa (Davies 2001). L'induzione dell'apoptosi ad opera della lenalidomide è innescata da differenti meccanismi: dall'attivazione della caspasi 8, che porta all'aumento della sensibilità delle cellule tumorali all'apoptosi indotta da FAS, alla "iporegolazione" dell'attività del fattore nucleare kB (NF-kB), all'espressione dell'inibitore cellulare della proteina apoptotica

2 e della proteina inibitoria di FLICE (FADD-like IL-1β-converting enzyme) (c-FLIP),

ed al potenziamento dell'apoptosi correlata al TNF (TRAIL/Apo2L).(Marriott 2002)

Azione antiangiogenetica

La lenalidomide (Revlimid®) inibisce l'angiogenesi, ovvero la formazione di nuovi

vasi sanguigni, blocca la formazione di microvasi e la proliferazione e migrazione delle

cellule endoteliali in modelli di angiogenesi *in vitro*.(Dredge 2002) In questi processi

è fondamentale anche l'inibizione della produzione di TNF-α, del fattore di crescita

fibroblastico basico (bFGF) ad opera delle BMSC, e del fattore VEGF da parte delle
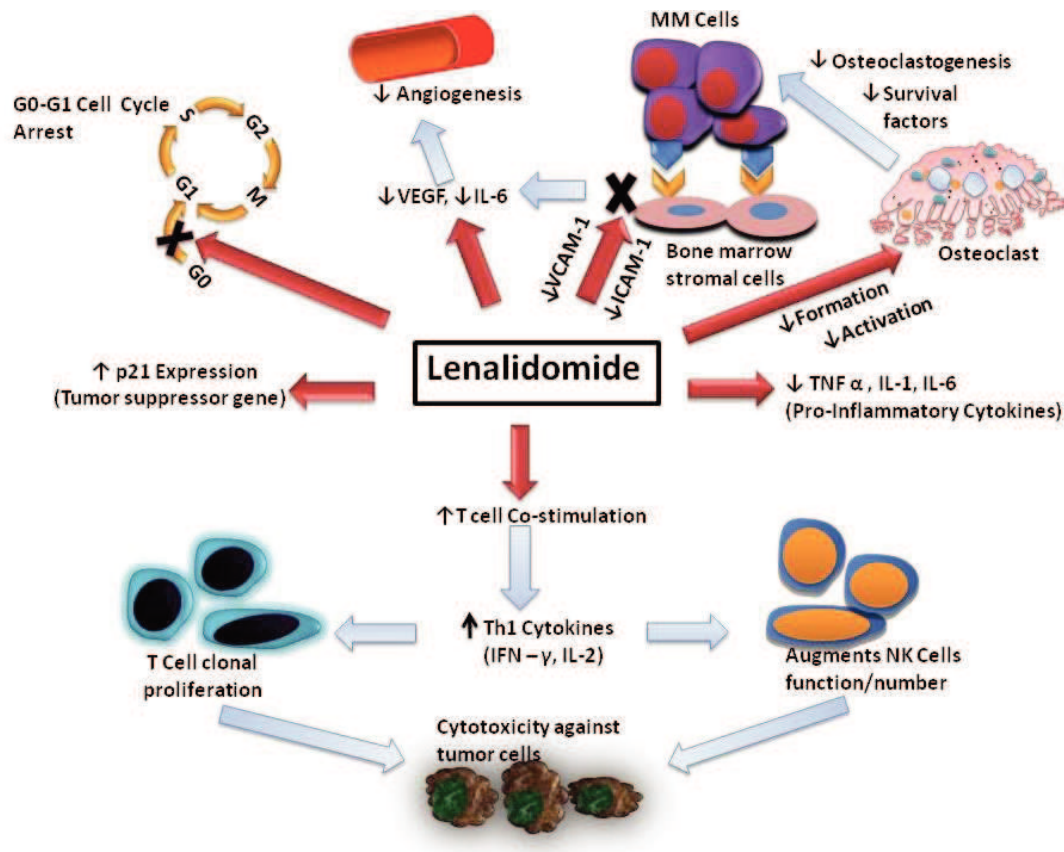
cellule tumorali.(Vacca 2005)

Fig 3 Meccanismo d'azione di lenalidomide

Lenalidomide nel mieloma multiplo

La lenalidomide è autorizzata da giugno 2007 nella terapia del mieloma multiplo refrattario alle terapie convenzionali.

Il mieloma è letteralmente un "oma," o tumore, che involve il "myelo," o le cellule produttrici di sangue del midollo osseo. Le cellule affette sono le plasmacellule (un tipo di globuli bianchi) che sono i nostri produttori di anticorpi (immunoglobuline).

La diagnosi è principalmente basata sull'infiltrazione di plasmacellule nel midollo osseo, lesioni osteolitiche e presenza di immunoglobulina (o frammenti nel siero o nelle urine). MM rappresenta circa il 10% dei tumori ematologici e l'1% di tutti i tumori. La diagnosi di MM è fatta in circa 21 000 pazienti ∕ anno in Europa con approssimativamente 16 000 morti all'anno.

MM è principalmente una malattia dell'anziano, con un età media di diagnosi di 68 anni. Prima dell'introduzione degli agenti alchilanti la media di sopravvivenza era di 12-17 mesi dalla diagnosi La combinazione melphalan più prednisone (MP) è rimasta il trattamento più efficace per decadi sebbene la remissione era rara (5%) e la media di sopravvivenza non superava i 3 anni. Negli anni 1990, chemioterapia ad alte dosi (HDT) associata a trapianto autologo di cellule staminali ha prolungato la sopravvivenza (di un anno in più con tasso di mortalità di 1–2%). L'avvento dei nuovi farmaci immunomodulanti ha raddoppiato la sopravvivenza mediana dei pazienti con MM fino a circa 5 anni e i pazienti hanno il 50% di probabilità di raggiungere la risposta completa ai trattamenti con un tasso di sopravvivenza fino a 10 anni.

Più recentemente è stata sviluppata la lenalidomide nel MM recidivato e refrattario. Le interazioni tra cellule di mieloma, le cellule stromali midollari e il microcircolo contribuiscono alla persistenza e all'insorgenza del tumore e la lenalidomide agisce alterando le interazioni chiave tra cellule tumorali e cellule stromali del midollo osseo superando la resistenza del tumore ai farmaci.(Ria 2000)

Obiettivi

Lo scopo del progetto di ricerca è stato quello di fornire nuove evidenze sul ruolo dei modulatori dell'angiogenesi e la loro connessione con i vari tipi di processi neurodegenerativi.

In collaborazione con l'azienda farmaceutica Celgene si è deciso di studiare l'attività antiangiogenica della lenalidomide e il relativo meccanismo molecolare coinvolto.

Poichè la trasduzione dei segnali legati all'interazione VEGF/VEGF-recettore-2 (VEGFR-2) contribuisce largamente all'angiogenesi e sviluppo del MM (Ria 2004), e media la proliferazione e capillarogenesi in MMECs attraverso un loop autocrino (Podar 2011); si è scelta come base di studio le cellule endoteliali isolate da aspirati midollari di pazienti affetti da mieloma multiplo (MMECs) per poi ampliare gli studi ad un nuovo modello cellulare, le cellule di neuroblastoma

Considerando il potenziale antiangiogenico di tale farmaco ad oggi autorizzato esclusivamente nella cura del mieloma multiplo l'idea è stata quella di testare la sua efficacia in vitro su linee di cellule di neuroblastoma Oggi è vivo un certo interesse in questo ambito d'applicazione.

Nonostante le recenti scoperte in neurochirurgia, radioterapia e chemioterapia la prognosi dei pazienti con glioma rimane infausta. Con il fallimento degli standard agenti citotossici c'è un crescente interesse nello sviluppo di nuove terapie con nuovi meccanismi d'azione.

Studi preclinici e clinici hanno dimostrato che i gliomi sono altamente angiogenici e che la terapia antiangiogenica rappresenta una potenziale nuova strategia di cura. La Talidomide è stato uno dei primi agenti antiangiogenici valutato nei pazienti con glioma

ricorrente. E' stato dimostrato in modelli murini che la talidomide riduce l'espressione di potenti fattori angiogenici come VEGF bFGF e TNF-α.(Marriott 2002,Vacca 2005)

La lenalidomide mostra in vivo proprietà antitumorali dovute a una forte stimolazione delle cellule T, alla produzione di IL-2/IFN-γ, e all'inibizione del TNF-α più marcata della talidomide (Richardson 2002, Corral 2004). L'effetto antitumorale della lenalidomide potrebbe essere correlato alla sua capacità di influire su processi diretti o indiretti legati alla crescita e sopravvivenza delle cellule tumorali. Il reale meccanismo antiangiogenico della lenalidomide non è ancora chiaro, sebbene sia stato visto che la lenalidomide può inibire il VEGF (Lu L 2009), il bFGF e la migrazione endoteliale indotta da TNF-α in modelli preclinici di MM (De Luisi 2010), ad oggi nessuna evidenza preclinica è stata osservata e/o compiuta su modelli neuronali.

Lenalidomide e gliomi

Trial di fase I e II (D'Amato 1997, Fine 2000) sono stati già condotti con la talidomide contro il glioma hanno mostrato un effetto di stabilizzazione della malattia e risposte positive, da qui la tendenza a testare molecole simili ma più efficaci come la lenalidomide. Dato il profilo di sicurezza già mostrato dalla lenalidomide nelle prove su altre forme di tumore, è stata valutata la possibile attività antiglioma della lenalidomide in uno studio di fase I in pazienti affetti da gliomi ricorrenti(Fine 2007). Su 36 pazienti 28 dei quali reclutati per valutare la tossicità primaria e finale, è stato dimostrato che la lenalidomide può essere somministrata a dosi fino a 20 mg/m2, con la sola tossicità legata ad un aumentato rischio di eventi trombo-embolici. Nessuna risposta radiografica è stata osservata nei pazienti che hanno avuto una media di

progressione di tumore <2 mesi e il 12.5% dei pazienti non hanno avuto progressione della malattia a 6 mesi.

Dati preliminari suggeriscono che il singolo farmaco può avere un indicazione limitata nel glioblastoma ricorrente alle dosi valutate, anche se queste osservazioni necessitano conferme in studi allargati.

Il meccanismo d'azione ancora non chiaro lascia ancora spazio per la ricerca del potenziale effetto del farmaco su altre patologie e modelli cellulari, fra cui i modelli neuronali.

Partendo dall'azione già confermata della lenalidomide sul VEGF e altre citochine circolanti si può ipotizzare un possibile effetto positivo della lenalidomide nei processi neurodegenerativi o patologie tumorali del SNC.

Lenalidomide e ipossia

L'ipossia è uno squilibrio tra domanda e offerta di ossigeno, che priva le cellule o i tessuti di ossigeno sufficiente. È consolidata che l'ipossia provoca risposte adattative, che contribuiscono a patologie a breve e lungo termine come l'infiammazione, le malattie cardiovascolari e il cancro. Indotta da ipossia del micro ambiente e mutazioni genetiche, l'elevata espressione della trascrizione di hypoxia-inducible factor-1 (HIF-1) e HIF-2 è una caratteristica chiave di molti tumori umani e ha dimostrato di promuovere processi cellulari, che facilitano la progressione del tumore. Fino ad oggi, sembra chiaro che vi è una conseguenza biologica fra un aberrante espressione di HIF in MM è l'angiogenesi. Gli studi hanno dimostrato che esiste una correlazione positiva tra espressione HIF-1a e 2a HIF e il livello dell'angiogenesi nel midollo osseo e

l'angiogenesi e l'espressione del fattore di crescita endoteliale vascolare (VEGF) e del recettore VEGF in campioni bioptici di pazienti (Giatromanolaki A. et al 2010) Un certo numero di farmaci anti-MM, come adaphostin, lenalidomide, bortezomib,(Tab 1) hanno anche dimostrato di mediare i loro effetti antiangiogenetici, almeno in parte, attraverso l'inibizione di HIF-1(LU L.et al 2009, Martin Sk et al 2011)

Table 1   Therapeutic inhibitors of hypoxia and HIFs

Agent

HIF inhibitors
   PX-478, EZN-2968, polyamide-2, YC-1

Hypoxia-activated pro-drug
   TH-302

Proteasome inhibitor
   Bortezomib

NSAIDs
   Celecoxib, NS-398, ibuprofen

Heat shock protein-90 inhibitors
   Geldanamycin

Tyrosine kinase inhibitors
   Imatinib, trastuzumab, erlotinib, cetuximab, sorafenib, sunitinib

Mammalian target of rapamycin inhibitors
   Rapamycin, CCI-779, RAD001

HDAC inhibitors
   Depsipeptide (FK228)

Immunomodulatory agents
   Lenalidomide

Microtubule inhibitors
   2-Methoxyestradiol, taxotere, vincristine

Chemotherapeutic agents
   Doxorubicin, daunorubicin, topotecan

Abbreviations: HDAC, histone deacetylase; HIF, hypoxia-induci transcription factor; NSAID, non-steroidal anti-inflammatory drug.

E' stato quindi dimostrato che l' ipossia è uno dei principali stimoli in grado di indurre l'over-espressione del VEGF e di conseguenza promuovere una patologica angiogenesi. Cosi si è deciso di indurre chimicamente delle condizioni ipossiche nelle cellule di neuroblastoma mediante trattamento con $CoCl_2$ (Wang et al 2012) e studiare

l effetto di modulazione di lenalidomide nell espressione di VEGF   Tuttavia, molto resta ancora da comprende circa i meccanismi che regolano il sistema del VEGF in condizioni ipossiche.

Saggio MTT

La citotossicità del farmaco è stata misurata attraverso la determinazione della vitalità delle cellule trattate rispetto al controllo le cellule non trattate, mediante test MTT [3-(4,5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromuro]. Il test si basa sulla capacità della citocromo c ossidasi, enzima mitocondriale e della succinato deidrogenasi, di ridurre il tetrazolo giallo in formazano viola che, non essendo in grado di attraversare la membrana plasmatica, si accumula all'interno delle cellule vitali nella coltura. La Solubilizzazione di formazano con un detergente appropriato darà un colore viola in proporzione alla quantità di formazano prodotta e di conseguenza dellla vitalità delle cellule. Dopo il trattamento con lenalidomide, le colture cellulari sono state incubate per 2 ore a 37 ° con una soluzione di MTT (1 mg/ml) in PBS; i cristalli formati si solubilizzzano con DMSO. Dopo la solubilizzazione dei cristalli di formazano, un'aliquota del surnatante è stata utilizzata per leggere l'assorbanza a 590nm con un lettore di micropiastre.
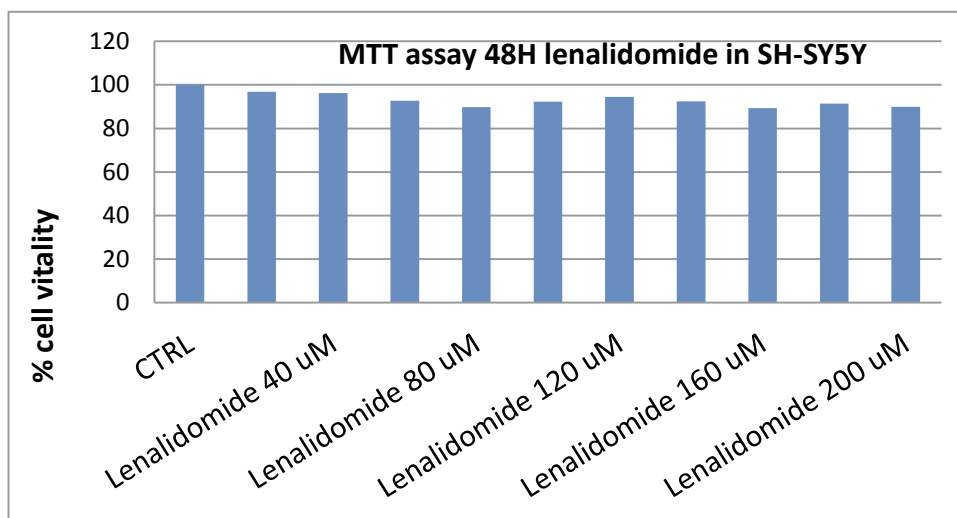
Saggio di ipossia con CoCl2

Cloruro Cobalto (II) esaidrato (CoCl2 • 6H2O, MW=237.9) è un induttore chimico dell hypoxia-inducible factor HIF-1α (Wang et al 2011) Questo prodotto è solubile in acqua (100 mg/ml), dando luogo ad una soluzione rossa E' stata preparata una soluzione stock 25mM di cloruro di cobalto in acqua sterile prima dell' uso ed è stata utilizzata alla concentrazione finale di 100μM per indurre la condizione di ipossia. La soluzione è stata aggiunta al terreno di coltura delle cellule e si è incubato per 24 ore in incubatore (37°C; 5% C02).

Risultati

Saggio di tossicità (MTT) della lenalidomide su neuroblastoma

La Lenalidomide non ha esercitato alcun effetto tossico su cellule di neuroblastoma (fig 9).

Effetto di lenalidomide su espressione di VEGF in condizioni di ipossia indotta da cloruro di cobalto.

Cellule di neuroblastoma sono state trattate a 24h con lenalidomide 50μM in condizioni di ipossia indotte da cloruro di cobalto 100 μM per studiare il ruolo di lenalidomide nella modulazione di VEGF.

Dopo 24h dopo estrazione di RNA secondo protocollo Trizol (Invitrogen) si evidenzia una riduzione nell'espressione di VEGF indotta dalla lenalidomide.
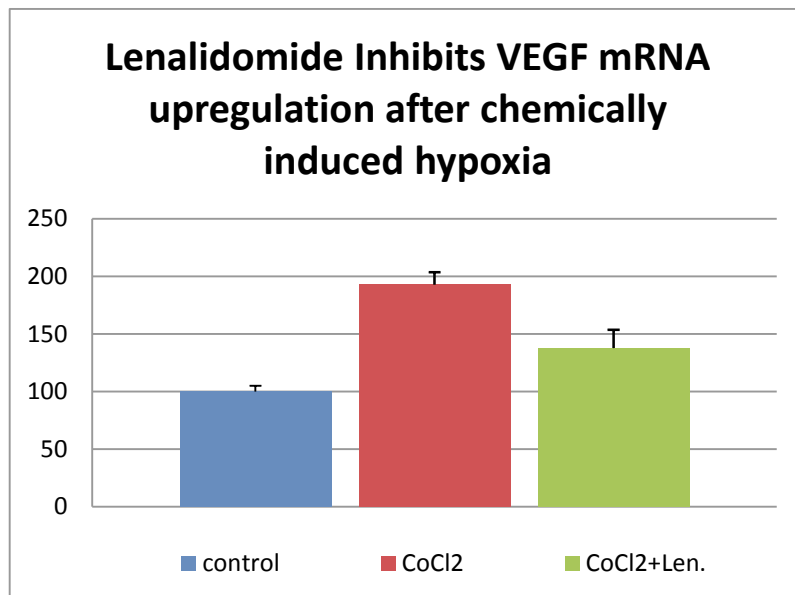
Lenalidomide Inhibits VEGF mRNA upregulation after chemically induced hypoxia

control   CoCl2   CoCl2+Len.

Fig.10 RT-PCR analysis of VEGF mRNA from neuroblastoma (SH-SY5Y) after chemically induced hypoxia by treatment with CoCl2 100 µM (24 h). Hypoxic conditions strongly upregulated VEGF mRNA expression (+95% vs control) and co-treatment with lenalidomide (50 µM) significantly reduced (-30% vs $CoCl_2$) VEGF expression

Discussione

Focalizzandosi sull'approvata indicazione terapeutica finora ottenuta da lenalidomide nel mieloma multiplo si è cercato di investigare lo studio del meccanismo d azione di lenalidomide in vitro su cellule neuroblastoma,

si è voluto quindi inizialmente testare l effetto citotossico di questo farmaco su un modello tumorale neuronale e si è mostrato che lenalidomide neanche a concentrazioni alte (200µM) sembra dare segni di citotossicità, al contrario di quanto largamente noto su cellule isolate di mieloma multiplo (Hideshima T 2000, Mitsiades N 2002).

Successivamente da esperimenti di ipossia effettuati utilizzando il saggio con CoCl2 si è notato che lenalidomide sembra aumentare l espressione genica di VEGF in

condizione di stress ipossico, svolgendo in questo modello cellulare un azione pro-angiogenica.

Una marcata vascolarizzazione è una caratteristica di molte neoplasie del sistema nervoso centrale e in numerosi studi si è evidenziato come il VEGF sia un potente mediatore dell angiogenesi in molti tumori cerebrali (glioblastoma, emangioblastoma, meningioma, neuroblastoma) e ci sia una significativa correlazione fra l' espressione del VEGF e la proliferazione dell' endotelio vascolare (Berkman RA, 1991).Comunque nel neuroblastoma, la forma più comune di tumore cerebrale pediatrico, l espressione del VEGF sembra influire sull angiogenesi nonchè direttamente sulla crescita delle cellule maligne di neuroblastoma (Takano S 1996).

L'RNA messaggero di VEGF è espresso in grande misura nel neuroblastoma come anche nei tumori solidi che dipendono per la loro crescita e disseminazione dai processi di neo-vascolarizzazione (Langer I 2000) per cui é possibile ipotizzare che l effetto inibitorio di lenalidomide verso l up-regolazione di VEGF qui osservato nelle colture cellulari di neuroblastoma possa aprire nuove prospettive nell utilizzo di questo farmaco anche in campo neurologico.

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INTRODUCTION

Neurodegeneration

Neurodegeneration is the umbrella term for the progressive loss of structure or function of neurons, including death of neurons. Many neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's occur as a result of neurodegenerative processes. As research progresses, many similarities appear which relate these diseases to one another on a sub-cellular level. Discovering these similarities offers hope for therapeutic advances that could ameliorate many diseases simultaneously.

The greatest risk factor for neurodegenerative diseases is aging. Mitochondrial DNA mutations as well as oxidative stress both contribute to aging (DiMauro S, Schon EA 2008) Many of these diseases are late-onset, meaning there is some factor that changes as a person ages for each disease (Rubinsztein DC October 2006).One constant factor is that in each disease, neurons gradually lose function as the disease progresses with age.

Alzheimer's disease (AD), in particular, is the leading cause of senile dementia in the elderly, is a complex neurodegenerative syndrome, which leads to the inexorable progression to a loss of cognitive abilities for which there is at present an effective cure. To date, in fact only certain medications are available, while lacking both symptomatic drugs able to revert the process of Neurodegeneration and effective diagnostic tools that allow a diagnosis in primary stages the gradual lengthening of life of the population suggests that the AD can reach epidemic proportions within the next twenty years, making it essential to develop new therapeutic strategies and reliable diagnostic tools for early detection of this disease. The emerging opinion is that at the base of the eziopatologico process there is a cerebrovascular dysregulation, which is a characteristic not only of purely diseases cerebrovascular, as the stroke, but also

neurodegenerative conditions Many neuropathologic studies have described, indeed, morphological alterations in brain capillaries and a reduction of blood flow in the brain of patients with the disease-causing importance of these vascular changes is difficult to interpret because it is unclear whether this is a consequence or a cause of neuronal dysfunction and neurovegetative process.

Angiogenesis

Angiogenesis means the set of functional processes that lead to the formation of new blood vessels from pre-existing vessels. Endothelial cells that make up the inner layer of all vessels, are central in the angiogenic phenomenon (Conway 2001). They are involved, according to a genetically determined program, mediated by specific factors and receptors, in a series of events such as proliferation, differentiation, migration and morphological organization in close connection with the surrounding tissue. The formation of a jar then passes through several well-defined stages, characterized by changes of endothelium and extracellular matrix. In the first stage of angiogenesis occurs a "destabilization" of existing vessels due to an increase in vessel permeability, and a loss of connections between endothelial cells. Therefore there is a second phase of the migration and proliferation of endothelial cells in the tissue where it is required the formation of new blood vessels. During this stage proteolytic enzymes are liberated which alter the density of extracellular matrix for the purpose of facilitating migratory activity of endothelial cells. The third stage of angiogenesis corresponds to the endothelial cell differentiation, and is characterized by inhibition of cell proliferation and formation of capillaries. The last stage of functional maturation is characterized by recruitment of periendoteliali cells, which support and smooth muscle cells periciti and reorganization of cellular interactions (Bussolino 1998)

Angiogenesis and Neurodegeneration

Angiogenesis-the development of new blood vessels--is a crucial step for the formation and remodeling of the nervous system and protect it from disease. Recent research has improved our understanding of how the brain and other tissues grow new blood vessels in normal and pathological conditions. Some factors of angiogenesis, particularly the VEGF (vascular endothelial growth factor) are known to have an involvement in neuronal proliferation (neurogenesis), prevention or protection from neuronal damage (neuroprotection) and in the pathogenesis of stroke and Alzheimer's disease.

Epidemiological studies show that anti angiogenic drugs bring benefits to patients with Alzheimer's disease (AD), indicating that the non-vascular regulation plays a critical role in the AD, which is then a disease "angiogenesis-dependent" (Harris 2005). In addition, the angiogenic growth factor, VEGF and bFGF are also included among those whose synthesis is stimulated by A-beta (Folkman 2007), and are also involved in neuronal plasticity due to their gliotrofic activities/controls (Mc Auslan 1980, Hu 1998). The study of relationship between A-beta and the angiogenic process (Glesne 2006, Gaggelli 2005) can clarify the process to etiopathology. Both the AD, as well as other neurodegenerative disorders are neurovascular diseases are, in fact, in recent years, it has emerged the concept of Neurovascular unit as a new paradigm for the study of the physiology and pathology of the CNS. This concept proposes that the focus purely neurocentric is not sufficient and emphasizes that all cell of Neurovascular unit i.e. vascular components, neurons and glia, should be examined in an integrated context. The signaling and cell-cell coupling between these different compartments form the basis for a normal functionality. A disordered communication and a neuro-vascular coupling disrupted gives away to disruption and disease. Besides a physical association the same micro-environment, these cells interact with one another through

feedback mechanisms positive and negative related to metabolic demands (Pogue 2004). Neurogenesis and angiogenesis revealed many similarities between these processes. These include growth factors and their receptors, similar signals for cell proliferation, cell fate, formation of contours and physical space as a result of a parallel model anatomical development (Cantara 2004). Angiogenesis is a multifactor complex process whose disomeostasis can be cause of several diseases with acute or chronic course. It has attracted the interest of many researchers who are involved in the study of various pathological processes, from cancer to neurodegenerative diseases. Then, one of the main objectives of this study concerns the search for common factors and correlations between chronic neurodegenerative diseases and vascular disorders, to highlight the role yet underestimated of angiogenesis and identify possible therapeutic interventions that have as primary target and then process the angiogenic endothelial cells.

Hypoxia and angiogenesis

Angiogenesis is not just a physiological process, but is also the basis of many pathological States as the progression of tumors, retinopathy of the premature and diabetic retinopathy. The growth factor of vascular endothelial cells (VEGF) is one of the most powerful pro-angiogenic factors. Pro-angiogenic action of VEGF is mediated by its interaction with two tyrosine kinase receptor (VEGFR-1, VEGFR-2) and it is known that over-expression of VEGF is one of the main causes of this. It has been shown that hypoxia is one of the main stimuli that can induce the over-expression of VEGF and therefore promote a pathological angiogenesis. However, much remains to be included about the mechanisms that regulate the system of VEGF in hypoxic conditions.

The term hypoxia indicates a State of insufficient oxygen in the cellular environment. This condition can affect the entire organism in situations of low concentration of oxygen in the environment (e.g. happens at high altitude), or it can develop in a bounded area of tissue as a result of ischemia or neoplastic growth (Jeong et al., 2011). Given the vital importance of oxygen for aerobic cellular metabolism in animal organisms have evolved complex homoeostatic mechanisms in order to prevent hypoxia. Specific oxygen sensors were already described in bacteria and yeast, but only recently has begun to shed light on regulation systems implemented by higher organisms including humans. Despite being known to exist some specialized cells in assessing the levels of oxygen, all mammalian cells are able to react to a local condition of hypoxia by activation of a transcriptional cascade involving numerous metabolic pathways. This phenomenon takes place in a few hours and is fundamental to the whole organism, much to be involved in many physiological and pathological processes (Gariboldi et al., 2010). A number of experimental evidence and in-depth analysis of the pathophysiology of vascular and neoplastic diseases have identified the availability of oxygen as an important regulator of angiogenesis, although adaptive systems are complex and still not quite clear (Cassavaugh and Lounsbury, 2011). Understanding the mechanisms that regulate cellular processes promoted by hypoxia may therefore assume a very important role in the development of therapeutic strategies aimed to combact major diseases such as cancer and proliferative retinopathy (Mazzone, 2010). Numerous research indicates that hypoxia induces an increase in VEGF expression in different cell types, including human endothelial cells (Avouac et al., 2008; Cho et al., 2008; Liu et al., 2009; Nilsson et al., 2004; Takata et al., 2008; Zheng et al., 2008). Hypoxia can also modulate the expression of VEGF receptors although the data riporati in human endothelial cells are sometimes very at odds with one another, both as regards

VEGFR-1 (Avouac et al., 2008; Cho et al., 2008; Gerber et al., 1997; Nilsson et al. 2004) and VEGFR-2 (Avouac et al., 2008; Takata et al., 2008; Waltenberger et al., 1996)
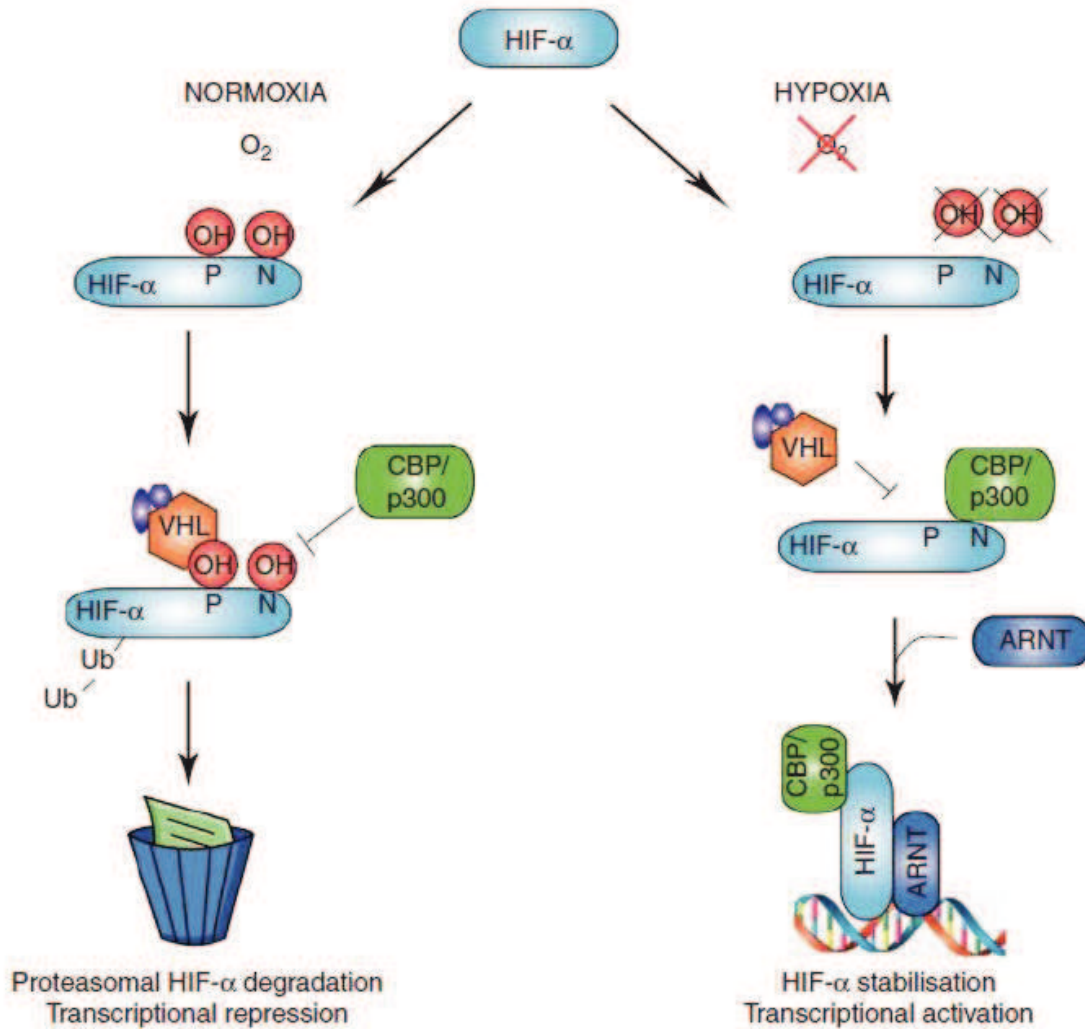
Transcriptional factors involved in angiogenesis

It's been done a lot of work to clarify the role of transcription factors in regulating target genes involved in angiogenesis. In this regard, the HIF hypoxia inducible factor-1 from and signal transducer and activator of transcription STAT3, are particularly interesting because their activation is coupled to the regulation of VEGF 15 (Chen and Han, 2008; Fong, 2009). HIF-1 is a transcription factor consisting of the α and β subunits. The HIF-1 β subunit is constitutively expressed while the expression and activity of HIF-1 α subunits are closely controlled by cellular concentrations of oxygen (Fig.1) .In fact, in conditions of normal oxygen levels, HIF-1 Alpha is quickly hydroxylated by prolyl hydroxylase enzymes that require oxygen and iron in order to carry on its business, prompting the binding of this protein with subunits of Von Hippel-Lindau a ubiquitin ligase E3-that determines the start to degradation by the proteasome. In condition of hypoxia HIF-1 α escapes this degradation due to inactivation of the hydroxylase and begins to accumulate in the cytoplasm of the cell. When its cytoplasmic concentration reaches a critical level, it moves into the nucleus where links HIF-1 β subunit (Takahashi and Shibuya, 2005). Once the dimerization of α and β subunits, HIF-1 promotes transcription of over 70 target genes (seed, 2004) depending on the type of cell (Kelly et al., 2003) and organ (Schneider et al., 2009). HIF-1 is able to regulate various physiological processes including erythropoiesis, Glycolysis and angiogenesis, which are the main responses to adaptation to hypoxia (Jiang et al., 1997). In particular, HIF-1 is the main factor responsible for the expression and secretion of VEGF by cells in hypoxic conditions and plays a key role in several diseases

characterized by abnormal development of blood vessels (Pagés and Pouysségur, 2005).

Therefore the investigation of the existing molecular correlation between angiogenesis and ipoxia has seemed interesting and challenging.

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Lenalidomide

Lenalidomide (Revlimid,[®] CC-5013) is an Immunomodulatory Drug (IMiD) structurally related to thalidomide. Development of lenalidomide and other IMiDs began in the 1990s (Hideshima 2000) to improve efficacy and reduce side effects of thalidomide.
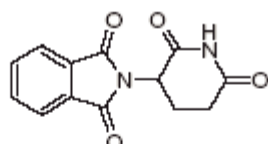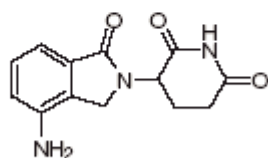

Fig. 1. Chemical structure of thalidomide.

Fig. 2. Chemical structure of lenalidomide.

Phase I, II, and III trials have been carried out with lenalidomide in patients with relapsed or refractory multiple myeloma (MM), and the drug has shown impressive response rates in the relapse (Dimopolous 2007). Two large multicentre phase III trials comparing the combination of lenalidomide plus dexamethasone to dexamethasone alone in patients with relapsed/refractory MM found prolonged time to progression with addition of lenalidomide. Furthermore a phase II trial showed promising activity in newly-diagnosed myeloma treated with lenalidomide plus dexamethasone (Dredge 2002).

Mechanism of action of Lenalidomide

The clinical evidence for therapeutic potential of lenalidomide in various malignant conditions is consistent with the multitude of pharmacodynamic effects that have been shown in vitro and in animal models. Studies have shown that lenalidomide may work through various mechanisms in different hematologic malignancies (Fig.3)

These mechanism involved direct cytotoxicity as well as through indirect effects on tumor immunity. Thus the differential efficacy noted with lenalidomide therapy among various disease states can possibly be explained individual's immune status and disease specific pathophysiology. Following are the different mechanisms explained by which lenalidomide acts in the body.

Immunomodulation

The immune system is comprised of cellular (macrophages, dendritic cells, NK cells, T cells and B cells), and humoral components (antibodies, cytokines). The immune system can prevent development of cancers by eliminating or suppressing oncogenic viral infections, altering the inflammatory milieu conducive to tumor genesis, and by immune surveillance by identifying and destroying transformed cells before they can cause harm [Hu 1998].

Lenalidomide has been shown to modulate different components of the immune system by altering cytokine production, regulating T cell co stimulation and augmenting the NK cell cytotoxicity. Immunomodulatory properties of Lenalidomide are implicated in its clinical efficacy in multiple myeloma, CLL and myelodysplastic syndromes; where the disease pathogenesis involves in part a deregulated immune system in the form of altered cytokine networks in tumor microenvironment, defective cell regulation of host-tumor immune interactions, and diminished NK cell activity.

Altering cytokine production

Cytokines are soluble proteins secreted by hematopoietic and non hematopoietic cell types and are critical for both innate and adaptive immune responses. The expression of cytokines by cells may be altered in immunological, inflammatory, infectious and neoplastic disease states.

Cytokines in turn exert their effects by influencing gene activation, growth, differentiation, functional cell surface molecule expression and cellular effector function. A coordinated cellular and humoral (cytokines, antibodies) interactions facilitate tumor destruction.

Lenalidomide has been shown to inhibit production of pro inflammatory cytokines TNF-α, IL-1, IL-6, IL-12 and elevate the production of anti-inflammatory cytokine IL-10 from human PBMCs[Corral 1999]. The downregulation of TNF-secretion is particularly striking and is up to 50,000 times more when compared to thalidomide[Muller 1999]. TNF-α is a highly pleiotropic cytokine produced primarily by monocytes and macrophages and plays an important role in protective immune responses against bacterial and viral infections. Elevated TNF-α production is implicated in the pathogenesis of various hematologic malignancies [Symeonidis 2001].

These reductions correlate with cytoreduction suggesting a casual relationship with tumor growth [Chanan Khan 2006].Similarly, reduction in IL-6 and TNF-α levels could explain the action of lenalidomide in multiple myeloma.IL-6 inhibits the apoptosis of malignant myeloma cells and helps in their proliferation[Lichestein 1995]. Lenalidomide downregulates the production of IL-6 directly and also by inhibiting multiple myeloma (MM) cells and bone marrow stromal cells (BMSC) interaction which augments the apoptosis of myeloma cells [Richardson 2002]. The precise mechanism of TNF-α downregulation by lenalidomide is not known, however thalidomide has been shown to increase the degradation of TNF-α mRNA [Moreira 1993,]. It is possible that lenalidomide may work through similar mechanisms.

Anti-angiogenesis activity

The growth of the primary and metastatic tumors requires the development of new blood vessels, a process described as angiogenesis. Tumors possess the ability topromote the formation of new blood vessels from preexisting host capillaries at a critical phase of the tumor development when the balance of pro- angiogenic and anti-angiogenic factors is altered. Vascular endothelial growth factor (VEGF) and its receptors are required for the formation of blood vessels during embryonic development, wound healing, and carcinogenesis. Tumors are more dependent on the VEGF-Receptor signaling for growth and survival compared to normal endothelial cells [D'amato 1994]. Early studies showed that Thalidomide had anti angiogenic activity in a rabbit model of corneal neovascularization that was induced as a response to bFGF. This report led to its use in Multiple Myeloma, where it demonstrated clinical benefit and was approved for use by the FDA. Thalidomide and the newer IMiDs have also been shown to significantly decrease the expression of angiogenic factors VEGF and Interleukin-6 (IL-6) in multiple myeloma; thereby reducing angiogenesis and hence contributing to clinical activity in multiple myeloma[Teo 2005]. The newer IMiDs were found to be 2–3 times more potent compared to thalidomide in antiangiogenic activity in various vivo assays [Gupta 2005] The antiangiogenic activity of both thalidomide and IMiDs has also been shown to be independent of immunomodulatory effects [Dredge 2001].

Apart from alteration in the levels of VEGF, analysis of signal transduction events show that lenalidomide partially inhibits Akt phosphorylation after VEGF stimulation in endothelial cells and also has inhibitory effects on phosphorylation of Gab1, a protein upstream of Akt 1[Dredge 2005]. These observations demonstrate that IMiDs may affect angiogenesis by multiple mechanisms.

Direct anti tumor activity

Lenalidomide treatment has also shown anti proliferative activity against MDS and MM cells in the absence of immune effector cells [Bartlett 2004]. Malignant plasma cells derived from refractory cases of myeloma were shown to be susceptible to IMiD induced growth arrest. Lenalidomide has also been shown to inhibit proliferation in Burkitt's Lymphoma cell lines by causing dose dependant cell cycle arrest in G0-G1 phase [Verhelle 2004]. Lenalidomide upregulated Cyclin dependant kinase (CDK) Inhibitor, p21 waf-1, a key cell cycle regulator that modulates the activity of CDKs. Similar reductions in CDK2 activity have been demonstrated in myeloma derived cell lines, U266 and LP-1. In contrast, the normal B cells obtained from healthy donors were immune from growth inhibition and did not show any upregulation of p21 expression after 3 days of lenalidomide treatment. In other studies, thalidomide and its analogues have also been shown to induce apoptosis in MM cell lines [Hideshima 2005].
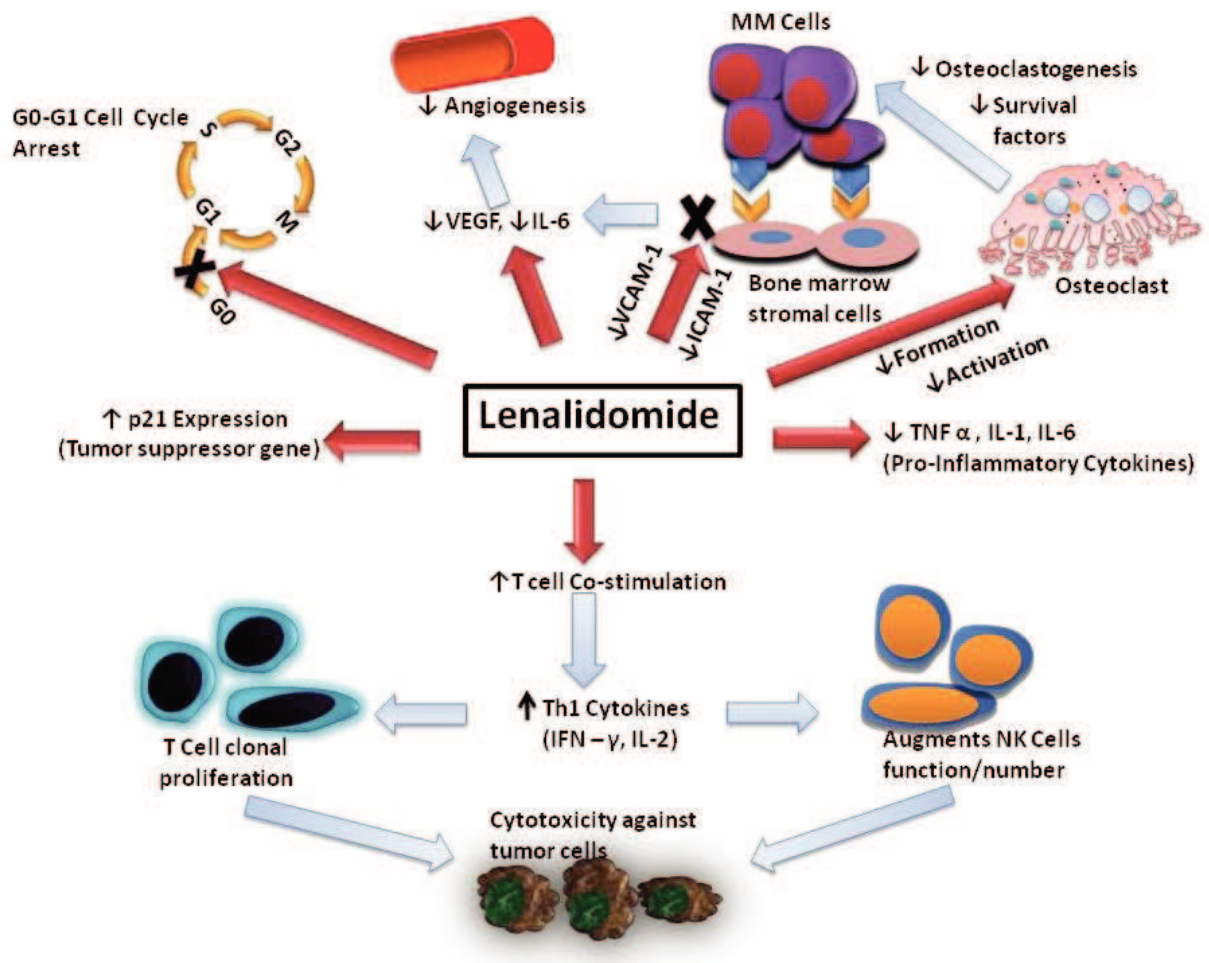
Fig.3 Mechanism of action of lenalidomide

Lenalidomide in myeloma

The lenalidomide is authorized by June 2007 in Myeloma therapy refractory to conventional therapy.(Dimopolous 2007, Lacy 2007) (Myeloma is literally a "oma, tumor, or which involve the" myelo, "or blood-producing cells of the bone marrow. The affected cells are plasma cells (a type of white blood cell) that are producers of antibodies (immunoglobulins). The diagnosis is primarily based on infiltration of plasma cells in the bone marrow, Osteolytic lesions and presence of immunoglobulin (or fragments in human serum or urine). MM represents approximately 10% of hematological cancers and 1% of all cancers. The diagnosis of MM is approximately 21.000 patients/year in Europe with approximately 16000 deaths per year.

MM is primarily a disease of the elderly, with an average age of diagnosis of 68 years. Before the introduction of alkylating agents average survival was 12-17 months after diagnosis plus melphalan prednisone combination (MP) remained the most effective treatment for decades although remission was rare (5%) and the average survival does not exceed 3 years. In 1990, high-dose chemotherapy (HDT) associated with Autologous stem cells has prolonged the survival (of one more year with the mortality rate of 1-2%). The advent of immunomodulating drug doubled the median survival of patients with MM up to about 5 years and patients have a 50% probability of achieving complete response to treatment with a survival rate of up to 10 years

More recently the lenalidomide has been developed in MM since relapsed and refractory. Interactions between myeloma cells, stromal cells and Medullary microcirculation contribute to persistence and the onset of cancer and the lenalidomide acts by altering key interactions between tumor cells and bone marrow stromal cells by overcoming the resistance of cancer drugs.

Lenalidomide and Hypoxia

Hypoxia is an imbalance between oxygen supply and demand, which deprives cells or tissues of sufficient oxygen. It is well established that hypoxia triggers adaptive responses, which contribute to short- and long-term pathologies such as inflammation, cardiovascular disease and cancer. Induced by both microenvironmental hypoxia and genetic mutations, the elevated expression of the hypoxia-inducible transcription factor-1 (HIF-1) and HIF-2 is a key feature of many human cancers and has been shown to promote cellular processes, which facilitate tumor progression.

. To date, the most well-documented biological consequence of aberrant HIF expression in MM is angiogenesis. Studies have shown that there is a positive correlation between

HIF-1a and HIF-2a expression, and the level of BM angiogenesis and expression of vascular endothelial growth factor (VEGF) and VEGF receptor in patient biopsy specimens. A number of anti- MM drugs, such as adaphostin, lenalidomide and bortezomib, have also been shown to mediate their antiangiogenic effects, at least in part, through inhibition of HIF-1.(Lu 2009).

The increasing importance of HIF-1a and HIF-2a in tumorigenesis (and indeed, numerous other disease states) raises the possibility that agents which specifically inhibit these transcription factors, would provide significant therapeutic benefit.

As showed in table 1 (Martin et al 2011) a growing number of conventional and novel anticancer agents included lenalidomide has been shown to indirectly inhibit HIF activity

Table 1   Therapeutic inhibitors of hypoxia and HIFs

Agent

HIF inhibitors
  PX-478, EZN-2968, polyamide-2, YC-1

Hypoxia-activated pro-drug
  TH-302

Proteasome inhibitor
  Bortezomib

NSAIDs
  Celecoxib, NS-398, ibuprofen

Heat shock protein-90 inhibitors
  Geldanamycin

Tyrosine kinase inhibitors
  Imatinib, trastuzumab, erlotinib, cetuximab,
  sorafenib, sunitinib

Mammalian target of rapamycin inhibitors
  Rapamycin, CCI-779, RAD001

HDAC inhibitors
  Depsipeptide (FK228)

Immunomodulatory agents
  Lenalidomide

Microtubule inhibitors
  2-Methoxyestradiol, taxotere, vincristine

Chemotherapeutic agents
  Doxorubicin, daunorubicin, topotecan

Abbreviations: HDAC, histone deacetylase; HIF, hypoxia-induci
transcription factor; NSAID, non-steroidal anti-inflammatory drug.

It has been shown that hypoxia is one of the main factor that can induce the over-expression of VEGF and therefore promote a pathological angiogenesis. So it was decided to induce chemically hypoxic conditions in neuroblastoma cells by treatment with $CoCl_2$ solution (Wang et al. 2012) and to study the effect of lenalidomide in modulation of VEGF expression However, much remains to be known about the mechanisms that regulate the system of VEGF in hypoxic conditions.

Objectives

Since the VEGF/VEGF receptor-2 (VEGFR-2) pathway greatly contributes to MM angiogenesis and growth (Ria 2004), and mediates proliferation and capillarogenesis in MMECs through an autocrine loop (Podar 2005); we aimed to investigate whether lenalidomide has an antiangiogenic activity on other cellular lines and to elucidate the molecular mechanisms involved.

Considering the antiangiogenic potential of the drug until now allowed only in the treatment of multiple myeloma, the idea is to test its efficacy in vitro on neuroblastoma cell lines and/or glioblastoma, isolated or co-culture with endothelial cells. Today is alive some interest in this scope. Despite recent advances in neurosurgery, radiotherapy and chemotherapy prognosis of patients with glioma remains poor. With the failure of standard cytotoxic agents there is a growing interest in the development of new therapies with novel mechanisms of action. Preclinical studies and clinical trials have demonstrated that gliomas are highly angiogenic and that antiangiogenic therapy represents a potential new treatment strategy. (Fine 2000, Bauman 2004). Thalidomide was one of the first antiangiogenic agents evaluated in patients with recurrent glioma. It has been shown in mouse models that Thalidomide reduces the expression of powerful factors as VEGF and bFGF TNF-α. The lenalidomide view in vivo anticancer properties due to a strong stimulation of T cells, the production of IL-2/IFN-γ, and the inhibition of TNF-α stronger of Thalidomide (Kotla 2009). The Antitumor effect of lenalidomide might be related to its ability to affect processes directly or indirectly related to the growth and survival of cancer cells. The real antiangiogenic mechanism of lenalidomide is not yet clear, although it has been seen that lenalidomide can inhibit VEGF, bFGF and endothelial migration induced by TNF-α in preclinical models of

MM, to date no preclinical evidence was observed and accomplished on neuronal models.

Therefore in accordance with Celgene we decided to study the mechanism of action of lenalidomide in neuroblastoma cells, to elucidate the influence of that drug in molecular mechanism involved in angiogenesis and neurodegeneration.

Lenalidomide and gliomas

In preclinical testing, lenalidomide demonstrates antiangiogenic, proapoptotic, and anti-inflammatory activities in addition to its immunomodulatory effects (Zhu 2008)

Lenalidomide is being evaluated in a number of adult solid tumors, including lymphomas (Wiernik 2008), renal cell carcinoma (Amato 2008), melanoma, and CNS tumors (Fine 2007). Although the exact antitumor mechanism is not completely understood, lenalidomide has direct effects on tumor cells, effects on the tumor microenvironment, and immunomodulatory effects that include alteration of ligand-induced cellular responses, modulation of cytokine responses, altered production of growth factors, and costimulation of T-cell activation (Crane 2005).

Clinical trials of lenalidomide have been performed in adults using several different schedules (Fine 2007, Patel 2008, Sharma 2007) Common adverse effects include neutropenia, thrombocytopenia, GI toxicities, skin toxicity, and fatigue (Bartlett 2004) Myelosuppression is the most common toxicity and the dose-limiting toxicity (DLT) and is more frequently observed at doses $\geq$ 50 mg/m^2/d. Other toxicities are generally mild, although a possible increased risk of thrombosis has been reported (Knight 2006)In the phase I dose-escalation trial of lenalidomide in adults with recurrent CNS tumors, a predetermined maximum dose of 40 mg daily for 21 days followed by a 1-

week rest was well-tolerated (Fine 2007) Children with recurrent, refractory, or progressive primary CNS tumors were treated with lenalidomide to estimate the maximum-tolerated dose (MTD), describe toxicities, and evaluate pharmacokinetics in this population.

Materials and methods

MTT Assay

The toxicity of the drug was measured through the determination of cell viability in the treated cells compared to control untreated cells, by MTT test [3-(4,5-dimethylthiazol-2-yl) -2 , 5-diphenyltetrazolium bromide]. The test is based on the ability of the mitochondrial enzyme, cytochrome c oxidase and succinate dehydrogenase, to reduce yellow tetrazole into purple formazan that, being unable to cross the plasma membrane, will accumulate within the viable cell in culture. Solubilization of formazan with an appropriate detergent will yield purple color in proportion to the amount of formazan produced and consequently to the viability of cells. After treatment with the drug, cell cultures are incubated for 2 hours at 37°C with a solution of MTT (1mg/ml) in PBS; the formed crystals are melted with DMSO. After the solubilization of the formazan crystals, a rate of supernatant was used to read the absorbance at 590nm using a microplate reader.

Hypoxia induced by CoCl2 solution

Cobalt (II) Chloride hexahydrate (CoCl2 • 6H2O, MW=237.9) is a chemical inducer of hypoxia-inducible factor-1. This product is soluble in water (100 mg/ml), yielding a clear, red solution.
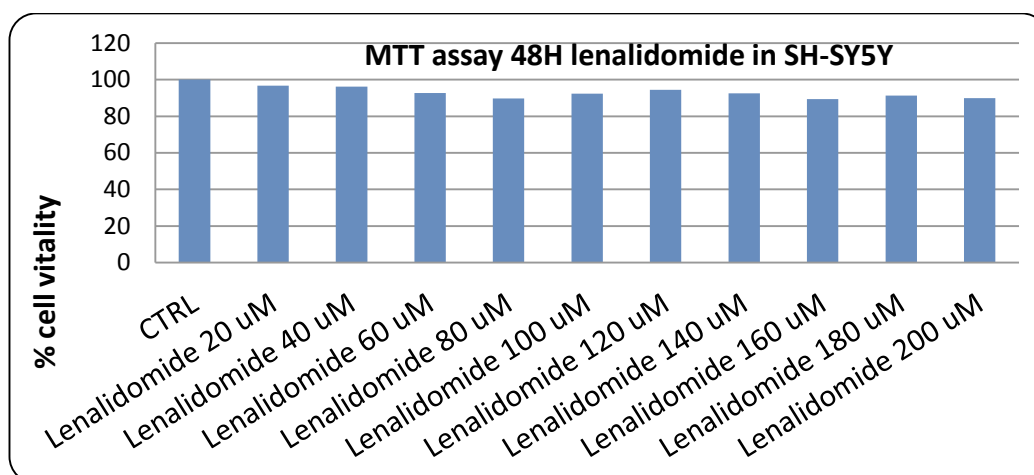
A 25mM stock solution was prepared in sterile dd water, (prepare immediately before use)

CoCl2 at the final concentration of 100μM was used in regular cell culture media to induce hypoxia .therefore we added the CoCl2 containing media to neuroblastoma cells and incubate the cultures for 24hours in a conventional incubator (37°C; 5% C02).

Results

MTT assay

Lenalidomide did not have any cytotoxic effect on neuroblastoma cells (fig 9)

Lenalidomide down regulates VEGF expression in hypoxic condition induced by CoCl2 solution

SHY5Y were treated with lenalidomide 50μM for 24 hours hipoxic conditions induced

by **CoCl2** 100 μM to study the effect of lenalidomide on VEGF expression

After RNA extraction by Trizol protocol and RT-PCR lenalidomide had a strong

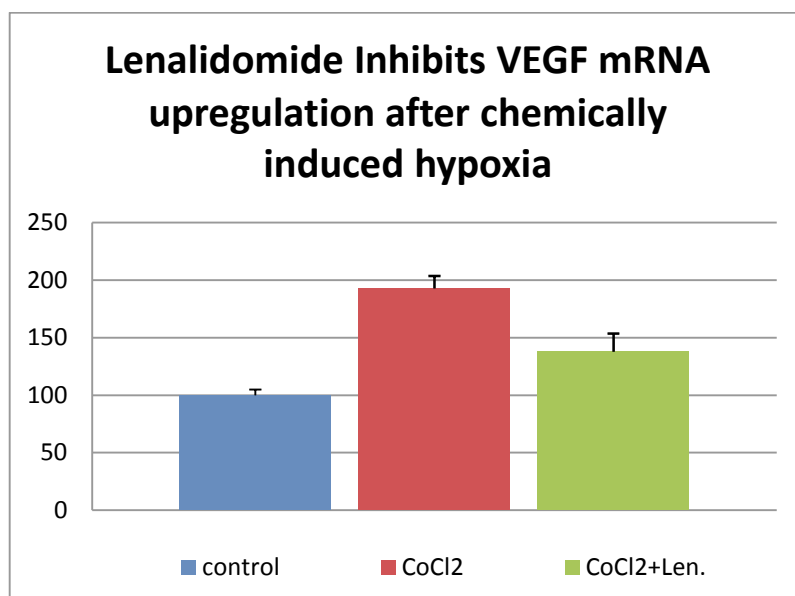inhibitory effect on VEGF mRNA expression (Fig.10)

Lenalidomide Inhibits VEGF mRNA upregulation after chemically induced hypoxia

FIG. 10
RT-PCR analysis of VEGF mRNA from neuroblastoma (SH-SY5Y) after chemically induced hypoxia by treatment with CoCl2 100 µM (24 h). Hypoxic conditions strongly upregulated VEGF mRNA expression (+95% vs control) and co-treatment with lenalidomide (50 µM) significantly reduced (-30% vs $CoCl_2$) VEGF expression.

Discussion

In this project we aimed to investigate the mechanism of action of lenalidomide in neuroblastoma cells thus firstly we tested the citotoxic effect of this drug in a tumour brain model such as neuroblastoma and we demonstrated that Lenalidomide has no citotoxic effect on this model neither at high concentration (200 μM), on the contrary in myeloma cells this drug exerts a strong tumoricidal effect (Hideshima 2000).

Subsequently in experiments of hypoxia induced by CoCl2 solution , lenalidomide seems to decrease VEGF mRNA expression in neuroblastoma cell coltures (SH5Y5Y), confirming its potential effect on VEGF molecular pattern also in stress conditions.

A marked vascularization is a hallmark of many neoplasms in the nervous system and VEGF has been investigated as a potent mediator of angiogenesis in many brain tumors (glioblastomas, hemangioblastomas, meningiomas and neuroblastomas) and there is a significant correlation between VEGF expression and vascular endothelial proliferation [Berkman 1993]. However, in human neuroblastomas, the most common pediatric neoplasms, VEGF expression has been shown to affect angiogenesis as well as neuroblastoma cell growth directly (Takano S 1996).

VEGF mRNA is expressed in highly malignant neuroblastoma tumors that, as well as many malignant solid tumors, depend on neo-vascularization for their growth and for dissemination [Langer 200] It is therefore possible to argue that the inhibitory effect of lenalidomide against VEGF upregulation, here observed in neuroblastoma cell cultures, can open new perspectives in the use of this drug even in the neurologic field.

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