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Original Communication

Mechanism-based drug combination targeting HIF-2α and VEGF in renal cancer xenografts

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ABSTRACT

Limited therapeutic efficacy, selectivity and resistance represent the major challenges of targeted-therapy. Unlike other solid tumors, clear cell renal cell carcinoma (ccRCC) constitutively expresses high incidence of hypoxia-inducible factors 1α and 2α (HIFs), and vascular endothelial growth factor (VEGF). This unique profile provided the opportunity to test the hypothesis that inhibition of these targets by optimal dose, schedule, and sequence of agents in combination would result in a treatment modality with greater efficacy and selectivity. This concept was evaluated in 786-0 ccRCC xenografts that constitutively express HIF-2α, but not HIF-1α, treated with methylselenocysteine, topotecan and VEGF/VEGFR-inhibitors alone and in combination. Results generated indicate that treatment of ccRCC xenografts with either methyl-selenocysteine, an inducer of HIF degradation and modulator of tumor vasculature, or with topotecan, a topoisomerase 1 poison and an inhibitor of HIF synthesis, resulted in significant but incomplete inhibition of HIFs and achieved limited therapeutic benefit. Treatment with a combination of MSC and topotecan administered sequentially when optimal inhibition of HIFs by MSC was achieved resulted in complete inhibition of HIFs and significant increase in response rate, but no cures. Although HIF was inhibited to undetectable levels in individual tumors with the sequential combination of the two agents, the level of VEGF was only partially down regulated. Since ccRCC is known to respond to VEGF/VEGFR-targeted agents, the sequential combination of MSC, prior to and concurrent with protracted and low doses of topotecan and VEGF/VEGFR-targeted agents, resulted in a highly selective and synergistic outcome, with 100% response rates, including 45% cures. In conclusion, the dose, sequence and schedule of HIFs and VEGF/VEGFR inhibitors are critical conditions for optimal therapeutic synergy.

KEYWORDS: methylselenocysteine, TKI-inhibitors, topotecan, mechanism-based combination, ccRCC xenografts

ABBREVIATIONS

786-0 ccRCC, Clear cell renal cell carcinoma; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; H/N, head and neck; CRC, colorectal cancer; PHD, prolyl hydroxylase domain; MSC, Methylselenocysteine.

INTRODUCTION

Clear cell renal cell carcinoma (ccRCC) represents 2-3% of adult cancers and the dominant type of kidney cancer [1]. A recent study by King *et al.* reported that in 342,501 diagnosed renal cell carcinoma patients, the incidence rate increased

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from 10.6/100,000 in 2001 to 12.40 in 2010, and the incidence in men was doubled that in women [2]. Unlike squamous cell carcinoma of the head and neck (H/N) and colorectal cancers (CRC), ccRCC is characterized by a unique molecular profile, which include: (1) high incidence and cellular distribution of the hypoxia-inducible factors HIF-1α and HIF-2α (HIFs) [3-5]; (2) the Von Hippel-Lindau (VHL) tumor suppression gene, which is located on chromosome 3, is deficient or mutated in majority of cases [6, 7]; (3) tumor cells expressing HIFs are associated with alteration of specific microRNAs (miRNA) [8, 9, Chintala and Rustum, unpublished preliminary data]; (4) stable expression of vascular endothelial growth factor (VEGF) is also considered as a critical factor [10]; (5) tumor cells are resistant to standard chemo-radiotherapy [11-13], but responsive to VEGF-targeted inhibitors [14-17] and mTOR inhibitors [18-21]; and (6) expresses low levels of circulating selenium molecules [22]. Stable, but not hypoxia related expression of HIF-α has been attributed, in part, to deletion of VHL function resulting in decreased degradation in the proteasome, and in part to enhanced synthesis through the PI3k/AKt pathways. Lack of prolyl hydroxylase 3 (PHD3) in ccRCC may also contribute to the high HIF-α expression. Stable expression of VEGF is likely due to a balance between the activity of the HIF-α-dependent and independent regulatory pathways.

Nutritional supplement of selenium has been described as a 'double-edged sword' for cancer, emphasizing its significance and the inverse relationship between blood selenium levels and prevalence of several types of cancers [23]. It is known that selenium impacts multiple anticancer pathways associated with tumor growth and metastasis. During the last thirty years, different types of selenium containing molecules have been evaluated extensively as chemopreventive agents in doses ranging from 50 to 200 µg with varying degree of success in different cancer types.

Recently the use of selenium as cancer preventive agent in the Selenium and Vitamin E Cancer Prevention Trial (SELECT) did not provide favorable results [24]. It is possible that the lack of demonstrable benefits is multifactorial, given that the population enrolled in the trial was only men with high basal level of selenium and high intake. The dose and type of selenium used in this prevention trial were based on research conducted at least thirty years ago. Though not known, the possible unwarranted interaction among seleno-L-methionine (SLM), vitamin E, and tocopherol, and variability in the pre-exiting plasma levels of selenium are also likely contributors to treatment outcome. We have determined previously that MSC and SLM are equally effective modulators of the therapeutic efficacy and selectivity of anticancer drugs in CRC and H/N tumor xenografts [25]. The observed synergy between MSC and anticancer drugs was associated with inhibition of HIF, utilizing standard dose and schedule of HIF-1α by PHD-dependent, VHL-independent mechanisms [3, 26]. The effects of MSC were also associated with stabilization of tumor microenvironment, enhanced pericytes coverage, and with selective increase of drug delivery to the tumors [27, 28]. We have also demonstrated that MSC offered selective protection against organ-specific toxicity induced by variety of anticancer agents in mice and rats [29].

Results generated to date provided the basis to expand and confirm that the dose, schedule, and sequence of drug administration used in combination are critical conditions to achieve optimal and sustained modulation of key molecular markers overexpressed in tumor cells, and responsible for tumor growth and resistance. With the demonstrated dual effects of selenium, offering selective protection against drug-induced toxicity [29], and HIFs mediating enhancement of the antitumor activity of multiple chemotherapeutic agents [3, 26, 29], the unique molecular profile of ccRCC tumors provided the opportunity to evaluate and confirm the therapeutic potential of this approach. The ultimate hope is to incorporate the concept, and the optimal conditions generated in preclinical models in the design of clinical trials aimed to validate this approach.

MATERIALS AND METHODS

Animals

Female athymic nude mice (nu/nu, body weight 20-25 g), 8-12 weeks of age were used [29].

Tumors

Clear cell renal cell carcinoma cells 786-0 which express HIF-2α were utilized for the studies.

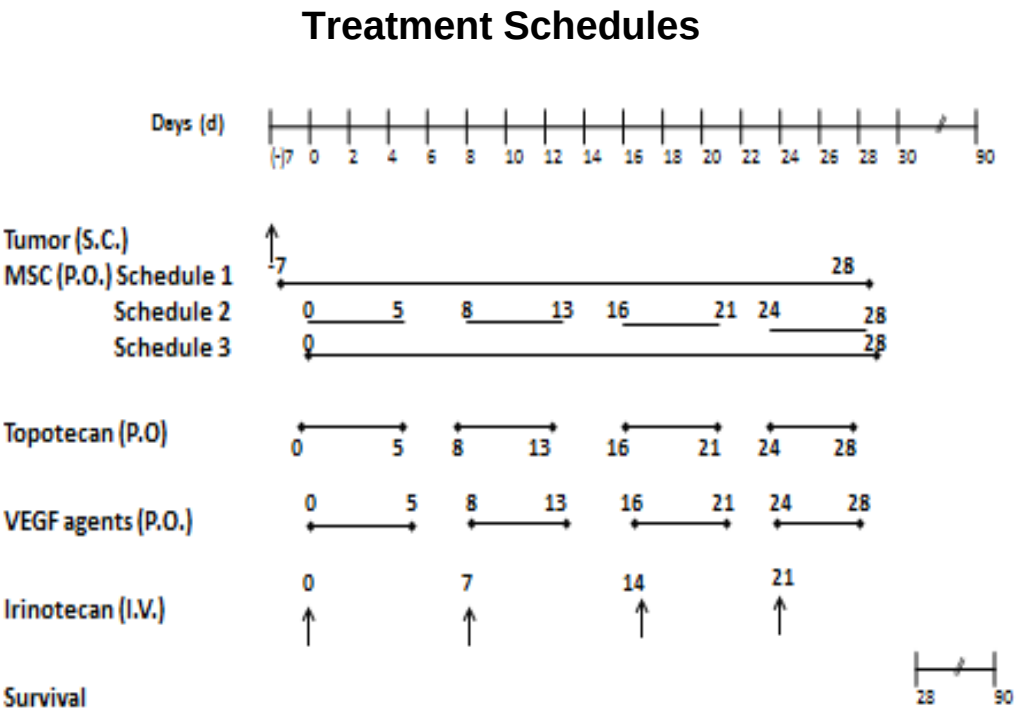
Treatments were initiated when the tumors reached approximately 200 mg.

Tumor measurement

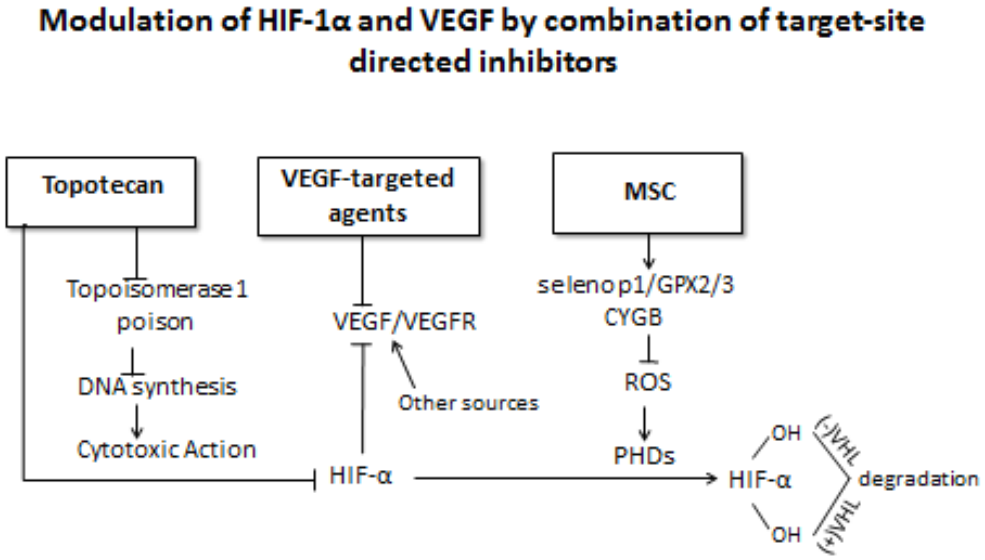
Two axes of the tumor (L, longest axis; W, shortest axis) were measured with a vernier caliper. Tumor weight (mg) was calculated as: ½ (L x W^2) [29].

Drug doses and schedules

Scheme 1 defines the three different schedules of MSC in combination with chemotherapy. Scheme 2 illustrates the proposed molecular targets for the three drug combinations. MSC, sunitinib and axitinib were administered by oral gavage (PO) and Avastin by IP. Irinotecan and topotecan were administered by the intravenous (IV) route. MSC was given at a dose of 0.2 mg/mouse (equivalent to 8-10 mg/kg/day. Three schedules of MSC were evaluated: Schedule 1 - pretreatment and concurrent treatment with MSC, daily for 35 days, with the first dose administered seven days prior to topotecan and VEGF/VEGFR-targeted agents; Schedule 2 - a total of 20 days treatment with MSC; xenografts were not treated with MSC prior to and between weekly courses of chemotherapy; and Schedule 3 - a total of 28 days of treatment with MSC; xenografts were not treated with MSC prior to chemotherapy but only during the five days of weekly and in between courses of chemotherapy. Topotecan, 2 mg/kg, daily for 5 days per week x 4 weeks; irinotecan, 100 mg per kg per wk. x 4 weeks; axitinib, 25 mg/kg, daily x 5/week x 4 weeks; sunitinib, 80 mg/kg, daily x 5 days/week x 4 weeks, and bevacizumab, 5 mg/kg, daily x 5/week x 4 weeks.



Scheme 1. Treatment schedule of drug administration: Approximately 50 mg 786-0 ccRCC tumor pieces were transplanted subcutaneously into each of 20-22 gram nude mice seven days prior to the initiation of treatment with topotecan, irinotecan and VEGF/VEGFR inhibitors (drugs). Three schedules of 0.2 mg MSC administered orally were evaluated: 1) daily for 35 days with the first dose administered seven days prior to drug administration (pre and concurrent schedule); 2) daily for 20 days and the first dose was administered daily for 5 days per week, seven days post tumor transplant, concurrent with drug treatment (0-5 days each week for 4 weeks (concurrent schedule)); and 3) daily for 28 days starting on day 0 through day 28 (concurrent and continuous schedule, no pretreatment). All other drugs were administered on day 0, seven days post tumor transplant. Antitumor activity and toxicity were assessed daily during treatment and twice weekly for up to 90 days when overall response rates were assessed.



Scheme 2. Diagrammatic representation of the mechanism-based combination of HIF-α and VEGF/VEGFR inhibitors based in part on data previously generated [3, 25].

Each experiment was repeated at least 2 times, with 5 mice included in each treatment group.

Antitumor activity

Mice were separated into different treatment groups with 5 mice in each group. Tumor responses were expressed as partial response (PR) when the tumor volume was temporarily reduced to 50% of saline treated xenografts, and complete response (CR) when tumor sizes were no longer detectable at the site of tumor transplant on day 90 post termination of treatment and were considered as cured, [29]. This study was carried out in accordance to a reviewed and approved protocol by the Institute Animal Care and Use Committee (IACUC).

Toxicity assessment

Toxicity was monitored using various treatment modalities daily during treatment and twice weekly thereafter by assessing whole body weight changes, and the incidence of diarrhea, mucositis, and alopecia. Protection against drug-induced toxicity by MSC was documented histologically and published [25].

Human tumors

Tissue micro assay (TMA) blocks were constructed separately from conventional blocks of primary and metastatic clear cell renal cell carcinoma (ccRCC), from squamous cell head and neck (H&N) carcinoma and from colorectal cancer (CRC). Surgical specimens were fixed in 10% buffered formalin embedded in paraffin. Each tumor was represented by one core (0.6 mm in diameter). TMAs were constructed in our Department of Pathology [4, 30].

Immunohistochemical methods

Paraffin sections (5 μm thickness) were cut from TMA blocks and immunostained with an automatic immunostainer for HIF-1α, PHD2 and PHD3 as described previously [3, 4, 29] utilizing a Catalyzed Signal Amplification (CAS) reagent that made the confined detection of HIF-1α possible. HIF-2α was detected with the same method using anti-HIF-2α from Novus Biologicals (Littleton, CO) at a concentration of 0.5 μg/ml [4, 30]. All immunohistochemical slides were reviewed by a board-certified, experienced pathologist.

Western blot

HIF-2α expression was determined in 786-0 tumor xenografts with and without the treatment of topotecan, MSC alone and in combination as described previously [3, 26].

RESULTS

Molecular profile of the patient's ccRCC, H/N and CRC tumors

Immunohistochemical (IHC) method was used to analyze the TMA blocks for the expression levels

of HIFs, PHDs and VEGF, and the results are summarized in Table 1. The data indicates that unlike the other two solid tumors, ccRCC expresses higher levels of HIFs, lower levels of PHD2 with no detectable levels of PHD3 protein, and lower levels and immune score of VEGF-A. It is possible that the low expression levels of PHDs may, in part, be the contributing factor to the stable high expression levels of HIFs in ccRCC, compared with H/N and CRC, which express significantly higher levels of PHDs. Further, since we demonstrated that HIF degradation by MSC is PHD-dependent [3, 26], it is possible that the observed therapeutic synergy between MSC and anticancer drugs may be the consequence of activation of PHDs by MSC, resulting in enhanced HIF degradation. In addition, the low levels of VEGF could serve as a basis for the sensitivity of ccRCC tumors to VEGF-targeted drugs. In contrast, in H/N and CRC tumors which express higher levels of VEGF, the active dose of VEGF-targeted agents utilized in ccRCC may not be as therapeutically beneficial as single agents for their treatment. Thus, agents such as MSC that can effectively inhibit HIFs and their transcriptionally regulated growth factors could result in the sensitization of tumor cells to chemotherapy.

Antitumor activity of selenium, topotecan, and VEGF-targeted inhibitors administered as single agent against human ccRCC tumor xenografts constitutively expressing HIF-2α

The data in figure 1 indicates that the tested agents demonstrated different antitumor responses against the 786-0 ccRCC tumor xenografts, with MSC and SLM exhibiting similar but limited efficacy, and sunitinib and topotecan being relatively more active. These data suggest that although HIF-2α is down regulated by MSC, SLM and topotecan, topotecan is more active, possibly due to its additional effects as a cytotoxic agent. The tyrosine kinase inhibitor sunitinib was the most active single agent in this model system. The greater therapeutic efficacy of sunitinib may be due to the fact that this agent also targets sites other than VEGFR. The therapeutic benefit gained with these agents is significant, but not durable. The diverse effects induced by these individual agents, however, may be sufficient to yield tumor cells with collateral sensitivity to these agents when used in sequential combination.

Therapeutic synergy among MSC, topotecan and sunitinib is highly scheduled and sequence-dependent

To confirm that the previously optimized dose, schedule and sequence of MSC used in combination with anticancer drugs against hypoxia-induced HIF tumor xenografts [3, 26] are also optimal against constitutively expressed HIF-2α xenografts, the three schedules outlined in scheme 1 were evaluated and the results are shown in figure 2. The results are consistent with our previous findings that pre- and continuous treatment with MSC (schedule 1 in scheme 1) is optimal in terms of kinetics of tumor response (Figure 2A) and tumor response rates (Figure 2B). Pre- and continuous treatment with MSC in combination with sunitinib resulted in a 100% response rate wherein 55% of the treated

Table 1. ccRCC expresses higher incidence of HIF-α, lower levels of PHD2 with no detectable PHD3 and lower immune score than squamous cell carcinoma of the head and neck (H/N) and colorectal cancer (CRC).

	HIF-α		PHDs		VEGF(A)	
Disease	**Primary**	**Met.**	**PHD2**	**PHD3**	**Incidence**	**Immuno score**
ccRCC	92% (81/88)	81% (46/57)	35% (31/88)	0% (0/88)	55% (48/88)	2.3*
H/N	38% (46/122)	ND	86% (180/210)	21% (32/153)	79% (136/173)	4.2^{+}
CRC	26% (17/64)	ND	90% (55/61)	50% (31/62)	97% (60/62)	$5.7^{\neq}$

*, weak; $^{+}$, moderate; $^{\neq}$, strong by IHC; ND, not done.

While MSC and SLM exhibited similar antitumor activity, sunitinib was the most active agent administered individually to xenografts bearing 786-0 ccRCC tumors.

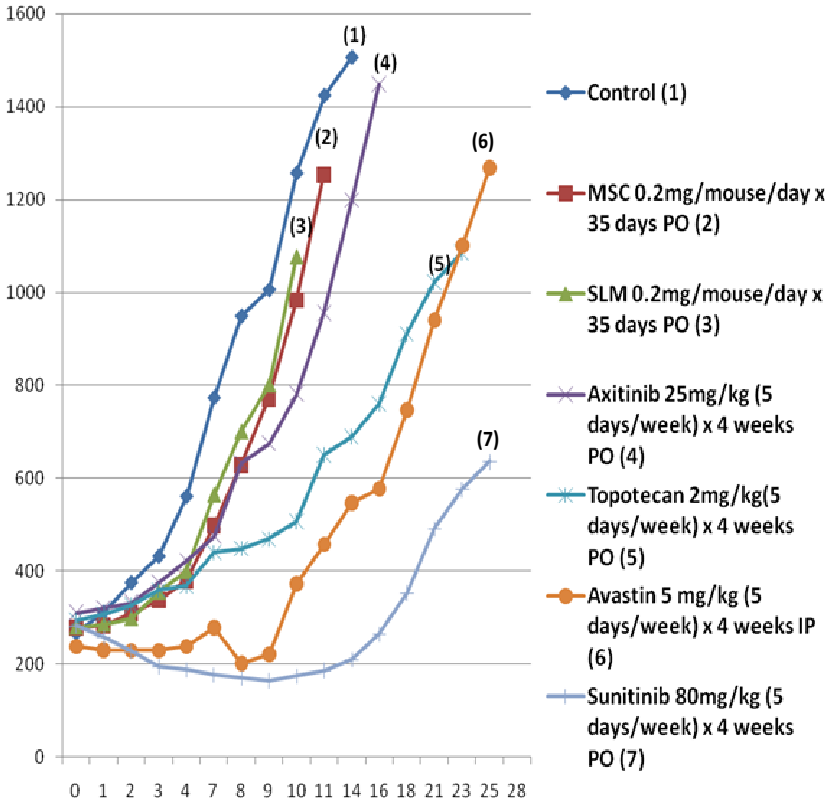


Figure 1. Comparative antitumor activity of MSC, SLM, avastin, axitinib, sunitinib and topotecan assessed individually in nude mice bearing ccRCC 786-0 xenografts expressing HIF-2α. MSC was administered orally as outlined in schedule 1 in scheme 1. All other agents were administered orally weekly as outlined in scheme 1.

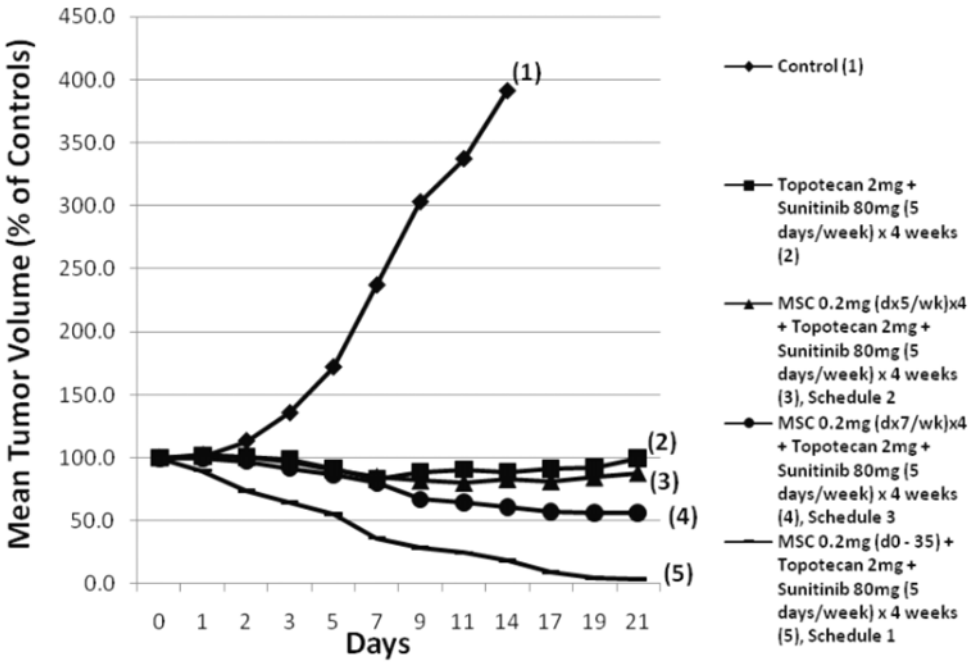
xenografts were tumor free for up to 90 days and considered as cured.

Sequential triple combination of MSC, topotecan and VEGF-targeted inhibitors is highly synergistic against 786-0 ccRCC tumor xenografts

The antitumor activity of the double and triple combinations was evaluated against human tumor xenografts bearing constitutively expressed HIF-2α and those deficient in Von Hipple-Lindau (VHL) protein expression. With all combinations, MSC was administered PO at 0.2 mg/mouse/day 7 days prior to and concurrent with the administration of topotecan, PO, 2 mg/kg/day x 5d/wk. x 4 weeks; sunitinib, PO, 80 mg/kg/day x 5d/wk. x 4 weeks avastin, PO, 5 mg/kg/day x 5d/wk. x 4 weeks; and axitinib, PO, 25 mg/kg/day x 5d/wk. x 4 weeks. The double combinations of MSC with tyrosine kinase-inhibitors (Figure 3A) and topotecan with TKI-inhibitors (Figure 3B) exhibited greater antitumor activity. The antitumor activity of the combination of MSC and sunitinib and the combination of topotecan and sunitinib were equally more active than the other treatment combinations outlined in figures 3A/B. The data in figures 3C and 3D indicate that greater antitumor activity can be achieved with the triple combination of MSC, topotecan and sunitinib. While the triple combination of MSC + topotecan + avastin yielded 60% partial response, the combination of MSC + topotecan + sunitinitib resulted in 55% complete tumor regression with no evidence of relapse at least 90 days post therapy. These results are consistent with the

Treatment with MSC daily for seven days prior to and concurrent with sunitinib and topotecan is optimal for therapeutic synergy.

A. Kinetics of response



B. Response rate

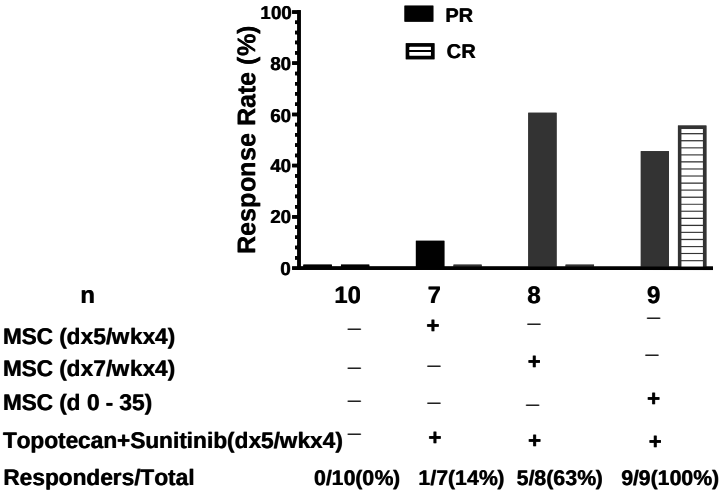


Figure 2. Antitumor activity of MSC, 0.2 mg/mouse/day (schedule 1) in combination with topotecan, 2 mg/kg/d x 5/wk. x 4 and sunitinib, 80 mg/kg/d x 5/wk. x 4: Role of MSC schedule and sequence in the modulation of the antitumor activity of sunitinib in xenografts bearing ccRCC 786-0, HIF-2α expressing tumors. **A.** Kinetics of response. **B.** Overall response rate (CR+PR) achieved post therapy. The number in parenthesis indicates the percentage responders in each treatment group. MSC was administered as outlined in scheme 1, schedule 1.

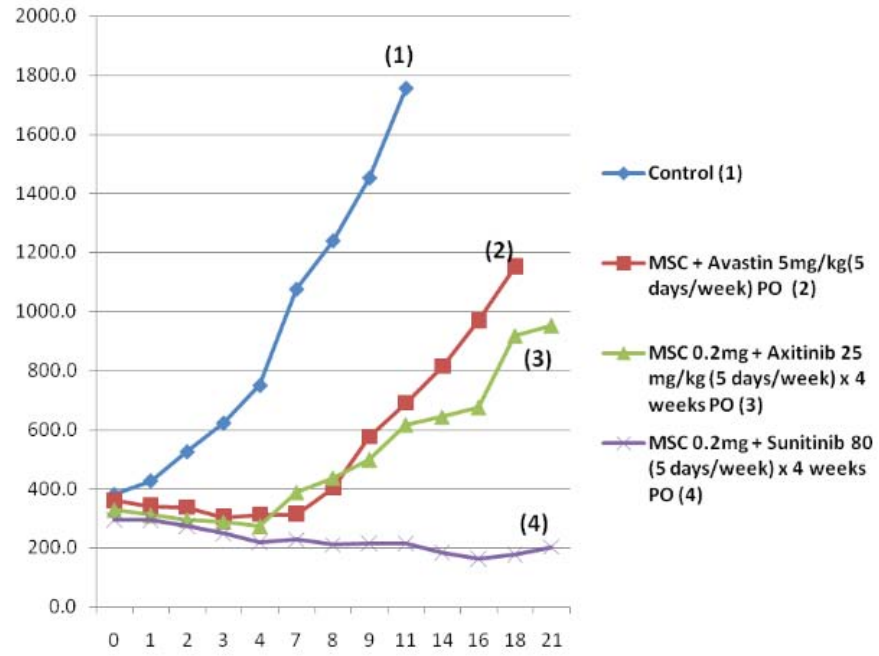
hypothesis that pre treatments with MSC and the administration of cytotoxics at the time when maximum inhibition of HIFs was maintained by concurrent treatment with MSC are critical conditions for optimal treatment outcome.

HIF-2α is down regulated more intensely and uniformly by the sequential combination of therapeutic doses of MSC and topotecan

Treatment with therapeutic doses of MSC or topotecan alone and in combination resulted in

Triple sequential combinatio of MSC, topotecan and sunitinib is highly synergistic.

(A)



(B)

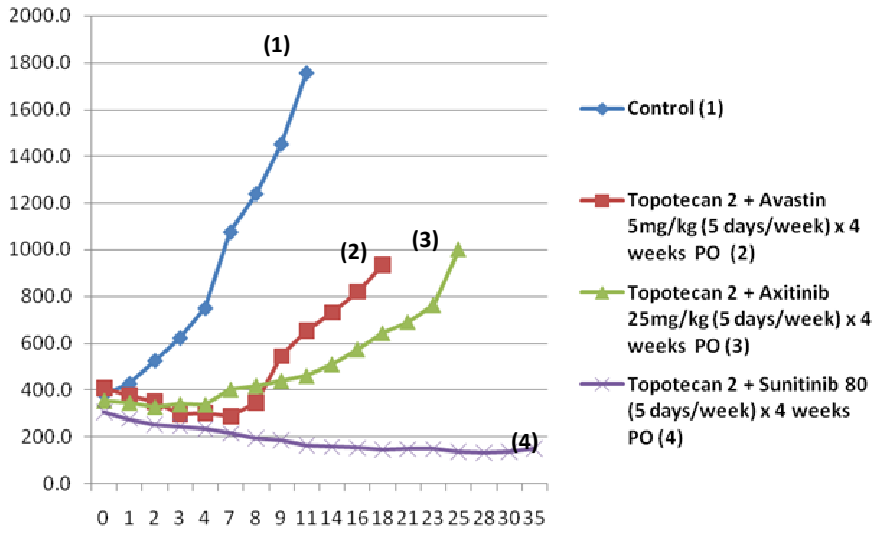
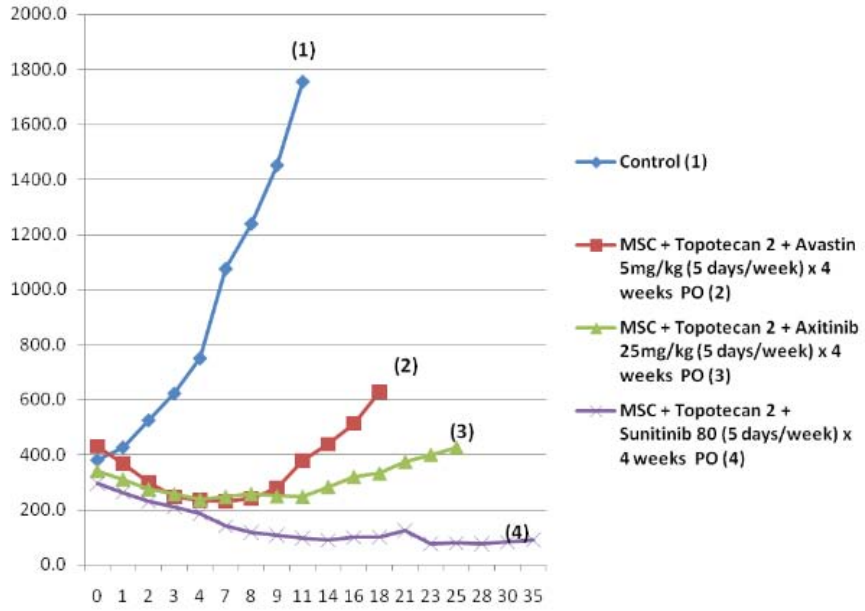


Figure 3

Figure 3 continued..

(C)



(D)

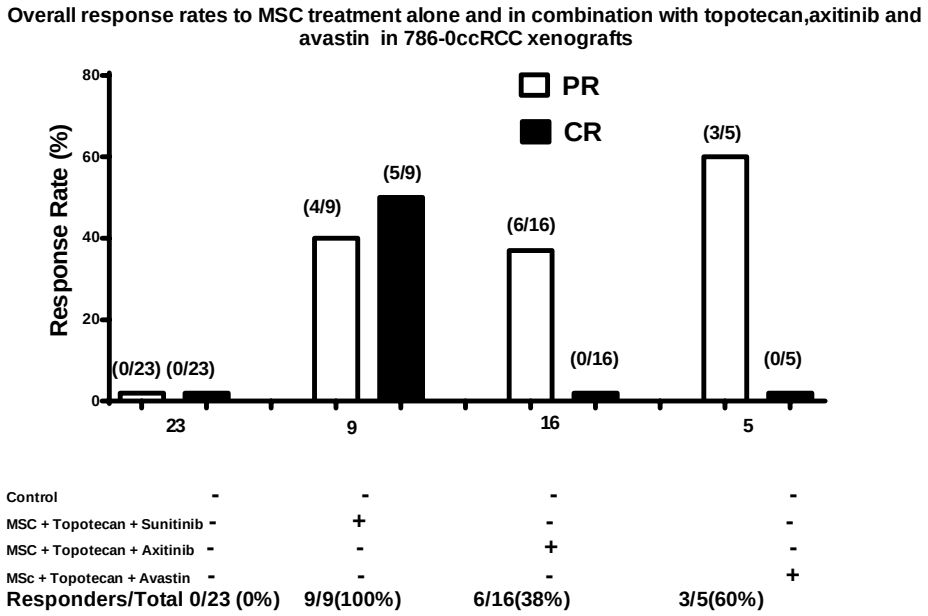


Figure 3. Antitumor activity of MSC in combination with VEGF-targeted agents and topotecan in nude mice bearing ccRCC 786-0 xenografts. **(A)** Antitumor activity of MSC in combination with sunitinib, axitinib and avastin; **(B)** Antitumor activity of topotecan in combination with sunitinib, axitinib and avastin; **(C)** Antitumor activity of MSC in combination with topotecan, sunitinib, axitinib and avastin and **(D)** Overall response rates PR and CR, to MSC treatment alone and in combination with topotecan and VEGF-targeted agents. Partial response (PR) and complete response (CR) were assessed at various times post termination of therapy. The numbers in parenthesis indicate the number of responding animals out of the total treated.

pronounced inhibition of the constitutively expressed HIF-2α in individual 786-0 tumor xenografts (Figure 4A). More pronounced and uniformed inhibition of HIF-2α in tumor xenografts, however, was only obtained with the sequential combination of daily x 7 days of MSC pretreatment followed by 5 days/wk. x 4 weeks with concurrent administration of MSC with topotecan. The pooled protein extracts were used to evaluate HIF-2α expression and we found consistent HIF-2α inhibition in the combination group (Figure 4B). The greater inhibition of HIF-2α by MSC/topotecan combination was associated with greater therapeutic efficacy (Figure 4C). The data indicate that while daily treatment with MSC is necessary and sufficient for inhibition of HIF-2α, treatment with topotecan, a known inhibitor of HIF-α synthesis resulted in complete inhibition of HIF-2α in all the mice treated. The data in figure 4D indicate that when the sequential administration of MSC and topotecan continued for four weeks, the observed pronounced HIF-2α inhibition was associated with greater antitumor activity. Interestingly, the antitumor response was observed with the combination of MSC with topotecan, agents that target HIFs, but not with irinotecan, an agent that is not known to

Pronounced inhibition of HIF–2α by the combination of MSC and topotecan correlated with enhanced antitumor activity in ccRCC 786-0 xenografts.

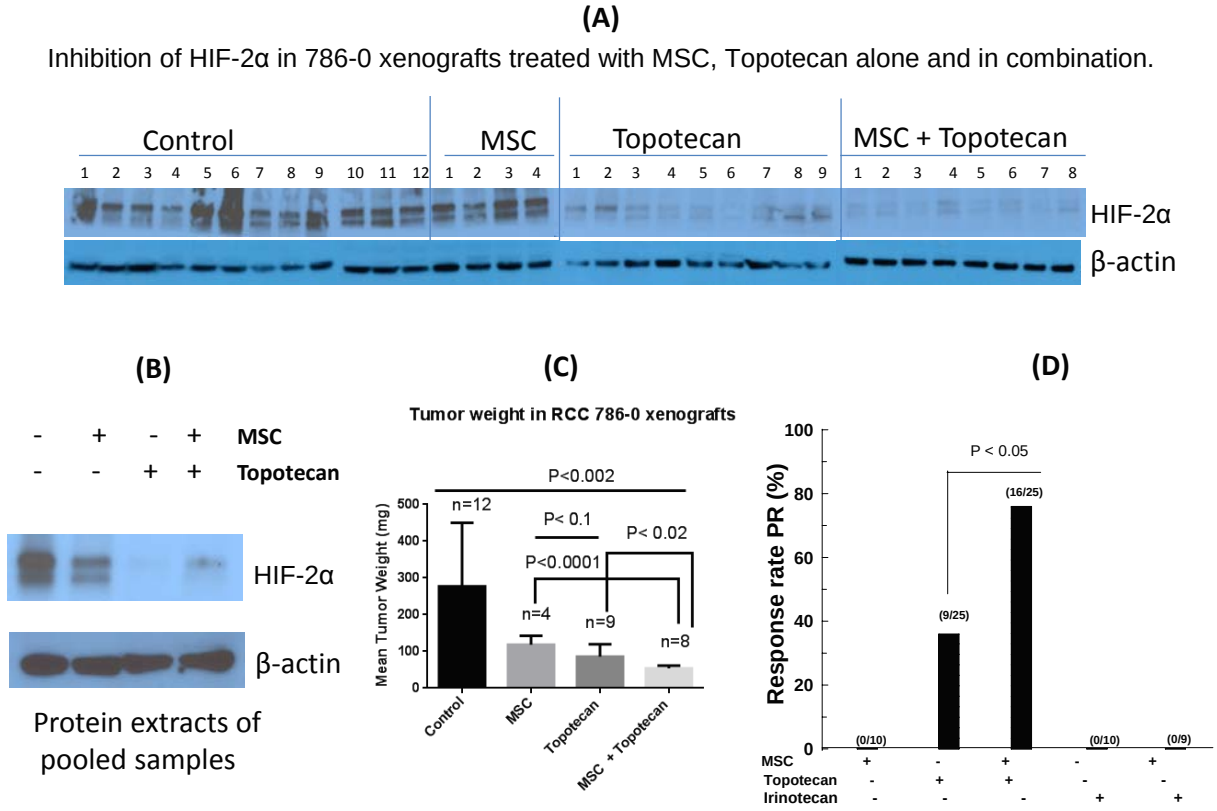


Figure 4. Effects of treatment of MSC and topotecan alone and in combination: **A)** levels of HIF-2α in the 12 individual controls, 4 MSC, 9 topotecan and 8 mice bearing 786-0 tumors treated with the combination of MSC and topotecan as outlined in scheme 1, schedule 1; **B)** pooled averaged expression levels of HIF-2α in each treated groups; **C)** calculated tumor growth inhibition by each treatment; and **D** represent partial response rate achieved with each treatment group. MSC was administered daily for seven days prior to and for fourteen days concurrent with the weekly administration of topotecan and irinotecan. The numbers in parenthesis represent the number of xenografts achieving partial response out of the total animals in each treated group. HIF expression in individual tumor was determined by Western blot on day 14 as previously described [3, 25].

target HIF. Since the best therapeutic benefit was achieved with the triple combination of MSC, topotecan and sunitinib, it is not known, at this time, whether sunitinib may have direct or indirect effects on the level of HIF protein. The data demonstrated that HIF-2α and VEGFR are critical therapeutic targets in ccRCC.

DISCUSSION

The ultimate goal of chemotherapy in the treatment of patients with advanced malignancies is to induce higher rates of complete tumor regression and overcome mechanisms responsible for innate and acquired resistance. A number of overlapping parameters including instability of tumor micro-environment, molecular heterogeneity of tumor cells, pharmacokinetics and pharmacodynamics of drugs, and immune response, collectively are critical factors that contribute to the efficacy and selectivity of treatment outcome. For a drug to be effective, it must be transported into the cancer cells, in some cases metabolized, and retained in sufficient concentrations for a period of time sufficient to inhibit the intended targets. For standard chemotherapy with cytotoxic and biologically directed agents, the dose and schedules are largely fixed independent of the disease type, yet the molecular target(s) expression and levels vary among tumors of the same histological type and among cancers of different histology. In case of advanced solid tumors, the unstable micro-environment expressing molecular markers essential for tumor growth, metastasis, and resistance may represent a potential barrier for drug delivery. Furthermore, the inter- and intracellular expression levels, intensity, and cellular distribution of the intended targets in solid tumors vary considerably.

Prolyl hydroxylases 2 and 3 are the enzymes responsible for hydroxylation of HIFs prior to their degradation in the proteasome. While ccRCC tumors are deficient in the expression of PHD3 protein, the incidence of PHD2 is reported as 35%, 86% and 90% in ccRCC, HNSCC and CRC tumors, respectively [3]. The incidence of HIFs is the lowest in CRC and H/N, and the highest in ccRCC. In contrast, the incidence of PHD2/3 and VEGF are the highest in CRC and H/N, and the lowest in ccRCC, which did not express PHD3 in 88 biopsies tested by IHC. These data indicate an inverse relationship between PHDs and HIFs, suggesting that stable expression of HIFs are partially regulated by the levels and activity of PHDs in addition to their regulation by the Von Hippel-Lindau gene. The VEGF immunosore is lower in ccRCC than CRC, and H/N tumor biopsies. Thus among the three cancers, ccRCC tumors appear to express higher HIFs, and lower VEGF and PHD levels than CRC and H/N tumors. Collectively, the results generated suggest that HIFs and VEGF are regulated by multiple pathways and could be amenable to a combination treatment strategy.

Clinically, agents that target HIFs and/or VEGF are generally evaluated using the same dose and schedule of the individual agent in different cancer types, regardless of the inter- and intra-heterogeneity in the incidence and intensity of the intended targets. Thus, in patients enrolled in phase 2 clinical trials, the observed limited treatment outcome with the targeted drugs [14-21] may be due to the fact that the incidence and intensity of the intended target(s) are expressed in the tumors of only a few patients. The relatively small number of patients evaluable for response assessment may not be sufficient to validate and/or confirm the predictive therapeutic value of the drug under evaluation.

The rationale for the choice of agents used in combination was based on the knowledge that these agents are therapeutically active as a single agent and act by different and non-overlapping mechanisms of action. In addition, we previously demonstrated that while treatment with selenium resulted in pronounced and similar inhibition of both HIF-1α and HIF-2α, VEGF-A was significantly down regulated in tumor cells expressing HIF-1α but not in HIF-2α expressing cells [3, 26]. Collectively, these data provided the rationale for the use of VEGF-targeted agents in combination with the HIF inhibitors with the hope of achieving complete inhibition of VEGFs which are dependently and independently regulated by HIF pathways.

The data summarized in figure 4 indicates that pretreatment of ccRCC xenografts with therapeutic and selective doses, schedule and sequence of MSC and topotecan, resulted in pronounced down regulation of HIF-2α, and enhanced antitumor. These data are consistent with the hypothesis that combined use of topotecan, an agent that inhibits HIF synthesis and also exerts cytotoxic effects,

with MSC that enhances HIF degradation and inhibits HIF-1α-derived VEGF, in the treatment of tumors expressing high incidence of HIFs, is necessary for the sensitization of tumor cells. The limited therapeutic efficacy of the combination of MSC and irinotecan (Figure 4D), an agent that does not inhibit HIFs, is consistent with the hypothesis that cytotoxic action of irinotecan alone is not sufficient to synergize with MSC in this high expressing HIF tumor mode. Collectively, the data generated suggest that tumors with high incidence and intensity of HIFs, such as those of ccRCC, and judicious use of the optimal dose, schedule, and sequence of inhibitor of the synthesis of HIFs with agents that enhance their degradation in sequence combination with agents that have direct intracellular cytotoxic effects are critical conditions for achieving durable responses. The data presented in figures 2-4 demonstrate that the triple combination of MSC and topotecan, with the agents targeting VEGF/VEGFR is highly synergistic with no dose-limiting toxicity in mice bearing tumor xenografts with high incidence and expression levels of HIFs.

Since the action of selenium is multi-targeted, and HIFs are critical therapeutic targets of selenium, data being generated in our laboratory and by others strongly link HIFs in the regulation of immune response biomarkers, such as the programmed death ligand-1, myeloid-derived suppressor cells and and Foxp3, among others. It is tempting therefore to suggest that inhibition of HIFs by MSC and/or topotecan would have profound modulatory effects on immune response elements possibly resulting in sensitization of tumor cells to treatment with agents that target immune response pathways. Studies are underway to evaluate the therapeutic potential and associated mechanisms for the combination of HIF inhibitors and immunotherapy.

CONCLUSION

In summary, MSC is orally bioavailable, and therapeutic doses are potent enhancers of HIF degradation by PHD-dependent, VHL-independent mechanisms [3, 26]. The observed therapeutic synergy with standard and biologically targeted agents was demonstrated to be highly dependent on the selenium dose, schedule and sequence. Collectively, the data generated demonstrated the pleiotropic effects of specific types of selenium-containing compounds as selective modulators of the antitumor activity of a variety of chemotherapeutic agents with different mechanisms of action in several xenograft tumors expressing hypoxia-induced and constitutively expressed HIFs. Furthermore, we demonstrated previously that MSC protects against host toxicity induced by a variety of chemotherapeutic agents [29]. The data generated here in ccRCC xenografts bearing constitutively expressed HIF-2α and data generated previously in our laboratory using hypoxia-induced HIF-1α head and neck and colorectal xenografts [3, 26, 29] demonstrated that therapeutic synergy was best achieved when MSC was combined with chemotherapy. It is likely that individual administration of a fixed dose and schedule of targeted agents to patients with heterogeneous expression of targets is responsible for their limited clinical efficacy. The data generated provided evidence on the need for continued preclinical and clinical development of selenium-containing molecules, preferably MSC, in different tumor models that use similar and different combination treatment modalities. Since the clinical efficacy of VEGF and mTOR-targeted therapy is also associated with diverse host toxicity, it is possible that MSC will offer selective protective effects against toxicity induced by these agents, similar to those obtained with cytotoxic drugs. While significant advances have been achieved clinically with VEGF-targeted agents, durable responses are limited. The data generated in several xenograft models demonstrated that judicious use of a combination of dose, sequence and schedule of agents that target HIFs and VEGF pathways are critical pharmacological parameters for optimal therapeutic outcome, and should be considered in the design of clinical trials.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to report.

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