bjh research paper

Desensitization to hydroxycarbamide following long-term treatment of thalassaemia intermedia as observed *in vivo* and in primary erythroid cultures from treated patients

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Summary

Hydroxycarbamide (HC) is a pharmacological agent capable of stimulating fetal haemoglobin (HbF) production during adult life. High levels of HbF may ameliorate the clinical course of β-thalassaemia and sickle cell disease. The efficacy of HC for the treatment of thalassaemia major and thalassaemia intermedia is variable. Although an increase of HbF has been observed in most patients, only some patients experience significant improvement in total haemoglobin levels. This study aimed to determine the effectiveness and safety of short- (1 year) and long-term (mean follow-up 68 months) HC treatment in 24 thalassaemia intermedia patients. Additionally, we evaluated if primary erythroid progenitor cells cultured from treated patients responded to HC treatment in a manner similar to that observed *in vivo*. Our results confirm a good response to HC after a short-term follow-up in 70% of thalassaemia intermedia patients and a reduction of clinical response in patients with a long follow-up. Erythroid cultures obtained from patients during treatment reproduced the observed *in vivo* response. Interestingly, haematopoietic stem cells from long-term treated patients showed reduced ability to develop into primary erythroid cultures some months before the reduction of the '*in vivo*' response. The mechanism of this loss of response to HC remains to be determined.

Keywords: hydroxycarbamide, thalassaemia intermedia, fetal haemoglobin, primary erythroid cultures, HBG mRNA.

Hydroxycarbamide (HC) is one of the main inductors of fetal haemoglobin (HbF) synthesis. It significantly increases HbF levels in patients with Sickle Cell Disease (SCD) and thalassaemia (Charache *et al*, 1992; Fucharoen *et al*, 1996; Fibach *et al*, 1993; Fathallah *et al*, 2005), in many cases alleviating the clinical severity of these diseases. In SCD, the beneficial effects of HbF are dilution of the HbS concentration and inhibition of HbS polymerization in the red blood cells. Recent studies on SCD patients suggested that long-term use of HC is safe and reduces mortality (Steinberg *et al*, 2010; Voskaridou *et al*, 2010). In β-thalassaemia, γ-globin chains can substitute for β-globin chains and reduce the excess of α-globin chains that cause damage to the membranes of red blood cells.

Different mechanisms have been proposed for the ability of HC to induce HbF production. According to one hypothesis, HC promotes HbF production by perturbing the kinetics of erythropoiesis. It does this by killing late erythroid progenitor cells, thus selecting for a minor pre-existing subpopulation of cells containing HbF (F cells) that has a growth and/or survival advantage (Dover & Charache, 1992; Atweh & Loukopoulos, 2001).

Another possible mode of HC action is through the activation of fetal gene transcription, resulting in an increase of HbF levels in all or the majority of the cell population. It has been shown that increases in *HBG* mRNA and protein strongly correlate with NO-radical donation by hydroxycarbamide acting through the sGC/cGMP pathway (Cokic *et al*, 2003). Several transcription factors, such as AP1 and SP1 (Moi & Kan, 1990; Safaya *et al*, 1994; Haby *et al*, 1994; Idriss *et al*, 1999; Fischer *et al*, 1993; Wang *et al*, 1999), are

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regulated by this biochemical pathway and might affect *HBG* expression.

More recently, we have demonstrated the activation of *HBG* transcription by HC in cultured erythroid precursors (Calzolari *et al*, 2008).

Although early reports showed a significant increase of Hb in thalassaemia Lepore patients (Olivieri *et al*, 1997; Rigano *et al*, 1997), further studies have suggested that the efficacy of HC for the treatment of thalassaemia intermedia is highly variable (Fucharoen *et al*, 1996; Loukopoulos *et al*, 1998; Bradai *et al*, 2007; Arruda *et al*, 1997; Karimi *et al*, 2010; Ehsani *et al*, 2009; Italia *et al*, 2009). A retrospective study on thalassaemia intermedia patients showed that HC treatment was protective for extramedullary haematopoiesis, pulmonary hypertension, leg ulcers, hypothyroidism and osteoporosis (Taher *et al*, 2010).

Additionally in a preliminary observational study the efficacy of HC treatment was evaluated on thalassaemia intermedia patients who were not undergoing periodical transfusions. In some patients who had shown a good clinical response, a decrease in the efficacy of treatment after long term follow up (range 70–144 months) was observed (Mancuso *et al*, 2006).

The aim of the current study was to determine the efficacy of short- and long-term HC treatment of thalassaemia intermedia patients in a larger group of patients who have been treated for both long and short periods of time (range 35–180 months). In addition to *in vivo* studies, we attempted to test the behaviour of erythroid precursors when treated *in vivo* for short or long periods of time. For this reason erythroid progenitors from a large group of HC-treated patients were cultured to determine if the response of the cultured cells to HC treatment correlated with the response *in vivo* and to evaluate whether HC might have any toxic effects on hematopoietic stem cells.

Patients and methods

For the study, we enrolled 24 patients with beta thalassaemia intermedia observed between 1995 and 2007 at the Haematology Unit, Ospedale V. Cervello. Thalassaemia intermedia was diagnosed if haemoglobin (Hb) levels were ≥70 g/l, without the need for regular blood transfusions.

The patients were considered eligible for the study if they met the following criteria: age ≥18 years, Hb ≤ 85 g/l, no blood transfusions during the past 2 years and if they had undergone splenectomy. Exclusion criteria were as follows: known intolerance to HC, age <18 years and >60 years, pregnancy, bone marrow hypoplasia, human immunodeficiency virus positivity, kidney insufficiency, aspartate transaminase and alanine transaminase levels more than 3× over baseline.

Molecular studies for genotype detection were performed according to procedures reported elsewhere (Maggio *et al*, 1993). Procreation was advised against in subjects of fertile age. All patients were informed about the side effects of HC and an informed consent form was obtained.

Intervention

The starting dose of HC administered was 10 mg/kg. If it was found necessary to increase total haemoglobin levels, the dose was adjusted upward in increments of 5 mg/kg every 3 months to a maximum of 30 mg/kg. The dose was adjusted downward if evidence of hematopoietic suppression was observed (i.e., neutrophil count $<2000 \times 10^9$/l, platelet count $<150\,000 \times 10^9$/l, or if haemoglobin levels were reduced by 10%).

The intervention was temporarily discontinued if neutrophil counts fell below 1500×10^9/l, platelet counts fell below $<80\,000 \times 10^9$/l, or haemoglobin levels were below 70 g/l, and was resumed at 50% of the starting dosage when haematological parameters returned to initial values. If significant suppression of bone marrow was found on three successive determinations, the treatment was stopped and the patient withdrawn from the study.

Each patient was advised on the possibility of blood transfusions if required. Compliance was assessed by counting the pills in each returned bag of HC and by questionnaires. All patients received folate supplementation during HC therapy. Patients showing a total Hb increase <10 g/l were considered non-responders, and the treatment was stopped after a period of 1 year. In responder patients (total Hb increase ≥10 g/l), a long-term follow-up for response and toxicity was evaluated.

Primary and secondary outcomes

The primary outcome of the study was to evaluate the proportion of thalassaemia intermedia patients that responded to HC treatment. The secondary outcomes were to assess the safety and effectiveness of long-term HC treatment.

Follow-up and data collection

Clinical visits and laboratory tests were performed weekly during the first month of treatment and monthly thereafter. Clinical and biochemical data, as well as complete blood cell counts, were registered in predefined data collection forms. A complete database for all included patients was established.

Ethics

The study protocol was performed in accordance with the Declaration of Helsinki and was approved by the local ethics committee for human investigations. The patients gave their written informed consent to participate in the study.

Statistical methods

The mean values are reported with standard deviations (SD). Hb levels were obtained from repeated observations over time

with the same patient. To model longitudinal data, we used the generalized estimating equation (GEE) model (Hedeker & Gibbons, 1996). This approach has been implemented in the 'xtgee' procedure of the Stata 9.2 software (StataCorp, College Station, TX, USA).

All statistical analyses were performed using Stata 9.2 (StataCorp). *P*-values <0·05 were considered significant.

Two phase liquid primary erythroid cultures

After informed consent was obtained, 20 ml of peripheral blood was withdrawn from 17 patients undergoing *in vivo* HC treatment. Primary cell cultures were performed as previously described (Di Marzo *et al*, 2005).

HC treatment in vitro

At day 6 of phase II, cells were washed with alpha-medium and the cell culture was split. Half of the culture was exposed to 100 μmol/l hydroxycarbamide (Teofarma Srl, Pavia, Italy), a dose that corresponded to the serum HC concentration during *in vivo* treatment at 20 mg/kg per d. As a control, the other half of the culture was grown without the drug. At day 10, the cells were harvested and analysed.

Flow cytometric analysis

The phase II cultured cells were monitored for erythroid differentiation by measuring cellular expression of transferrin receptor (CD71) (Immunotech, Marseille Cedex 9, France) and glycophorin A antigen (GPA) (Immunotech) as previously described (Calzolari *et al*, 2008). Cells were analysed using the Cytomics FC 500 (Beckman Coulter Inc., Fullerton, CA, USA).

Real-time quantitative polymerase chain reaction (PCR)

RNA was isolated with TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. 200 U Moloney Murine Leukaemia Virus (Invitrogen) was used to synthesize cDNA from 1 μg of total RNA after priming with random examers (Invitrogen). Quantitative real-time PCR assay of transcripts was carried out with the use of gene-specific double fluorescently-labelled probes in a 7900 Sequence Detector (Applied Biosystems, Norwalk, CT, USA). All samples were assayed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) (Calzolari *et al*, 2008).

The experiments were performed in triplicate to ensure the reproducibility of the results.

Results

All 24 enrolled patients continued treatment for at least 1 year. As such, all were included in the evaluated dataset. The patients consisted of 13 males and 11 females with a median age of 37 years (range 18–59). Patients' genotypes are shown in Table I. The mean dosage of HC administered was 14·6 mg/kg (range 5–30), and the mean follow-up time was 52 months (range 12–180).

Short-term follow-up

An average Hb increase of 15 g/l ($P < 0·001$) was observed after 1 year of treatment. Overall, 17 of the 24 (70%) patients had an Hb increase ≥10 g/l and were considered responders. Among these, 12 had an increase ≥15 g/l. Moreover, the three patients with a βLepore/$\beta^0$39 genotype had an average Hb increase of 31 g/l (range 20–45). Overall, patients had a statistically significant increase in MCV ($P < 0·001$), HbF ($P < 0·001$) and a decrease in reticulocytes ($P < 0·05$) (Table II). Responder patients were followed long-term. Only seven patients were considered non-responders (total Hb increase <10 g/l) and were withdrawn from the study. Five of these patients were homozygotes for the IVS1nt6 mutation (Table I).

Long-term follow-up

All 17 responder patients continued the treatment. The mean follow-up for these patients was 68 months (range 35–180). In

Table I. Patients genotype.

Non responders	*n*	Responders	*n*
IVS1,6/IVS 1,6	5	βLepore/$\beta^0$39	3
IVS1,1/-101	1	βLepore/βLepore	1
IVS1,110/-87	1	IVS1,6/IVS1,6	4
		δβ/$\beta^0$39	3
		IVS1,6/-101	1
		IVS1,6/IVS1,110	1
		IVS1,6/$\beta^0$39	1
		IVS2,1/$\beta^0$39	1
		$\beta^0$39/CD6	1
		$\beta^0$39/IVS2,745	1

Table II. Haematological effects of short term HC treatment in 24 thalassaemia intermedia patients.

Test	Baseline value (mean ± SD)	1 year HC treatment (mean ± SD)	*P*-value
Hb (g/l)	78 ± 9·0	93 ± 13	<0·05
MCV (fl)	72·0 ± 7·2	82·0 ± 6·1	<0·05
MCH (pg)	25·2 ± 3·4	29·6 ± 3·2	<0·05
WBC ($\times10^9$/l)	15·8 ± 9·6	9·8 ± 5·2	<0·05
Reticulocytes (%)	15·3 ± 13·4	8·3 ± 8·8	<0·05
nRBC ($\times10^3$)	5·0 ± 4·2	3·2 ± 2·4	<0·05
HbF (%)	46·5 ± 40·2	57·2 ± 41	<0·05

nRBC, nucleated red blood cells.
Baseline values were obtained within 1 month before the initiation of HC treatment. *P*-value determined by Student's *t*-test.

nine responder patients, long-term follow-up response did not differ significantly from their 1-year response. Their Hb levels remained stable over the period of observation. However, a trend towards a reduction of response was observed in the eight other responder patients (47%) (Fig 1). These were the patients with the longest follow-up (mean follow-up 104 months, range 95–180). In seven of these patients, the treatment was stopped, while the remaining patient's response to HC treatment was considered still clinically relevant despite a decrease in Hb levels.

Effects on extramedullary erythropoiesis

Two patients entered the study with severe symptomatic cord compression by extramedullary erythropoiesis. In these patients, a disappearance both of erythropoietic masses and of clinical symptoms was observed after 3 months of treatment. In six patients with non symptomatic extramedullary erythropoiesis, a reduction of erythropoietic masses was observed during the treatment.

Toxicity

No severe complications were observed during the treatment. Five patients had mild dyspepsia at the start of treatment that was tolerable and did not necessitate drug discontinuation. No leukaemia cases or other malignancies were observed during the long-term follow-up.

HC treatment in vitro

During the long-term follow-up of the clinical study, primary erythroid cultures from patients under treatment were obtained to determine if HC might have any effects on erythroid precursor *HBG* mRNA output *in vitro* that might correlate with *in vivo* total haemoglobin and HbF increase.

To analyse erythroid progenitor cultured cells from 17 patients (12 responders and 5 non-responders), we used a fluorescent-based real-time PCR assay to determine both fetal *HBG* and adult *HBB* gene expression after normalization to housekeeping *GAPDH* gene expression levels. As summarized in Table III, the results showed a correlation in that there was no statistical significant difference (*P*-value 0·9751) between *in vivo* and *in vitro* responses to HC treatment both for responder and non-responder patients. In fact, in all patients studied, the *in vitro HBG* mRNA fold increase was very similar to the *in vivo* HbF fold increase. The increase in HbF g/l corresponded to the increase in total haemoglobin (g/l) in all but one patients (Patient 13 in Table III). This patient was clinically defined as a non-responder owing to a slight increase in total haemoglobin (only 7·0 g/l) despite a 13 g/l increase in HbF. This particular patient had a threefold increase in *in vitro HBG* mRNA that was similar to an *in vivo* 2·7-fold increase of HbF, and was also associated with a threefold reduction in *HBB* mRNA. These observations may indicate a simultaneous decrease *in vivo* of adult haemoglobin (HbA), considering that the patient presented a genotype homozygous for a mutation (IVS1 nt6) and produced correct *HBB* mRNA. No difference in *HBB* gene expression was found in the other patients.

Interestingly, the peripheral blood haemopoietic stem cells (PBHSC) obtained from a group of patients under long-term HC treatment did not proliferate nor differentiate into erythroid cells in liquid cultures, and some months later the same patients showed a reduction in clinical response. These were the seven patients with the longest follow-up (mean follow-up 104 months, range 95–180).

Discussion

Patients with thalassaemia have previously been reported to respond clinically to HC, as assessed by total Hb increase. HC has been administered to thalassaemia patients alone or in

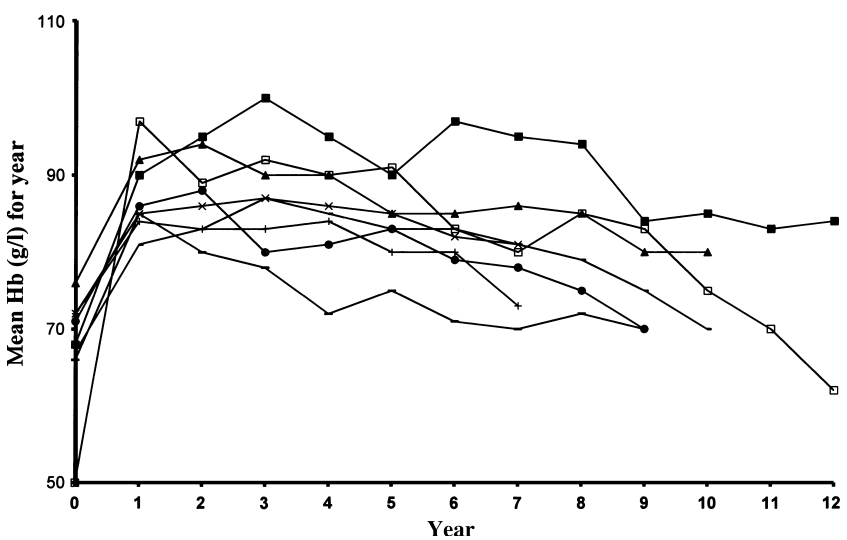

Fig 1. Progressive decrease of haemoglobin (Hb) (mean/year) during long-term follow up in eight patients treated with hydroxycarbamide.

Table III. Correlation between the response to HC treatment *in vivo* and *in vitro*.

		In vivo response			*In vitro* response	
Patient number	Genotype	Total Hb increase (g/l)	Fetal Hb increase (g/l)	HbF fold increase	*HBG* mRNA fold increase	*HBB* mRNA fold increase
Responder patients						
1	βLepore/$\beta^0$39	40	40	2	1·9	–
2	IVS1,6/IVS1,6	10	10	1·6	1·4	–
3	βLepore/$\beta^0$39	35	35	1·5	1·5	–
4	IVS1,6/$\beta^0$39	20	20	1·6	1·8	–
5	IVS1,6/IVS1,6	20	21	3·4	3	–
6	IVS1,6/IVS1,6	12	13	1·4	1·5	–
7	$\beta^0$39/CD6	10	10	1·1	1	–
8	βLepore/βLepore	20	20	1·6	1·8	–
9	δβ/$\beta^0$39	16	16	1·2	1·4	–
10	δβ/$\beta^0$39	14	14	1·2	1·3	–
11	δβ/$\beta^0$39	17	17	1·3	1·4	–
12	IVS 2,745/$\beta^0$39	23	23	1·3	1·2	–
Non responder patients						
13	IVS1,6/IVS1,6	7·0	13	2·7	3	−3
14	IVS1,6/IVS1,6	5·0	5·0	1·7	1·5	–
15	IVS1,6/IVS1,6	6·0	7·0	2·1	2	–
16	IVS1,6/IVS1,6	5·0	8·0	1·5	1·7	–
17	IVS1,6/IVS1,6	3·0	3·0	1·2	1·4	–

combination with erythropoietin or butyrate (Hoppe *et al*, 1999). In some studies, the response was impressive, with significant increases both in HbF and total Hb (Arruda *et al*, 1997; Bradai *et al*, 2007), while an increase of HbF and only a mild effect on the total haemoglobin concentration was documented in others (Loukopoulos *et al*, 1998; Fucharoen *et al*, 1996).

Additionally, in a preliminary study conducted by our group, a decrease in the efficacy of treatment during long-term follow-up was observed (Mancuso *et al*, 2006). As such, we investigated the efficacy of short- and long-term HC treatments in thalassaemia intermedia patients.

In this study, we confirmed a good short-term response to HC in 70% of thalassaemia intermedia patients. Patients with Lepore or δβ thalassaemia genotypes showed a better response, while non-responder patients mainly had homozygous IVS1.6 genotypes. However, a reduction in clinical response was observed in 47% of patients with a very long follow-up (mean 104 months), and it was necessary to stop the treatment in seven patients. Finally, the impairment in the clinical response correlated with the time of exposure to HC, instead of dosage administered.

Whether or not HC exerts long-term toxic effects on bone marrow stem cells that lead to drug-related bone marrow failure remains an unresolved hypothesis. Our observation that PBHSCs from patients under long-term treatment lose the ability to develop primary erythroid cultures suggests that this hypothesis might be correct. The drug could affect HSC proliferation, making it more difficult for these cells to be committed for differentiation into cells of the erythroid lineage. This reduced ability of HSCs to proliferate and differentiate was already present some months before the reduction of the *in vivo* response, suggesting that periodical primary erythroid cultures from PBHSC during HC treatment might predict any decrease in clinical response to HC.

Human erythroid liquid cultures, combined with the use of quantitative real-time PCR, have been shown to be a useful tools for the investigation of pharmacological agents that induce HbF production on cells derived from SCD and β-thalassaemia patients (Fibach *et al*, 1993). To date, this approach has been used in cultures derived from HSCs not treated *in vivo* to predict the response before starting the *in vivo* administration (Watanapokasin *et al*, 2005; Smith *et al*, 2000). The results reported here also showed a correlation between the response to HC treatment *in vivo* and *in vitro* (Table III) in cultures from patients under *in vivo* treatment. Indeed, all patients studied showed a *HBG* mRNA fold increase *in vitro* comparable to the HbF fold increase observed *in vivo* (Table III). In one patient, classified in the study as non-responder, the drug caused an increase in *HBG* and a concomitant reduction in *HBB* gene expression that appeared to ultimately result in a failure to increase the total haemoglobin level *in vivo*.

In conclusion, these findings suggest that although HC might be an effective and safe treatment in some patients with thalassaemia intermedia, its long-term effectiveness may be attenuated, probably by the reduced potential of HSC towards erythroid differentiation.

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