RESEARCH ARTICLE

A novel unified model of fetal hemoglobin induction by pharmacological agents: An *in-silico* approach

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Abstract

Background: In β-thalassemia patients or patients of sickle cell disease (SCD), the mutant β globin gene is the absolute cause of alpha-beta globin chain imbalance, resulting in severe anemia. This condition can be ameliorated by inducing the production of γ globin chain, which is mainly expressed in the fetus and thereby producing the fetal hemoglobin ($\alpha_2\gamma_2$) in adults. In normal adults, a very low amount of fetal hemoglobin is usually present since the transcription of the γ globin gene is gradually repressed as the development progresses. However, several pharmacological agents have been reported to derepress the γ globin gene transcription or induce fetal hemoglobin. Still, the precise molecular mechanism underlying this potential is yet to be clear. *Objective:* Here, we have performed a bioinformatic study with the aim of revealing the molecular pathways involved in fetal hemoglobin induction following exposure to hydroxyurea – the only FDA-approved drug for this purpose. *Methods:* Microarray gene expression data from bone marrow samples of rats exposed to hydroxyurea have been analyzed through bioinformatic methods. *Results:* Our study revealed that a novel signal transduction pathway downstream to G-protein coupled neurotransmitter receptors is possibly involved. Along with that, several other pathways reported in earlier studies were also activated and interrelated. *Conclusion:* Combining these findings and linking the identified molecular pathways we have formulated a comprehensive model of fetal hemoglobin induction by pharmacological agents. We hope this model will light up the way for developing targeted drugs for thalassemia and SCD.

Keywords: Fetal hemoglobin, Gamma-globin, GPCR pathway, Hydroxyurea, MeCP2, Neurotransmitter, Reactome analysis.

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Introduction

The β-like globin chains of human hemoglobin are encoded by five genes located in β globin gene cluster of chromosome 11. These five genes are sequentially arranged as 5'-epsilon(ε)-gamma G (Gγ)-gamma A (Aγ)-delta (δ)-beta (β)-3', and their order of expressions also follow the same sequence during human development, *i.e.*, ε gene is expressed only in embryo, Gγ, Aγ genes are mainly expressed in fetus and δ, β genes are expressed in adults.[1] In normal adults, hemoglobin A (HbA), having two α and two β chains, comprises about 97% of total hemoglobin, whereas hemoglobin A2 (HbA2), consisting of two α and two δ comprises only 2% of total hemoglobin. The fetal hemoglobin (HbF) consisting of two α and two γ chains, is the main oxygen carrier of the fetus but its level in baby blood remains elevated up to one year after birth. Adult blood also contains a small amount (about 1%) of fetal hemoglobins.[2]

Mutation of β globin gene is associated with two common genetic disorders. β thalassemia, where the presence of mutant beta globin gene in homozygous condition results in less or no production of the β globin chains,[3] and sickle cell disease (SCD), where abnormal β chains are produced by mutant β globin gene.[4] Both conditions ultimately lead to severe anemia; therefore, the common palliative care for those patients is repeated blood transfusions. Bone marrow or hematopoietic stem cell (HSC) transplantation though is a successful treatment strategy, but it is not always feasible.[5]

Another effective treatment strategy has also emerged, which targets the increased expression of γ globin chains, i.e., induction of fetal hemoglobin by pharmacological compounds. 5-azacitidine a nucleoside analog, is the first such compound reported to have the potential of fetal hemoglobin induction.[6] To date more than 70 pharmacological compounds have been identified with this potential. Among them, the few most studied compounds are -hydroxyurea, decitabine, cytosine arabinoside, butyrate etc.[7] Hydroxyurea is the only compound approved by the United State Food and Drug Administration (FDA) for treating SCD and beta-thalassemia patients. Despite such progress in drug discovery, the molecular mechanism of actions of these drugs that lead to increased transcription of the gamma-globin gene or induction of Hbf is still not fully understood. However, currently, five models partly explain the possible mechanism of actions of these pharmacological compounds. First model proposes that the compounds that inhibit DNA methyltransferase enzymes and thus prevent methylation of CpG present at the promoter of

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gamma-globin gene allow binding of transcription factors to the promoter leading to transcriptional activation of the gene.[8] The second model opposes this hypothesis and argues that cytotoxic properties of the pharmacological compounds are actually responsible for Hbf induction. Cytotoxic agents deplete the late progenitor cells responsible for regular hemoglobin production and therefore to meet the erythropoietic demand of the body, the total time of erythroid cell differentiation is reduced in a process known as stress erythropoiesis. As a result the number of early progenitors in which the repression of the γ globin gene is incomplete increases along with increased production of γ chains.[9,10] The third model proposes that pharmacological agents with histone deacetylase (HDAC) inhibitory effect block the activity of HDAC enzymes and leave the histone protein of nucleosome in hyperacetylated condition. This prevents global condensation of chromatin, so also in γ globin gene locus and thus promotes transcription of γ chains.[11,12] The activation of p38 MAPK signaling pathway by these pharmacological compounds is the key essence of the fourth model. Different agents by different means, generate reactive oxygen species (ROS) or nitric oxide (NO) which ultimately activate p38 MAPK pathway and lead to downstream phosphorylation and activation of cAMP response element binding (CREB) and other transcription factors. The transcription factors then bind to the promoter of the γ globin gene and induce their transcription.[13,14] A unified stress signaling model has been proposed in a review as a consensus which proposes that various cellular stress (oxidative stress, DNA damage, heat shock, NO, ER unfolded protein response) induce cellular signaling pathways like p38 MAPK, c-Jun or extracellular signal mediated kinase pathways and lead to downstream activation of different transcription factors like CREB, ATF2, MYC and GADD34, thereby increasing γ gene transcription.[7]

Several experimental evidences are present in support or in opposition to each of the models but none of these current models can describe the exact mechanism. In this connection, we have performed a bioinformatic study with a specific aim of revealing the molecular mechanism of hydroxyurea mediated induction of Hbf. In this study, we propose that hydroxyurea activates different G protein-coupled neurotransmitter receptors in early erythroid progenitor cells and thus recruits key transcription factors for the transcription of γ globin gene.

This novel pathway also unites other existing hypotheses, encouraging us to formulate a new unified model.

Materials And Methods

Microarray Data Collection

The microarray data (Drugmatrix database, Accession: GSE59894)[15] based on GPL5425 platform (CodeLink Arrays) was acquired from gene expression omnibus (GEO)[16]-a free public database of gene expression profile (https://www.ncbi.nlm.nih.gov/gds/). The dataset included 7 bone marrow samples from rats (Rattus norvegicus) treated with hydroxyurea (59 mg/kg and 400 mg/kg of body weight) *via* oral gavage and 4 bone marrow samples from control rats that received only water in the same manner. As the bone marrow was collected from both control and hydroxyurea-treated rats after 1 and 3 days of treatment, the samples were divided into three groups (59 mg/1 day, 59 mg/3 days and 400 mg/3 days).

Identification of Differentially Expressed Genes

The primary list of differentially expressed genes (DEGs) between control and hydroxyurea treated bone marrow samples in each group was obtained via online tool - GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/). The list of DEGs was then downloaded and imported into XLS files (Microsoft Office Excel 2016) for further processing. Protein coding genes with adjusted *p-value* < 0.05 (using Benjamini and Hochberg false discovery rate controlling method) and log2 (Fold change) >1 have been considered as DEGs and selected for further analysis. To identify the common upregulated and downregulated DEGs present in each group, an online tool Draw Venn Diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) has been used.

Reactome Pathway Enrichment Analysis

The list of upregulated and downregulated DEGs of each group and the upregulated and downregulated common DEGs of all groups were analyzed separately in reactome[17,18] to determine the functional association among different gene products and to identify the molecular pathways in which these DEGs are involved. In reactome, a molecular pathway is significantly enriched or overrepresented only when a considerable number of input genes are mapped in that pathway. In this study, *p-value* < 0.01 has been selected to consider each pathway as significantly enriched.

Construction of Protein-protein Interaction Network (PPI) and Identification of Hub Genes

To predict the interactions among 365 common DEGs of all groups, the STRING database (http://www.string-db.org/)[19] was used and combined interaction score > 0.4 was used as cut-off to detect significant interaction. The protein-protein network was then exported to Cytoscape software (version: 3.8.2)[20] for further analysis and to visualize the PPI network for DEGs. MCODE- a plug-in of Cytoscape,[21] was used to identify the most significant modules (score ≥4) and Cytohubba-another plug-in[22] was used to identify top ten hub genes in the network with 11 topological methods.

Results

Identification of DEGs

GEO2R analysis identified 3044, 3452 and 713 DEGs in 59 mg 1-day, 59 mg/3 day and 400 mg/3 day groups, respectively. The box plots of the gene expression data and volcano plots of differentially expressed genes are also obtained through

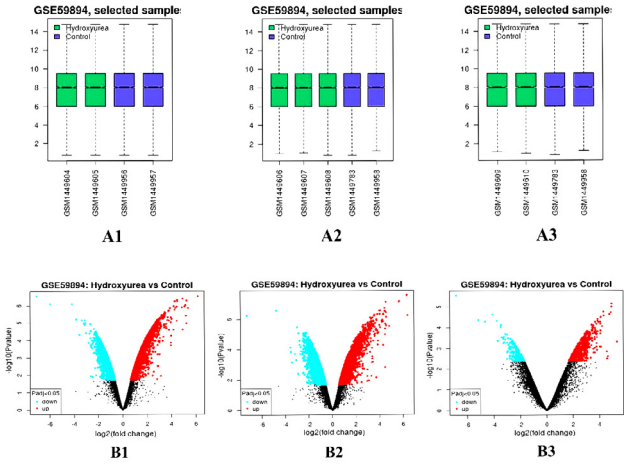

Figure 1: (A1-A3) Box plots of normalized gene expression data of selected samples A1) 59 mg hydroxyurea /1 day vs control A2) 59 mg hydroxyurea /3 day vs control and A3) 400 mg hydroxyurea vs control from GSE59894 microarray dataset. (B1-B3) Volcano plots representing upregulated and downregulated DEGs between hydroxyurea treated and control samples B1) 59 mg hydroxyurea /1 day vs control B2) 59 mg hydroxyurea /3 day vs control and B3) 400 mg hydroxyurea vs control from GSE59894 microarray dataset.

GEO2R, which shows that data are normalized and significant DEGs are present between control and test samples in each group (Figure 1). After processing the three lists of DEGs on the basis of log2 FC cutoff and presence of annotated genes, 1267, 1280 and 461 DEGs were selected from 59 mg/1 day, 59 mg/3 days and 400 mg/3 days groups, respectively for further analysis. Out of 1267 DEGs in the 59 mg/1 day group, 759 upregulated and 508 downregulated genes are included. In the 59 mg 3 days group 779 upregulated and 501 downregulated genes are included. Likewise, the 400 mg/3 days group includes 372 upregulated and 89 downregulated genes. As shown in Venn diagram (Figure 2) 337 upregulated and 28 downregulated genes are common in all the groups.

Reactome Pathway Enrichment Analysis

Significantly enriched or overrepresented reactome pathways by upregulated and downregulated DEGs of three groups and the common group are listed in Table 1**.** In the reactome database, pathways are clustered into few broad categories, so one gene product involved in more than one molecular pathway naturally may be mapped in different reactome pathways in the same or under different broad categories at the same time. As listed in Table 1, all enriched pathways of our reactome analysis are mapped under fifteen broad categories and we intentionally have not listed the mapped pathways under the disease category. Among the fifteen broad categories, signal transduction, gene expression, transport of small molecules, metabolism of proteins, immune system and neuronal system are most important as most of the enriched pathways, common pathways and as per our analysis the relevant pathways to Hbf induction are included under these categories.

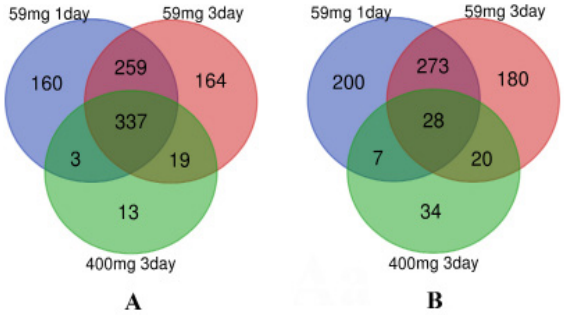

Figure 2: Venn diagrams based on overlapping upregulated (A) and downregulated (B) DEGs between three treatment groups compared with controls.

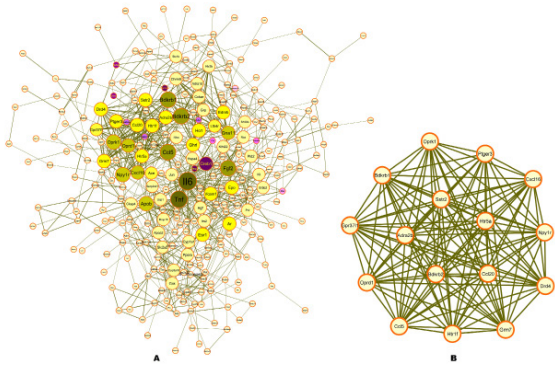

Figure 3: A) Constructed protein-protein interaction network for the common DEGs of three treatment groups (59 mg/1day, 59mg/3day and 400mg/3day hydroxyurea) compared to controls. Sizes of the nodes and width of edges are proportional to the scores. The important nodes are highlighted by colors. B) The most significant module (MCODE score=16) obtained from MCODE network analysis.

Construction of Protein-protein Interaction Network (PPI) and Identification of Hub Genes

Using common DEGs, the PPI network has been constructed, which consists of 357 nodes (genes) and 942 edges (interactions). The PPI enrichment *p-value* as per string db is $<1.0e\text{-}^{16}$ which is highly significant. The most significant module identified by MCODE consists of 16 nodes and 120 edges. The top ten hub genes identified by cytohubba are Htr1f, Adra2b, Npy1r, Bdkrb1, Bdkrb2, ccl20, Ccl5, Cxcl16, Sstr2, and Ptger3. All of them are included in the most significant module (Figure 3).

Discussion

The specific molecular mechanism of fetal hemoglobin induction by different pharmaceutical agents is not clearly understood from the proposed mechanisms as described in various existing models. Even, these models develop contradiction. To unveil the facts, we did a bioinformatic analysis of transcriptomes from bone marrow cells of rats exposed to one of the pharmaceutical agents - hydroxyurea.

Oral hydroxyurea exposure to rats has resulted in transcriptional up and down-regulation of several genes in bone marrow cells. As a result, many cellular pathways involving these gene products are either upregulated or

Table 1: Reactome pathway enrichment analysis of differentially expressed genes.

Pathway identifier	*Pathway name*	*p value of enriched pathways with DEGs of dose group 59 mg for 1day*		*p value of enriched pathways with DEGs of dose group 59 mg for 3days*		*p value enriched pathways with DEGs of dose group 400 mg for 3days*		*p value of enriched pathways with common DEGs of all dose groups*	
		Up regulated	Down regulated	Up regulated	Down regulated	Up regulated	Down regulated	Up regulated	Down regulated
	Cellular responses to stimuli								
R-HSA-2262752	Cellular responses to stress								0.002277
R-HSA-1234158	Regulation of gene expression by Hypoxia-inducible Factor							0.009285	
	Transport of small molecules								
R-HSA-425407	SLC-mediated transmembrane transport					0.002047		0.000940	
R-HSA-425366	Transport of bile salts and organic acids, metal ions and amine compounds					0.006457		0.002642	
R-HSA-8949664	Processing of SMDT1						0.007667		
	Immune system								
R-HSA-449147	Signaling by Interleukins	0.000013		0.000027	0.006156	0.000227			0.005330
R-HSA-6783783	Interleukin-10 signaling	0.000000		0.000008	0.009766	0.000521	0.004435		0.000069
R-HSA-6785807	Interleukin-4 and Interleukin-13 signaling	0.000005		0.000009		0.000006		0.008105	
R-HSA-9014843	Interleukin-33 signaling					0.007409			
R-HSA-9008059	Interleukin-37 signaling				0.000023				
R-HSA-1059683	Interleukin-6 signaling				0.000743				
R-HSA-1266695	Interleukin-7 signaling								0.002880
R-HSA-5660668	CLEC7A/inflammasome pathway	0.001968							
R-HSA-5676594	TNF receptor superfamily (TNFSF) members -mediating non-canonical NF-kB pathway						0.007667		
R-HSA-168927	TICAM1, RIP1-mediated IKK complex recruitment						0.009483		
	Signal transduction								
R-HSA-372790	Signaling by GPCR	0.000008		0.000034		0.000146		0.000282	
R-HSA-500792	GPCR ligand binding	0.000006		0.000008		0.000003		0.000006	
R-HSA-373076	Class A/1 (Rhodopsin-like receptors)	0.000001		0.000006		0.000000		0.000001	
R-HSA-375280	Amine ligand-binding receptors	0.000615		0.002532		0.000272		0.000126	
R-HSA-390648	Muscarinic acetylcholine-receptors	0.007273							
R-HSA-390666	Serotonin receptors					0.008602		0.006299	
R-HSA-390696	Adrenoceptors					0.005459		0.003978	

Continued...

Identifier	Pathway name	59mg/1day		59mg/3day		400mg/3day		common DEGs	
		Up	Down	Up	Down	Up	Down	Up	Down
R-HSA-375276	Peptide ligand-binding receptors	0.000468		0.000137		0.000012		0.000287	
R-HSA-380108	Chemokine receptors bind-chemokines					0.002590			
R-HSA-388396	GPCR downstream signalling	0.000006		0.000026		0.000230		0.000291	
R-HSA-418594	G alpha (i) signalling events	0.000632		0.002959		0.001362		0.005567	
R-HSA-418555	G alpha (s) signalling events	0.004980		0.006468					
R-HSA-166520	Signaling by NTRKs	0.000699		0.002100					
R-HSA-187037	Signaling by NTRK1 (TRKA)	0.000230		0.001789					
R-HSA-198725	Nuclear Events (kinase and transcription factor activation)	0.000305		0.003184					
R-HSA-9031628	NGF-stimulated transcription	0.000025		0.000128					
R-HSA-199418	Negative regulation of the PI3K/AKT network	0.009289				0.001733		0.002140	
R-HSA-6811558	PI5P, PP2A and IER3 Regulate PI3K/AKT-Signaling	0.005317				0.001053		0.001346	
R-HSA-6806942	MET Receptor Activation					0.003133		0.002271	
R-HSA-8874081	MET activates PTK2 signaling	0.005356							
R-HSA-8875513	MET interacts with TNS proteins							0.009116	
R-HSA-8865999	MET activates PTPN11							0.009116	
R-HSA-8875791	MET activates STAT3							0.009116	
R-HSA-5626978	TNFR1-mediated ceramide production	0.007273							
R-HSA-9027276	Erythropoietin activates Phosphoinositide-3-kinase (PI3K)						0.006825		
R-HSA-1236394	Signaling by ERBB4		0.008783						
R-HSA-1251985	Nuclear signaling by ERBB4		0.004164						
R-HSA-190241	FGFR2 ligand binding and activation		0.005231						
R-HSA-5654221	Phospholipase C-mediated cascade; FGFR2		0.004448						
R-HSA-5654695	PI-3K cascade:FGFR2				0.009455				
R-HSA-9006936	Signaling by TGFB family members		0.003774						0.003067
R-HSA-201451	Signaling by BMP		0.009349						
R-HSA-2173789	TGF-beta receptor signaling activates SMADs		0.004574						
R-HSA-2173788	Downregulation of TGF-beta receptor-signaling		0.007083						
	Gene expression (Transcription)								

Continued...

Identifier	Pathway name	59mg/1day		59mg/3day		400mg/3day		common DEGs	
		Up	Down	Up	Down	Up	Down	Up	Down
R-HSA-383280	Nuclear Receptor transcription pathway	0.000000		0.000000		0.000124		0.000045	
R-HSA-8986944	Transcriptional Regulation by MECP2	0.000132		0.000000	0.003157	0.000026		0.000168	
R-HSA-9022927	MECP2 regulates transcription of genes involved in GABA signaling	0.000689		0.000754					
R-HSA-9022702	MECP2 regulates transcription of neuronal ligands	0.001734		0.000032		0.000070		0.006299	
R-HSA-9022699	MECP2 regulates neuronal receptors and channels	0.005356		0.000072		0.000089		0.000044	
R-HSA-9022707	MECP2 regulates transcription factors	0.004343		0.004726		0.000326		0.000212	
R-HSA-9022692	Regulation of MECP2 expression and activity					0.001724		0.000967	
R-HSA-8864260	Transcriptional regulation by the AP-2 (TFAP2) family of transcription factors	0.007619		0.008828		0.000309		0.004007	
R-HSA-8866910	TFAP2 (AP-2) family regulates transcription -of growth factors and their receptors	0.002730		0.003071		0.000068		0.000036	
R-HSA-9614085	FOXO-mediated transcription	0.006340				0.003146			
R-HSA-9617828	FOXO-mediated transcription of cell cycle -genes	0.008951							
R-HSA-8951911	RUNX3 regulates RUNX1-mediated transcription				0.000637				
R-HSA-8931987	RUNX1 regulates estrogen receptor mediated transcription		0.009020		0.000827				
R-HSA-8939246	RUNX1 regulates transcription of genes involved in differentiation of myeloid cell				0.001172				
R-HSA-8939242	RUNX1 regulates transcription of genes involved in differentiation of keratinocytes				0.001172				
R-HSA-8939245	RUNX1 regulates transcription of genes involved in BCR signaling						0.001366		
R-HSA-5628897	TP53 Regulates Metabolic Genes								0.004056
R-HSA-6796648	TP53 Regulates Transcription of DNA Repair Genes						0.000643		
	Metabolism of protein								
R-HSA-422085	Synthesis, secretion, and deacylation of Ghrelin	0.001737		0.000414		0.001597		0.000967	
R-HSA-5688426	Deubiquitination						0.006910		
R-HSA-8957275	Post-translational protein phosphorylation	0.000976		0.007221		0.008991			

Continued...

Identifier	Pathway name	59mg/1day		59mg/3day		400mg/3day		common DEGs	
		Up	Down	Up	Down	Up	Down	Up	Down
R-HSA-4090294	SUMOylation of intracellular receptors			0.001654		0.001955		0.001099	
R-HSA-381426	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	0.000122		0.001088		0.008305			
R-HSA-3247509	Chromatin modifying enzymes								
R-HSA-3214842	HDMs demethylate histones								0.002880
	Metabolism								
R-HSA-400206	Regulation of lipid metabolism by PPARalpha			0.002152					
R-HSA-1989781	PPARA activates gene expression			0.001871					
R-HSA-71384	Ethanol oxidation							0.008745	
R-HSA-8957322	Metabolism of steroids		0.006220						
R-HSA-1655829	Regulation of cholesterol biosynthesis by SREBP (SREBF)		0.000000						
R-HSA-2426168	Activation of gene expression by SREBF		0.000000						
R-HSA-200425	Carnitine metabolism		0.007083						
R-HSA-8964539	Glutamate and glutamine metabolism								0.004727
	Cell cycle								
R-HSA-68877	Mitotic Prometaphase						0.005790		
R-HSA-69560	Transcriptional activation of p53 responsive genes	0.000689				0.000996			
R-HSA-69895	Transcriptional activation of cell cycle inhibitor p21	0.000689				0.000996			
	Neuronal System								
R-HSA-112316	Neuronal System	0.001923		0.000069		0.001106		0.000859	
R-HSA-112315	Transmission across Chemical Synapses	0.009953		0.005039		0.007275		0.009592	
R-HSA-112314	Neurotransmitter receptors and postsynaptic signal transmission			0.007359					
R-HSA-210500	Glutamate Neurotransmitter Release Cycle								0.003064
R-HSA-1299316	TWIK-releated acid-sensitive K+ channel (TASK)					0.004255		0.003406	
	Cell-Cell communication								
R-HSA-1500931	Cell-Cell communication				0.009604				

Continued...

Identifier	Pathway name	59mg/1day		59mg/3day		400mg/3day		common DEGs	
		Up	Down	Up	Down	Up	Down	Up	Down
	Developmental biology								
R-HSA-186712	Regulation of beta-cell development	0.000164		0.002089		0.001563		0.000758	
R-HSA-210745	Regulation of gene expression in beta cells	0.002308				0.001001		0.000555	
	Hemostasis								
R-HSA-75205	Dissolution of Fibrin Clot						0.005278		
	mRNA metabolism				0.003146				
R-HSA-77042	Formation of editosomes by ADAR proteins	0.003334							
	Apoptosis								
R-HSA-5218859	Regulated Necrosis						0.003002		
R-HSA-5213460	RIPK1-mediated regulated necrosis						0.005444		
R-HSA-5675482	Regulation of necroptotic cell death						0.003207		

Table 2: List of common upregulated neurotransmitter receptors and SLC transporter.

Gene name	*Function*
Neurotransmitter receptors	
Drd4	Dopamine receptor
Htr1f	Serotonin receptor
Htr2b	Serotonin receptor
Htr2c	Serotonin receptor
Htr4	Serotonin receptor
Htr5a	Serotonin receptor
Adra1b	Adrenergic receptor
Adra1d	Adrenergic receptor
Adra2b	Adrenergic receptor
Adora2b	Adenosine receptor
Chrm3	Cholinergic receptor
Chrm5	Cholinergic receptor
Solute carrier family transporters	
Slc6a3	Dopamine importer
Slc6a5	Glycin importer
Slc6a11	GABA importer
Slc6a18	Neurotransmitter importer
Slc14a1	Urea transporter
Slc14a2	Urea transporter
Slc22a12	Urate transporter
Slc13a2	Dicarboxylate transporter

downregulated. To identify these pathways, the reactome analysis has been performed. However, the biggest challenge was to find the relationship among diverse cellular pathways enriched in reactome analysis (listed in Table 1) and to reveal the sequential events in hydroxyurea-induced gamma-globin expression.

Upregulation of neurotransmitter receptor pathways like transmission across chemical synapses and amine ligand binding receptor pathways primarily drew our attention. The upregulated genes that enriched the pathways are found to encode several neurotransmitter receptors for serotonin, dopamine, acetylcholine, GABA and glutamine. We then conducted a literature survey to determine whether hematopoietic stem cells express these kinds of neurotransmitter receptors. The survey revealed that in the past few years, the expression of numerous neurotransmitter receptors and signaling molecules have been confirmed in hematopoietic cells. The more immature CD34+ progenitor cells are of particular interest as they are reported to express various G protein–coupled neurotransmitter receptors like dopamine receptor, serotonin receptor (HTR1F), GABA receptor, adenosine A2B receptor corticotropin-releasing hormone (CRH 1 and CRH 2) receptors, orexin/hypocretin receptors, opioid receptors.[23,24] Therefore, one explanation for enrichment of this pathway is the expression of a subset of

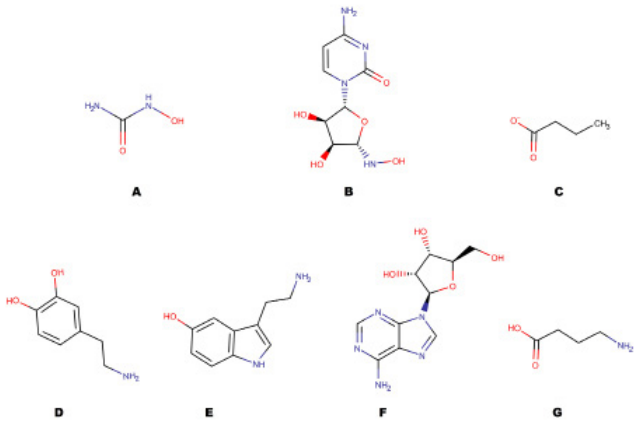

Figure 4: First row represents the 2D chemical structure of three Hbf inducing agents- hydroxyurea (A), 5-azacytidine (B) and butyrate(C). Second row represents the 2D chemical structure of four corresponding neurotransmitters - Dopamine (D), Serotonin (E), Adenosine (F) and GABA (G).

neurotransmitter receptors in hematopoietic cells in response to hydroxyurea. Moreover, few studies have demonstrated that bone marrow is innervated by sympathetic nerves and these nerves possibly regulate the hematopoietic stem cell (HSCs) differentiation.[25,26] If the nervous system influences the hematopoiesis, the possible way to do this would be through neurotransmitter-mediated crosstalk. Hence, it can be assumed that the neurotransmitter receptors expressed in HSCs act as receivers of neuronal command. Furthermore, we found that most of the upregulated neurotransmitter receptors in our study matched with the receptors of one of the two communities identified by a computational study where two community detection methods (modularity algorithm and clique percolation algorithm) were used to detect the transcriptional associations among 100 G-protein coupled neurotransmitter receptors in brain regions.[27]

Enrichment of solute carrier family (SLC) mediated transport of small molecule pathways has further sparked one more speculation. For example, the SLC of membrane transporters that are upregulated, are known to be explicitly involved in uptake of extracellular dopamine, GABA, glycine, urea and dicarboxylate (Table 2) and thus terminate neurotransmitter signals or maintain homeostasis. Such a type of cellular response to elevated hydroxyurea level indicates that either hydroxyurea enhances the level of urea, a few amino acids or neurotransmitter levels in the extracellular environment or itself mimics those molecules structurally or functionally. A study has reported that hydroxyurea is less permeable to plasma membranes and enters cells through urea transporters.[28] This report supports the second assumption and exemplifies a phenomenon of molecular mimicry.

Similarly, we can assume that HSCs express these neurotransmitter importers, since hydroxyurea mimics some of the neurotransmitters. The phenomenon of molecular mimicry is not restricted only to SLC transporters. Several biomolecules have been found to mimic various neurotransmitters and interfere with their activity. For example, heroin resembles the natural opioids endorphin and enkephalin; nicotine attaches to acetylcholine receptors, ergoline mimics dopamine.[29,30] Furthermore, the structural comparison of hydroxyurea with dopamine and serotonin shows that at least they share some common groups (Figure 4). Therefore, we hypothesize that hydroxyurea too mimics any amine neurotransmitters acting through GPCR signaling.

The activation of G-protein coupled neurotransmitter receptors after hydroxyurea exposure is apparent from enrichment of downstream signaling pathways which include mainly three second messenger cascades. $G\alpha_s$ mediated activation of adenylate cyclase leading to cAMP production, $G\alpha_i$ mediated inactivation of adenylate cyclase leading to inhibition of cAMP production and $G\alpha_q$ mediated activation of phospholipase C leading to generation of inositol triphosphate (IP3) and diacylglycerol (DAG) through cleavage of membrane phosphatidylinositol 4,5 bisphosphate (PIP2).[31] The activation of $G\alpha_i$ pathway is most significantly enriched. Out of seven GPCRs included under top ten hub genes, six of them, irrespective of their ligands function through $G\alpha_i$. The activation of such inhibitory GPCRs possibly act as feedback regulation following the $G\alpha_s$ mediated cAMP production. $G\alpha_q$ mediated pathway does not cross the *p-value* cutoff; its activation can be traced by linking the downstream events. The rise of intracellular Ca^{++} level through IP3-mediated Ca^{++} release from endoplasmic reticulum results in the formation of Ca-calmodulin complex, which is critical for activation of CamKII.[32] Similarly, the rise of the intracellular cAMP level following the $G\alpha_s$ mediated signaling is responsible for the activation of protein kinase A (PKA). The PKA is primarily involved in the phosphorylation and activation of cAMP response element binding (CREB1)[33] - the transcription factor which lies in the centre of each hypothesis related to the pharmacological induction of Hbf.

The enrichment of transcriptional regulation by Methyl-CpG-binding protein 2 (MeCP2) pathway as observed in reactome analysis is possibly the most important cue about the connection and sequence of cellular events after hydroxyurea exposure. MeCP2 is a nuclear protein which can either repress or activate transcription of genes by specifically binding to methylated CpG sequences in the gene promoter and the repression or activation of the genes depends on the presence of 5 methylcytosine (5mC DNA) or 5 hydroxymethylcytosine (5hmC DNA) in the promoter of that genes, respectively.[34] Therefore, to sort out whether MeCP2 participates in the repression or activation of the gamma-globin gene through methylated promoter, we looked into the regulatory mechanism of MeCP2 activity. The activity of MeCP2 is found to be controlled by CamKII and CamKIV through phosphorylation.[35] Studies have shown that phosphorylation at Tyrosine-308 involves dissociation of NCoR/SMRT co-repressor complex from MeCP2 repressor

domain and as a result the transcriptional repression ability of MeCP2 is suppressed.[36]

Furthermore, MeCP2 also binds to the SIN3A co-repressor complex with the same repressor domain along with HDAC1 and HDAC2.[37] So, it is possible that after hydroxyurea exposure, CamKII mediated phosphorylation of MeCP2 ultimately inhibit the gamma-globin gene repression by releasing histone deacetylases (HDAC1 and HDAC2) from the MeCP2 repression complex. This observation coincides with the hypothesis of the third model in which inhibition of HDACs by pharmacological compounds have been proposed and also indicates the involvement of epigenetic mechanism in gamma-globin gene repression. Moreover, MeCP2 is shown to upregulate the transcription of CREB1, and the expression of MeCP2 is downregulated post-transcriptionally by CREB1-induced microRNA-132.[38,39] They can also form a complex that stimulates the transcription of other genes, like somatostatin.[40,38] In a study, rats treated with 2,4,6-trinitrobenzene sulfonic acid (TNBS) to induce colonic inflammation showed that the inflammation triggers elevation of phosphorylated MeCP2 with a significant decrease of MeCP2 level and the majority of MeCP2- positive cells co-expressed CREB.[41] Therefore, the mutual functional relationship between CREB and MeCP2 is of great importance.

Many more signaling pathways are also activated after hydroxyurea exposure, few of which as per our analysis, occurs as a result of MeCP2-regulated gene expression. The expression of MeCP2-regulated receptor tyrosine kinase (Met), opioid receptors (oprk1, oprm1), transcription factor (creb1), peptide ligand somatostatin (sst) and delta (dll1) as observed in our study, may initiates a group of secondary signaling cascades. In other words, MeCP2 sensitizes the hematopoietic cell to various kinds of extracellular regulatory ligands. For example, the upregulation of PIP3/Akt second messenger signaling pathway is a sequel of MET receptor activation.[42] Met - a receptor tyrosine kinase (RTK) is activated after binding with its ligand- hepatocyte growth factor (HGF) and in turn activates an enzyme phosphoinositide-3-kinase (PI3K). PI3K kinase then generates a second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP3) through phosphorylation of the membrane lipid phosphatidylinositol (4,5)-bisphosphate (PIP2). PIP3 induces downstream activation of Akt kinase which can also induce the activation of the transcription factor CREB1 through phosphorylation. Activated RTKs also send downstream signals through the Ras/Raf/ERK1,2 pathway.[43]

NTRKs - another such RTK[44] mediated signaling pathway is enriched in the reactome study. The transcriptional upregulation of immediate early genes (IEG) like fos, junb, jund and egr indicates that possibly the nerve growth factor (NGF) stimulated receptor NTRK1(TRKA) has been activated.[45,46] The NTRK activation is regulated by controlling the cell surface level of the receptors and after ligand binding the NTRK-ligand complex is internalized in the endosome for more sustained activation.[44,47] Studies have also shown that the elevated intracellular cAMP and Ca^{++} level promotes the movement of endosomal NTRKs to the cell surface and after ligand binding favors the internalization of the ligand-receptor complex.[48,49] Hence, it seems that the progenitor cells become sensitive to NGF after GPCR mediated signaling. Moreover, even in absence of NGF, the Trka can also be activated through adcyap1r1 - a GPCR which binds to a neuropeptide adenylate cyclase activating peptide 1(adcyap1).[50] Like NGF the adcyap1 is a neurotrophic peptide that is secreted by neurons after neuronal injury or neurotrophin deprivation. The upregulation of adcyap1r1 after hydroxyurea exposure thus indicates the second possibility also. Within the endosome the activated adcyap1r1 transactivates the TRKA through tyrosine phosphorylation which is also dependent on elevated intracellular Ca^{++} level.[51]

We also observed separate ways through which hydroxyurea may induce fetal hemoglobin and these pathways connect our observations with the hypothesis of the fourth and fifth model, *i.e.,* involvement of oxidative and other stress response related pathways in fetal hemoglobin induction. Enrichment of stress response signaling pathways like oxidative stress, DNA damage and hypoxia related stress are evident from our study and also can be traced by enrichment of downstream events, like TP53 mediated cell cycle arrest and secretion of proinflammatory cytokines (IL1a, IL1b, IL6)- an indication of cellular senescence or growth arrest induced by oxidative stress.[52–54] The stress response pathways either restore the homeostasis of the cells or activate apoptotic pathways when the damage is irreversible. Different chemokines (Ccl5, Ccl20 and TNFα) are secreted by damaged cells and their crosstalk attracts and activates macrophage and lymphocytes, leading to clearance of damaged cells.[55] However, in response to ROS, MAP3K5 (ASK1) becomes catalytically active[56] and activates MAP2K3 (MKK3) and MAP2K6 (MKK6) through phosphorylation.[57] MKK3 and MKK6 activate both the p38 MAPK pathway[56,58] as well as JNK pathway.[57,59] These pathways are involved in activation of CREB, TP53 and induce cellular senescence.
The enrichment of anti-inflammatory cytokines pathways (IL4, IL13 and IL10) and their downstream events give a clear indication that in response to proinflammatory cytokines and chemokines secreted by the senescent or damaged cells, the immune cells, particularly helper T2 (TH2) cells come to action.[60] The anti-inflammatory cytokines secreted by the TH2 then attempt to restore cellular homeostasis by suppressing the proinflammatory cytokines[61,62] and chemokines synthesis[63,64] and inducing the synthesis of antioxidant enzymes, transcription factors (Foxo3)[65] and several growth factors like HGF, TGF beta and FGF2.[66–68] The FOXO3 transcription factor regulates the transcription of oxidative stress response genes, metabolic genes and cell cycle genes.[69] We also observed activation of another transcription factor AP2 which regulates the expression of growth factors and their receptors like tyrosine-protein kinase KIT,[70] estrogen receptor 1 (ESR1)71 and Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2).[72] But the exact mechanism of

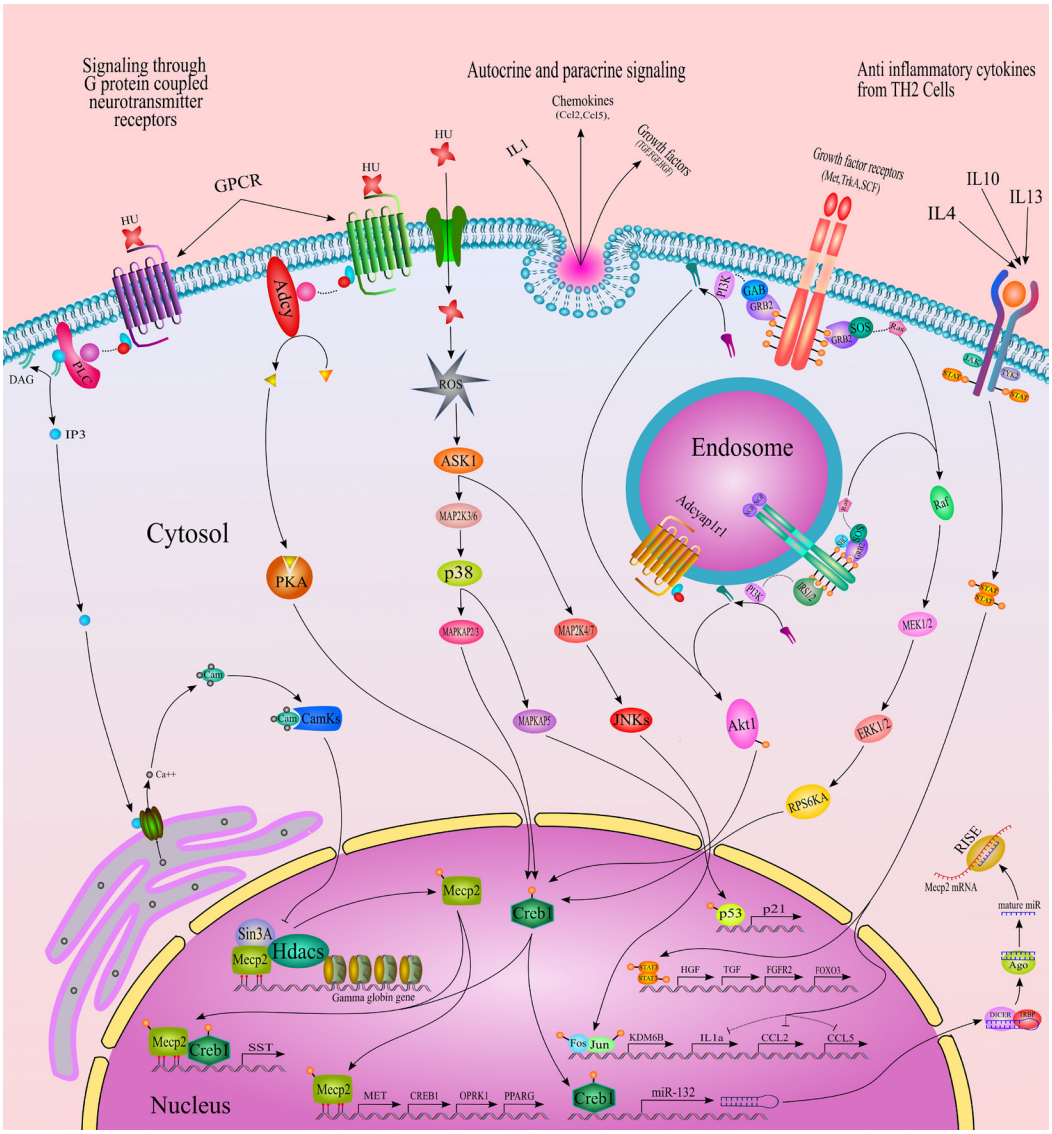

Figure 5: Schematic illustration of intracellular signal transduction pathways in bone marrow hematopoietic stem cells following hydroxyurea exposure. On cell surface hydroxyurea binds and stimulates G-protein coupled amine neurotransmitter receptors. The stimulated receptors then activate downstream effector enzymes- protein kinase A (PKA) and Phospholipase C (PLC). PKA phosphorylates a key transcription factor CREB1 and PLC is responsible for intracellular rise of Ca^{++} level. Activation of the CamK enzyme following elevated Ca^{++} level results in phosphorylation of MeCP2 and consequently dissociation of HDACs occurs from MeCP2-Sin3A-HDACs gene repression complex - the reason for gamma globin gene expression. Phosphorylated MeCP2 and phosphorylated CREB1, separately or in a complex, regulate transcription of several genes. Phosphorylated MeCP2 increases the transcription of CREB1, whereas CREB1 decreases MeCP2 expression through microRNA-132 in a feedback.Thus, the complex interrelation between Mecp2 and Creb1 is the key to solving the mystery of fetal hemoglobin induction by pharmacological agents. Hydroxyurea also may enter into HSC through SLC transporter and within cytosol through metabolic processes generate reactive oxygen species (ROS). Cellular enzyme ASK1 senses the ROS and activates two major stress response pathways- p38 MAPK and JNK pathway. p38 MAPK pathway activation is associated with CREB1 activation and also with cell cycle arrest through p53 mediated DNA damage response. The activation of the JNK pathway is associated with cellular senescence and secretion of proinflammatory cytokines and chemokines. The immune cells attracted by chemokines besides eliminating damaged cells also restore the normal cellular function of partly damaged cells by secreting anti-inflammatory cytokines. The anti inflammatory cytokines (IL4,IL13 and IL10) signal through their cell surface receptors and increase transcription of several growth factors. The growth factors initiate a second loop of signal transduction through second messengers like Akt1 or Raf and ultimately activate the CREB1 transcription factor.

activation of TFAP2 could not be traced. Possibly there might be a role of FOXO3-induced expression of CITED2 which is also known to inhibit transactivation of HIF1 inducible genes like erythropoietin.[73,74] Few studies have shown that immunomodulatory agents,[75] erythropoietin,[76] TGFβ[77] and KIT ligand[78] can induce Hbf. Our study also has come up with such findings and reveals that rather than a single signaling pathway, a complex network of interdependent signaling pathways are actually responsible for Hbf induction.

Briefly, on the HSC surface, hydroxyurea binds and stimulates G-protein coupled amine neurotransmitter receptors. The stimulated receptors then activate downstream

effector enzymes- protein kinase A (PKA) and phospholipase C (PLC). PKA phosphorylates a key transcription factor CREB1 and PLC is responsible for the intracellular rise of Ca^{++} level. Activation of the CamK enzyme following elevated Ca^{++} level results in phosphorylation of MeCP2 and consequently, dissociation of HDACs occurs from MeCP2-Sin3 A-HDACs gene repression complex- the reason for gamma-globin gene expression. Phosphorylated MeCP2 and phosphorylated CREB1, separately or in a complex, regulate the transcription of several genes. Phosphorylated MeCP2 increases the transcription of CREB1, whereas CREB1 decreases MeCP2 expression through microRNA-132 in feedback. Thus, the complex interrelation between Mecp2 and Creb1 is the key to solving the mystery of Hbf induction by pharmacological agents. Hydroxyurea also may enter into the HSC through SLC transporter and within cytosol through metabolic processes ROS is generated. Cellular enzyme ASK1 senses the ROS and activates two major stress response pathways- p38 MAPK and JNK pathway. p38 MAPK pathway activation is associated with CREB1 activation and also with cell cycle arrest through p53-mediated DNA damage response. The activation of the JNK pathway is associated with cellular senescence and secretion of proinflammatory cytokines and chemokines. Besides eliminating damaged cells, the immune cells attracted by chemokines also restore the normal cellular function of partly damaged cells by secreting anti-inflammatory cytokines. The anti-inflammatory cytokines (IL4, IL13 and IL10) signal through their cell surface receptors and increase transcription of several growth factors. The growth factors initiate a second loop of signal transduction through second messengers like Akt1 or Raf and ultimately activate the CREB1 transcription factor.

A Comprehensive Model of Hbf Induction by Hydroxyurea

Although our study is based on hydroxyurea exposure in rats, the findings can solve many mysteries related to Hbf induction by other compounds. The key components of the model has been illustrated in Figure 5. Our model emphasizes the modulation of G-protein coupled neurotransmitter receptor signaling pathways and supports and integrates other existing models. We have placed MeCP2 along with the CREB1 transcription factor in the centre of our model. Several ways of CREB1 activation have been described. Among them, the growth factor-mediated RTKs activation and downstream second messengers pathways like PI3K/Akt pathway and p38 MAPK are of great importance.

Conclusion

Using an *in-silico* approach, we attempted to perceive the cellular events that occur after hydroxyurea exposure. The observations have prompted us to construct a new comprehensive model that can relate all other existing models of fetal hemoglobin induction by pharmacological agents and point out several proteins and pathways that can be targeted for future drug development. We acknowledge that our study is based on a limited sample size. Besides, *in-vitro* experiments needed to validate the vast finding which were beyond the scope of the present study. Therefore, considering the scale of the outcome and their possible impact on future research, we thought it appropriate to formulate a hypothesis that could be tested further. We hope that our model will show a new direction in future research.

Conflicts Of Interest

The authors declare no competing financial interests.

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During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.